# Effects of acute exercise and voluntary freewheel exercise in mice on pro-inflammatory cytokines and markers of apoptosis in the hippocampus

by

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## Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## ABSTRACT

**Introduction:** Alzheimer's disease (AD) and dementias constitute a significant public health burden and it is estimated that one in 85 people may be living with AD by 2050. Dementias are a spectrum of diseases with common traits including amyloid protein growth, neurodegradation, neurofibrillary plaque and tangle formation, and which may be influenced by pro- and anti-inflammatory immune mechanisms. Even a modest delay in onset could result in significant reductions in the social and economic burdens of dementias. An important lifestyle factor identified in risk reduction is physical activity (PA). Although the association between dementia risk and PA has been established, the exact physiological mechanisms through which protection occurs are not known. This research consists of two experiments that were designed to explore the effects of physical activity on pro- and anti-inflammatory cytokines and apoptosis in the mouse hippocampus, a brain region implicated in learning, memory, and cognition.

**Methods:** Study #1: Female C57BL/6 mice, 4-5 months of age, were divided into three groups: sedentary controls (NOTREAD) (n = 22), treadmill exercise with immediate sacrifice (TREAD-Imm) (n = 21), or treadmill exercise with sacrifice after 2 hours (TREAD-2h) (n = 20). TNF-α, IL-6, and IL-1β expression in the hippocampus and intestinal lymphocytes were measured by Western blot analysis. Percentages of hippocampal cells undergoing apoptosis ($Annexin^{+}$) or necrosis ($Propidium\ Iodide^{+}$) were determined through flow cytometry. Plasma levels of 8-isoprostane and corticosterone were measured using commercially available EIA kits. Study # 2: Female C57BL/6 mice, 3-4 weeks of age, were assigned to wheel running (WR; n = 20) or a control condition (No WR; n = 22) and sacrificed after the 16 weeks. Data collected included measures of training status (running volume, body weight, run-to-exhaustion time, and skeletal

muscle cytochrome c oxidase activity), flow cytometric analysis of hippocampal cell phenotypes and apoptosis ($CD45^+$, $CD11b^+$, $Annexin^+$, $Annexin^+/PI^+$, $PI^+$), and cytokine concentrations (TNF-α, IL-1β, IL-12, IL-6, IL-1ra, and IL-10) in cell lysates.

**Results:** Study #1: Acute treadmill exercise lead to significant decreases in TNF-α ($p<0.05$) and increases in IL-6 ($p<0.05$) expression in the hippocampus of healthy mice. No effects of acute exercise on the apoptotic status of hippocampal cells were observed. In intestinal lymphocytes, the exercise bout lead to significant increases in TNF-α ($p<0.05$), IL-6 ($p<0.05$), and IL-1β ($p<0.05$). Acute exercise was associated with a significant increase in both plasma 8-isoprostane ($p<0.05$) and corticosterone ($p<0.05$) levels. Study #2: WR mice had measurable training effects and significantly lower TNF-α ($p<0.05$) and higher IL-6 ($p<0.05$), IL-1ra ($p<0.05$) and IL-12 ($p<0.05$) expression in the hippocampus compared to controls. IL-1β, IL-10, and the percent of apoptotic, dead cells, and cell phenotypes did not change due to training.

**Conclusion:** Exercise chronicity (acute vs. chronic), stress characteristics of the exercise (forced vs. voluntary) and tissue location (systemic vs. central) emerged as important variables with effects on both cytokine concentrations and plasma levels of stress hormones. Physical activity may protect the hippocampus against inflammatory damage caused by TNF-α, and the suppression of this cytokine may be due to increased glucocorticoid secretion during acute exercise. It is also proposed that elevated IL-6 expression (central and systemic) may mediate this protection by creating an anti-apoptotic environment in the hippocampus. Less apoptosis may also contribute to maintenance of cognitive function during acute and long-term physical activity.

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# INTRODUCTION

Alzheimer's disease (AD) and dementias constitute a significant public health burden and it is estimated that one in 85 people worldwide may be living with AD by 2050 (Brookmeyer et al., 2007). Dementias are a spectrum of diseases with common traits including amyloid protein growth, neurodegradation, neurofibrillary plaque and tangle formation, and inflammation in the central nervous system (CNS) (Pope et al., 2003). The pathogenesis of dementias may be influenced by pro- and anti- inflammatory immune mechanisms (Packer et al., 2010; Maccioni et al., 2009). For instance, levels of the pro-inflammatory cytokine TNF-α are increased in the frontal, temporal, and parietal cortices (Grammas and Ovase, 2001) and serum IL-1β levels are elevated in AD patients (Alvarez et al., 1996). Neurofibrillary plaques and tangles and risk of cognitive decline are correlated with serum levels of IL-1β and plasma levels of TNF-α (Krabbe et al., 2009). Stimulation of microglia (CNS phagocytes) by lipopolysaccharide (LPS) also increases TNF-α and IL-1β release, generation of reactive oxygen species (ROS), and oxidative stress that damages cells and tissues (Schwab and McGeer, 2008). Age-related amyloid-β formation induces a response similar to LPS, resulting in a pro-inflammatory environment in the brain, a reduction in microglial phagocytic capacity, and the inability to clear abnormal protein aggregates (Kramer et al., 1999).

Most dementias have long asymptomatic periods. Even a modest delay in onset could result in significant reductions in the social and economic burdens that accompany disease (Larson, 2010). One factor identified in risk reduction for dementias is physical exercise (Coley et al., 2008). Furthermore, it has been shown that exercise lowered risks for "cognitive-impairment-not-dementia" in adults compared to those with no physical activity (Laurin et al.,

2001). Physical training improved executive control tasks and decreased reaction time in older adults (Kramer et al., 1999), promoted neuronal growth, survival and differentiation, and improved cognitive performance (Cotman et al., 2007).

In contrast to regular moderate physical activity, acute exhaustive exercise has been associated with reduced immune function and increased risk of infection (Kakanis et al., 2010; Mars et al., 1998; Nieman, 2003). Moreover, high intensity acute exercise leads to leukocytosis, immediately followed by lymphocytopenia (Pedersen and Hoffman-Goetz, 2000), in part due to exercise-induced DNA fragmentation and apoptosis (Lin et al., 1999; Mooren et al., 2002; Navalta et al., 2010). These effects are thought to be the result of increased oxidative stress in the affected tissues. Oxidative stress is an imbalance between endogenous antioxidants, such as superoxide dismutase and glutathione peroxidase, and ROS (Sies, 1997). Acute exercise increases oxygen consumption, resulting in ROS formation and consumption of intracellular antioxidants, alterations in mitochondrial membrane potential, and DNA damage leading to apoptosis in lymphocytes and skeletal muscle (Mooren et al., 2002; Phaneuf and Leeuwenburgh, 2001). Markers of apoptotic cell death, including caspase-3 and caspase-7, are significantly elevated in mouse intestinal lymphocytes after prolonged exhaustive exercise (Hoffman-Goetz and Spagnuolo, 2007; Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). Acute exercise also alters the cytokine balance systemically. Activity at intensities greater than 70% $VO_{2max}$ increases the levels of TNF-α in plasma of young adult males (Ostrowski et al., 1999; Steinacker et al., 2004; Starkie et al., 2000; Zaldivar et al., 2006) and the expression of TNF-α in colonic lymphocytes of mice (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). Increases were also observed in men in response to acute exercise for the cytokines IL-1β and IL-6 (Ostrowski et al., 1999; Starkie et al., 2000; Zaldivar et al., 2006) suggesting that strenuous exercise may

lead to inflammation. Little is known, however, about the impact of acute exhaustive exercise on cytokine expression and apoptosis in the central nervous system, especially when examining areas of the brain that play a role in cognition, learning, and memory, such as the hippocampus.

This thesis addresses some of the gaps in the current understanding of acute exercise and exercise training and their effects on pro- and anti-inflammatory cytokine expression and apoptosis levels in the central nervous system, and more specifically, the hippocampus. The first chapter is a brief review of the literature concerning the effects of cytokines on immune function and their role in the CNS, the mechanisms involved in apoptosis, how these processes are regulated, and current knowledge on the impact of acute exercise and exercise training on cytokines and apoptosis both systemically and centrally. Chapter 2 outlines the overall project rationale and objectives for the experiments presented in this thesis. Chapter 3 relates to the effects of acute exercise on pro-inflammatory cytokines, apoptosis, and oxidative stress in the hippocampus of healthy mice. Chapter 4 describes the effects of long-term voluntary exercise training on pro- and anti-inflammatory cytokines, apoptosis, and cell subsets in the hippocampus of healthy mice, as well as measures of training status. Chapter 5 is the last chapter, and contains a general discussion that takes into consideration the results of both individual studies, integrates these results, and illustrates the implications, limitations, and future directions for experiments in this field of research.

# CHAPTER 1:
# LITERATURE REVIEW

## 1.1 Introduction

The purpose of this literature review is to provide a concise background on: 1) the role of cytokines in "brain health," 2) the role of cellular apoptosis in "brain health," 3) the effects of acute exercise on cytokines and apoptosis in both systemic and central tissues, and 4) the effects of exercise training on cytokines and apoptosis in systemic and central compartments. The review will first address the role of cytokines as immunomodulators, with an emphasis on pro-inflammatory and anti-inflammatory classifications, cellular sources in the central nervous system, and their effects on cognition. Following this, the process of apoptosis will be briefly explained, along with the effects that this mechanism exerts in the aging brain. Lastly, the effects of both acute exercise and long-term exercise training on systemic and central cytokine expression and apoptosis will be considered.

## 1.2 Cytokines, Inflammation, and Brain Health

Cytokines are a complex and heterogeneous group of small proteins whose functions include the regulation of immunity and inflammation. Acting over short distances, and at very low concentrations, cytokines can bind to specific receptors with high affinity found on the target cell membranes, and are thus usually considered to be paracrine or autocrine. Receptor binding leads to signal transduction via second messengers (tyrosine kinases) which alter the behaviour of the target cell through new gene transcription (Corwin, 2000). Cytokine action often results in a production cascade, as the stimulated target cell produces additional cytokines of either the same or a different type, which can act synergistically or antagonistically. Over 100 different

cytokines have been identified, with most having pleiotropic effects on cells (Ozaki and Leonard, 2002). Some of the major cytokines types include: interleukins (such as IL-1, IL-2, IL-6) which are cytokines produced by leukocytes that act on other leukocytes; interferons (such as IFN-γ) which are cytokines produced by a variety of immune cells including macrophages; and tumour necrosis factor (such as TNF-α) which acts on macrophages and on tumour cells to stimulate cell death (Corwin, 2000).

Cytokines are often classified into two functional categories: pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines include IL-1, TNF-α, and IFN-γ and anti-inflammatory cytokines include IL-4, IL-10, and IL-1ra. IL-6 is also considered to be pro-inflammatory as it is produced through the stimulation of IL-1 and TNF-α during inflammatory responses (Dinarello, 1996; Corwin, 2000). IL-6, however, is also regarded as an anti-inflammatory cytokine because it participates in a negative feedback mechanism to reduce the production of both IL-1 and TNF-α depending on the surrounding cytokine environment (Corwin, 2000; de Gonzalo-Calvo et al., 2010).

There are the three major types of non-neuronal cells in the central nervous system (astrocytes, oligodendrocytes, and microglia) (Hanisch and Kettenmann, 2007). Each of these cells has different biological functions. Astrocytes provide structural and metabolic support to neurons. Oligodendrocytes support neuronal signal transduction by providing insulation to the axon portion of the neuron. Microglia play a more complex role, however, and synthesize proinflammatory cytokines to carry out immune and regulatory functions. These cells constitute approximately 10% of the CNS glia (Hanisch and Kettenmann, 2007) and act as monitors of the extracellular environment to respond to and relay information about changes or damage to surrounding neurons or other immune cells (Davalos et al., 2005). A recent review by Barres

(2008) identified that microglia have some phagocytic ability and are tissue-specific macrophages, but are also involved in initiating and maintaining inflammatory processes. Activated microglia release high levels of cytokines, including TNF-α, which communicate directly with lymphocytes and macrophages to regulate their function, control generation of new oligodendrocytes, and reduce the stability of the blood brain barrier during inflammation.

Pro-inflammatory cytokines are thought to contribute to age-related cognitive decline. Healthy octogenarians who had single nucleotide polymorphisms (SNPs) within TNF-α and IL-6 promoter regions were found to be at higher risk of cognitive decline, as indicated by poor performance IQ (perceptual organization and processing speed) and verbal IQ (verbal comprehension and working memory) scores on the Wechsler Adult Intelligence Scale (WAIS) in comparison to those without such SNPs (Krabbe et al., 2009). Circulating levels of TNF-α and IL-6 were negatively correlated with IQ at age 85, suggesting that a pro-inflammatory environment is a risk factor for reduced cognitive function in this population. Tan et al. (2007), using data from the Framingham Study, reported that higher spontaneous production of TNF-α and IL-1β by peripheral blood mononuclear cells can be considered as markers of future risk of cognitive decline leading towards mild cognitive impairment and subsequent AD. Schwab and McGeer (2008) found that microglia (i.e., the immune effector/phagocytic cells of the CNS) can be stimulated to release the pro-inflammatory cytokines, TNF-α and IL-1β, in response to inducers such as bacterial lipopolysaccharide (LPS). Activation of microglia results in the generation of toxic reactive oxygen species through the phagocytic respiratory burst, and these free radicals result in oxidative stress with subsequent damage to surrounding cells and tissues. What is interesting is that age-related amyloid-β formation produces a microglial response similar to that of LPS, and leads to a heightened pro-inflammatory environment within the brain.

Chronic inflammation reduces the phagocytic capacity of microglia and subsequently their ability to clear abnormal protein deposits that contribute to neurodegeneration (Koenigsknecht-Talboo and Landreth, 2005).

### 1.3 Apoptosis and Brain Health

Apoptosis, or "programmed cell death", is a genetically controlled event which is essential for a number of biological processes including embryogenesis, morphogenesis, tissue homeostasis, and immune function (Phaneuf and Leeuwenburgh, 2001). Apoptosis can be contrasted to other forms of cell death, such as necrosis (i.e., premature cell death caused by external factors and which involves inflammation). Apoptosis is a highly regulated process. Cells are efficiently endocytosized by healthy cells or phagocytosized by macrophages and neutrophils that have the ability to recognize hallmark signals on the surface of apoptotic cells. Within the immune system, such cells are those that have proliferated in response to an antigen, and upon carrying out their function, must be eliminated in order to control the total lymphocyte population. This subsequently aids in the avoidance of chronic inflammation (Avula et al., 2001) due to excessive neutrophil accumulation as well as in selection of T-lymphocytes and B-lymphocytes (Chen et al., 1998).

Apoptosis results in irreversible DNA fragmentation arising from nuclear and intranucleosomal cleavage and ends in the formation of membrane-bound apoptotic bodies and the cleavage of intracellular proteins that maintain cell growth and function (Hoffman-Goetz et al., 1999; Hoffman-Goetz et al., 2005). A variety of factors can trigger cellular apoptosis, including radiation, heat, hormonal (glucocorticoid) signalling, TNF-α, reactive oxygen species, as well as intense physical exercise (Phaneuf and Leeuwenburgh, 2001; Hoffman-Goetz et al.,

2005). There are two main pathways through which apoptosis is activated: the intrinsic or death-by-neglect pathway and the extrinsic or receptor-ligand mediated pathway (Hoffman-Goetz et al., 2005). In the former, cytochrome c is released from the mitochondrial membrane into the cytosol and binds to caspase-9 (one of many cytosolic cysteine proteases that cleave proteins at specific amino-acid sites) and other cofactors to form an apoptosome (Ashe and Berry, 2003). This initiates a cascade in which caspase-9 activates caspase-3, with the proteolysis of downstream targets. The initial activation is controlled by the formation of a mitochondrial permeability transition (MPT) pore and results in the loss of mitochondrial transmembrane potential. This, in turn, is regulated by the Bcl protein family, which inhibits the permeability of the mitochondria and subsequent cytochrome c loss (Bredesen, 2007).

The extrinsic pathway involves the binding of ligand to a death receptor located at the cell membrane. Apoptosis Stimulating Factor (FAS) and tumour necrosis factor receptor-1 (TNFR-1) are such receptors expressed at the surface of target cells and interact with FAS-Ligand (FASL) (on cytotoxic T-cells) and TNF-α, respectively. When these ligands bind their respective receptors, the activation of an intracellular "death domain" occurs which transduces the signal to initiator caspases (caspase-8 and capase-10), leading to the activation of caspase-3 and downstream proteolysis (Walczak and Krammer, 2000). Activation of caspase-3 is a key signalling/cascade event in both the intrinsic and extrinsic pathways of apoptosis (Degterev et al., 2003).

The control of apoptosis is dependent on the ratio of initiating proteins (such as FAS or the pro-apoptotic Bcl proteins Bax and Bak) to inhibiting proteins (such as Bcl-2 and Bcl-xL) (Phaneuf and Leeuwenbergh, 2001). When the inhibitory protein concentrations are high, mitochondrial permeability is low and cytochrome c is not released; this prevents the activation

of caspase-9 and the proteolytic cascade. In addition, there may be decoy receptors that bind available FASL and TNF and render them unable to bind to FAS or TNFR1 and inhibit apoptosis via the extrinsic pathway. Of interest with respect to aging, memory and apoptosis is a recent study by Wang et al. (2009). Accelerated aging in mice was associated with declines in spatial memory as well as increased protein levels of caspase-3 in the hippocampus. This finding implies that hippocampal neurons in aging mice are committed to apoptosis, and that this may contribute to age-related decreases in cognition especially in contextual and spatial memory (Bartolini et al., 1996).

### 1.4 Systemic and Central Effects of Acute Exercise

Physical activity (PA) is a lifestyle behaviour with positive and negative influences on the immune system. The influence of PA on the immune system reflects the duration and intensity of the exercise. Acute, exhausting exercise is implicated in decreased immune function and increased susceptibility to infectious illness (Mars et al., 1998). Acute exhausting exercise initiates leukocytosis (increase in circulating leukocytes), immediately followed by lymphocytopenia (loss of lymphocytes) due to exercise-induced apoptosis in humans (Mooren et al., 2002) and animals (Hoffman-Goetz and Quadrilatero, 2003). Concordet and Ferry (1993) were the first to demonstrate in rats an increase in DNA fragmentation in thymocytes following two run-to-exhaustion protocols separated by 24 hours. Lin et al. (1999) indicated that DNA fragmentation and apoptosis were greater in rats that underwent two consecutive days of heavy exercise in comparison to sedentary controls; 1 hour of exhaustive exercise (80% $VO_2$ max) resulted in decreased circulating lymphocyte numbers and increased apoptotic cells compared to

pre-exercise baseline levels and this effect was not observed with moderate exercise (60% $VO_2$ max).

Exhaustive exercise is coupled with an increase in oxygen consumption that leads to the formation of reactive oxygen species (ROS) (Mooren et al., 2002). ROS generation leads to a decrease (or consumption) of intracellular antioxidant levels (such as glutathione), changes in mitochondrial membrane potential, or DNA damage. Exhausting, aerobic exercise also increases activation in blood lymphocytes of the transcription factor NFκB for the pro-inflammatory cytokine TNF-α (Vider et al., 2001), an increase in capase-3 activity in thymocytes (Patel and Hoffman-Goetz, 2002), and an increase in lymphocytes expressing early markers of apoptosis on their cell surfaces, such as externalization of phosphatidylserine (Hoffman-Goetz and Quadrilatero, 2003). Hoffman-Goetz and Spagnuolo (2007) reported an increased expression of caspase-3 and decreased expression of Bcl-2 proteins after repeated bouts of exhaustive treadmill exercise in young female C57BL/6 mice. Muscle damaging (eccentric) exercise (such as downhill running) increased the expression of TNF-α in both humans and rats (Steinacker et al., 2004) which has the possibility of activating the extrinsic pathway of apoptosis. A similar increase in plasma TNF-α levels, as well as soluble FAS ligand, was observed in men taking part in acute high-intensity exercise (85% $VO_2$ max), with TNF-α levels returning to normal after 72 hours. Ostrowski and colleagues (1999) reported that high-intensity marathon running (median running time 3 hours 27 min) was associated with significant increases in plasma TNF-α, IL-1β, and IL-6 immediately after the race. The elevated levels of these 3 cytokines were also observed in men who participated in a 2 hour cycling challenge at 70% $VO_2$peak, both immediately and 2 hours post-exercise (Starkie et al., 2000). Similar findings were obtained by Zaldivar et al. (2006) in which 30 min of heavy exercise in healthy 18 to 30 year old males led to significant

increases in plasma levels of IL-1α, TNF-α, IL-6, and IFN-γ immediately following the exercise bout. Kimura and colleagues (2001) found that submaximal exercise at 50% $VO_2$max on a cycle ergometer resulted in a significant increase in plasma IFN-γ 2 hours after the challenge, but this change was not observed at 70% $VO_2$max. This finding is partially explained by the fact that exhaustive exercise is thought to suppress IFN production (Kohut et al., 1998; Northoff et al., 1998) and that an increase in IFN-γ may occur more than 2 hours after heavy exercise or not at all. The latter point has yet to be systematically investigated (Kimura et al., 2001).

Although most studies have focused on exercise-associated apoptotic and antioxidant changes systematically, exercise may also affect these processes in the central nervous system. There have been few studies which assessed the effects of an acute exercise stressor on apoptosis or pro-inflammatory cytokines in the brain. Scopel et al. (2006) found that treadmill exercise at 60% $VO_2$max worsens existing damage to hippocampal mitochondria induced by oxygen and glucose depletion in rats. In contrast, Ackigoz et al. (2006) determined that exhaustive treadmill running at a speed of 25 m/min and slope of 5° was not associated with significant lipid peroxidation in the hippocampus, prefrontal cortex, or striatum of Wistar rats. No changes in superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities were observed. Radák et al. (1995) reported that exhaustive treadmill exercise at 24 m/min and 15% incline did not alter levels of SOD, catalase, or GPx in the hippocampus and cerebellum of rats. Furthermore, Somani et al. (1996) showed no changes in SOD activity in the cortex, striatum, cerebellum, medulla, and hypothalamus of rats following treadmill exercise at 100% $VO_{2max}$ for 40 min. These results imply that the brain may be protected (at least to some extent) from the systemic inflammatory damage related to oxidative stress which occurs with high-intensity aerobic exercise.

Packer et al. (2010), in a recent systematic review, suggest that acute exercise may still carry inflammatory "risks" in the CNS. This review found that acute exercise increased pro-inflammatory cytokine levels within central tissues. Steensberg et al. (2006) found that cerebrospinal fluid (CSF) levels of HSP72 (an indicator of oxidative stress) were increased 5-fold in healthy men who underwent 2 hours of cycle ergometry at 60% $VO_{2max}$; no changes in CSF IL-6 concentrations were noted and TNF-α levels remained undetectable before and after the exercise bout. In several studies using animal models, Carmichael et al. (2005; 2006; 2010) found that muscle-damaging downhill treadmill exercise in mice leads to elevations in IL-1β in the cortex and cerebellum through activation of perivascular and meningeal macrophages. Thus, and in contrast to the generally pro-inflammatory and apoptotic responses observed with exercise in the periphery, the direction of effects of acute exercise on CNS brain inflammatory cytokine responses is unclear. In addition, the matter of exercise-induced apoptosis in brain microglial cells has gone unexplored.

### 1.5 Systemic and Central Effects of Exercise Training

Voluntary exercise in rodents has consistently been shown to enhance various components of the innate and acquired immune responses. For example, a 5-6 week freewheel training protocol increased splenic natural killer (NK) cell activity in mice and rats (MacNeil and Hoffman-Goetz, 1993; Jonsdottir et al., 1996). Eight weeks of voluntary activity in mice resulted in greater peritoneal macrophage function in response to the mitogens concanvalin A and phytohaemagglutinin (Suguira, 2000). Campisi and Fleshner (2003) and Fleshner et al. (2002) showed that the immunosuppressive effects of an acute heterotypic stress on the innate immune system can be lessened by voluntary exercise training. *E. coli* induced inflammation was less in

the spleens and dorsal cutaneous surfaces of trained rats after inescapable tail shock compared to sedentary controls.

Freewheel training has been associated with reductions in the levels of the pro-inflammatory cytokines, IL-1 and IL-12, in white adipose tissue of rats (Gomez-Merino et al., 2007) and in TNF-α in intestinal lymphocytes of mice (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). Kohut et al. (2006) found that long-term aerobic exercise (treadmill running, stationary cycling) (45 min/day, 3 times a week over 10 months) in people was associated with reduced circulating levels of IL-6 and IL-18. A recent systematic review of clinical trials reported that exercise training decreases the systemic (circulating) concentration of pro-inflammatory cytokines, soluble TNF-α receptors I and II, and IL-1 receptor antagonist (Haaland et al., 2008).

Physical activity and exercise are thought to have distinct effects on immune mechanisms in the central nervous system as compared with effects systemically. Physical activity may lessen age-related cognitive impairment and dementias by altering the cytokine environment in the CNS (Packer et al., 2010). Chennaoui et al. (2008) found that 7 weeks of treadmill training in rats decreased the expression of the inflammatory cytokine IL-1β in the hippocampus, IL-6 concentration in the cerebellum, and IL-1ra in the pituitary. Nybo et al. (2002) observed a small release of IL-6 in cerebral blood (collected via paired arterial and jugular venous blood samples) of endurance-trained male athletes after 60 min of exercise on a cycle ergometer at 60% maximal oxygen uptake. Upon a second exercise exposure, the concentration of IL-6 released was 5X greater than in the previous bout. As noted earlier, IL-6 is pleiotropic and can act as both a pro- and anti-inflammatory cytokine, depending on the surrounding cytokine environment. An increase in IL-6 following 3 hours of bicycle exercise at 75% $VO_{2max}$, as well as the intravenous

infusion of recombinant human IL-6 over 3 hours, inhibits circulating levels of endotoxin-induced TNF-α in healthy human males (Starkie et al., 2003). Rats given 8 weeks of swim training had elevated plasma IL-6 without increases in either TNF-α or IL-1 (Bonyadi et al, 2009). As such, IL-6 induction may offer protection against TNF-α related changes in post-exercise inflammation and other inflammatory conditions both systemically and centrally through negative feedback to reduce TNF-α secretion. In support of this, sedentary Tg2576 mice (a strain that is highly susceptible to plaque formation and used as a model of AD) had significantly higher baseline levels of hippocampal IL-1β and TNF-α than did the sedentary wild type C57BL/6 mice. After a 3-week running wheel protocol, these differences were no longer significant. In fact, IL-1β levels in the trained Tg2576 mice were significantly lower than in the sedentary Tg2576 group. Sedentary Tg2576 mice also exhibited increased gene expression in the brain for inflammatory markers, including IL1 and TNF-α receptors (Nichol et al., 2008).

Freewheel running by mice for 3 weeks restored Tg2567 cognitive deficits to non-transgenic levels (Parachikova et al., 2008). Interpreting these studies of transgenic mice can be complicated because it is difficult to distinguish age-independent deficits from age-dependent cognitive deficit in these models. Furthermore, Tg2576 mice lack neurofibrillary tangles and are only a partial model of AD (Westerman et al., 2002). Decreases in mRNA and protein levels of TNF-α and IL-1β were found in the forebrain of healthy male Wistar rats after 12 weeks of treadmill running (Ang et al., 2004). There may be some correlation between these findings and those of behavioural studies showing that voluntary exercise training improves performance on cognitive tasks (i.e., spatial memory and maze performance) involving the hippocampus (Anderson et al., 2000; Churchill et al., 2002) as these cytokines inhibit long term potentiation in the dentate gyrus (Butler et al., 2004).

Exercise also protects against oxidative stress pathways in the central nervous system through the regulation of antioxidant enzyme expression (Radák et al., 2007). Female TgCRND8 mice (a strain that exhibits amyloid-β early in life) with access for 4 months to running wheels had in the cerebrum 1) lower levels of oxidative stress biomarkers (nitrotyrosine and protein carbonylation) (ELISA), 2) downregulation of caspase-3 (PCR), and 3) upregulation of SOD1 and SOD2 (Western blot) (Herring et al., 2008). Neither acute nor chronic exercise was associated with significant oxidative stress in rat cortex or hippocampus; however, long term (chronic) exercise increased SOD activity in the hippocampus and may facilitate the reduction in free radical formation observed in other tissues as a result of aerobic training (Aksu et al., 2009). Navarro et al. (2004) found an increase in whole brain Mn-SOD and Cu-SOD activity as a result of treadmill training in aged mice. Hippocampal and cortical glutathione peroxidise (GPx) activity increased in aging rats in response to 12 weeks of swim training compared to sedentary controls (Devi et al., 2004; Devi and Kiran, 2004). Similarly, Somani et al. (1996) found an increase in GPx activity in rat hypothalamus and cortex after a 6.5 week treadmill exercise protocol. It may be possible that the cytokine and antioxidant changes observed in long-term exercise training in systemic lymphocytes and other tissues (spleen, thymus, and muscle) produce similar changes in the brain.

### 1.6 Conclusion

The literature indicates that 1) exhaustive exercise increases oxidative stress, apoptosis, and pro-inflammatory cytokine expression systemically as well as centrally and 2) voluntary exercise enhances immune function against acute stressors, decreases pro-inflammatory cytokine expression, enhances immune cell proliferation, and reduces oxidative stress by enhancing the

activity or level of antioxidant enzymes. These effects have been documented in systemic organs and tissues, but have not been systematically evaluated for components of the central nervous system. The animal studies which described outcomes of exercise training on pro-inflammatory cytokine expression and antioxidant activity in the brain have significant limitations, especially with respect to documentation of exercise/training effects. In fact, none of the "training" studies document whether training effects actually occurred. Moreover, training periods and protocols varied from study to study (ranging from 10 days to 4 months and voluntary vs. forced training) adding to the difficulty in making generalizations about duration and type of exercise training on the collective outcome measures. Studies involving transgenic mice do not reflect what occurs in healthy animals, much less human populations. It is, thus, important to design experiments that avoid these shortcomings and provide a clearer understanding of the impact of acute exhaustive exercise and long-term exercise training on pro-inflammatory cytokines and apoptosis in the brain.

# CHAPTER 2:
# RESEARCH OBJECTIVES, THESIS COMPONENTS AND RATIONALE

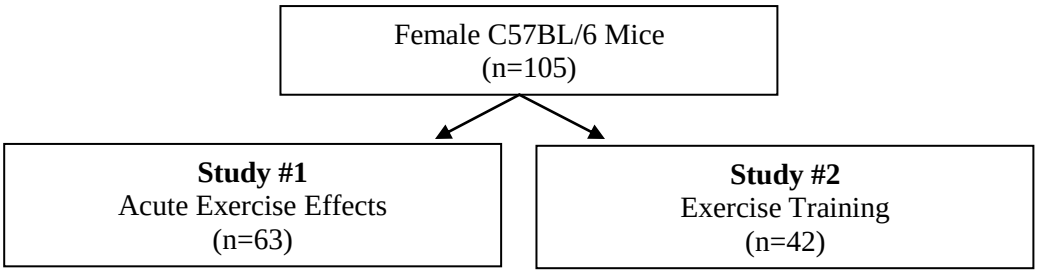
## 2.1 Research Objectives

The first objective of the thesis experiments was to describe the effects of a single bout of acute exhaustive exercise on the concentration of pro-inflammatory cytokines and percentage of apoptotic cells within the hippocampi of healthy female C57BL/6 mice, and to compare these findings to those in systemic tissues (utilizing an internal control and previous studies). The second objective was to describe the effects of long-term voluntary exercise training on the concentration of pro- and anti-inflammatory cytokines and percentage of apoptotic cells within the hippocampi of healthy female C57BL/6 mice. In addition, differences in cell phenotypes as a result of training were to be assessed. The overall goal of this research was to describe the impact of two different exercise modalities on cytokine expression and apoptosis and to consider the results with regard to possible implications for cognitive decline. These experiments were considered hypothesis-generating.

## 2.2 Thesis Components and Rationale

As outlined in **Figure 1**, two separate studies were conducted to address the research objectives.

**Figure 1.** Thesis study components



### 2.2.1 Study #1

In Study #1, the impact of acute exhaustive exercise in female C57BL/6 mice on pro-inflammatory cytokine expression and the apoptotic status of cells within the hippocampus were examined. In addition, pro-inflammatory cytokine expression in the intestinal lymphocytes of these mice was also considered as a positive (internal) control, in order to compare central versus systemic differences. Intestinal lymphocyte cytokine responses to exercise in C57BL/6 mice have been repeatedly described by this (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2008) and other (Rogers et al., 2008) laboratories.

#### *2.2.1.1 Research Questions*

1) Does acute exhaustive exercise in female C57BL/6 mice affect pro-inflammatory cytokine expression in the hippocampus and intestinal lymphocytes?

2) Does acute exhaustive exercise in female C57BL/6 mice affect apoptotic status of immune cells within the hippocampus?

#### *2.2.1.2 Rationale*

Acute exercise leads to increases in oxygen consumption, which then results in the formation of ROS and consumption of intracellular antioxidant levels, alterations in mitochondrial membrane potential, and DNA damage leading to apoptosis (Hoffman-Goetz et al., 2005; Mooren et al., 2002; Phaneuf and Leeuwenburgh, 2001). In addition to changes in oxygen metabolism, acute exercise alters systemic cytokine balance. For example, physical activity at intensities greater than 70% $VO_{2max}$ increases the levels of TNF-α (a cytokine that can activate apoptosis) in plasma (Ostrowski et al., 1999; Steinacker et al., 2004; Starkie et al., 2000;

Zaldivar et al., 2006) and colonic lymphocytes (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). Similar exercise-induced increases were also observed for the cytokines IL-1β and IL-6 (Ostrowski et al., 1999; Starkie et al., 2000; Zaldivar et al., 2006) suggesting that strenuous exercise (and especially eccentric exercise with muscle damage) results in inflammation.

Though there is ample literature that has focused on acute exercise effects on cytokines and apoptosis in systemic tissues, very few studies describe these phenomena in central compartments, including the hippocampus, a primary tissue involved in cognition, memory, and the stress response (Cotman et al., 2007; Neves et al., 2008).

#### *2.2.1.3 Hypotheses*

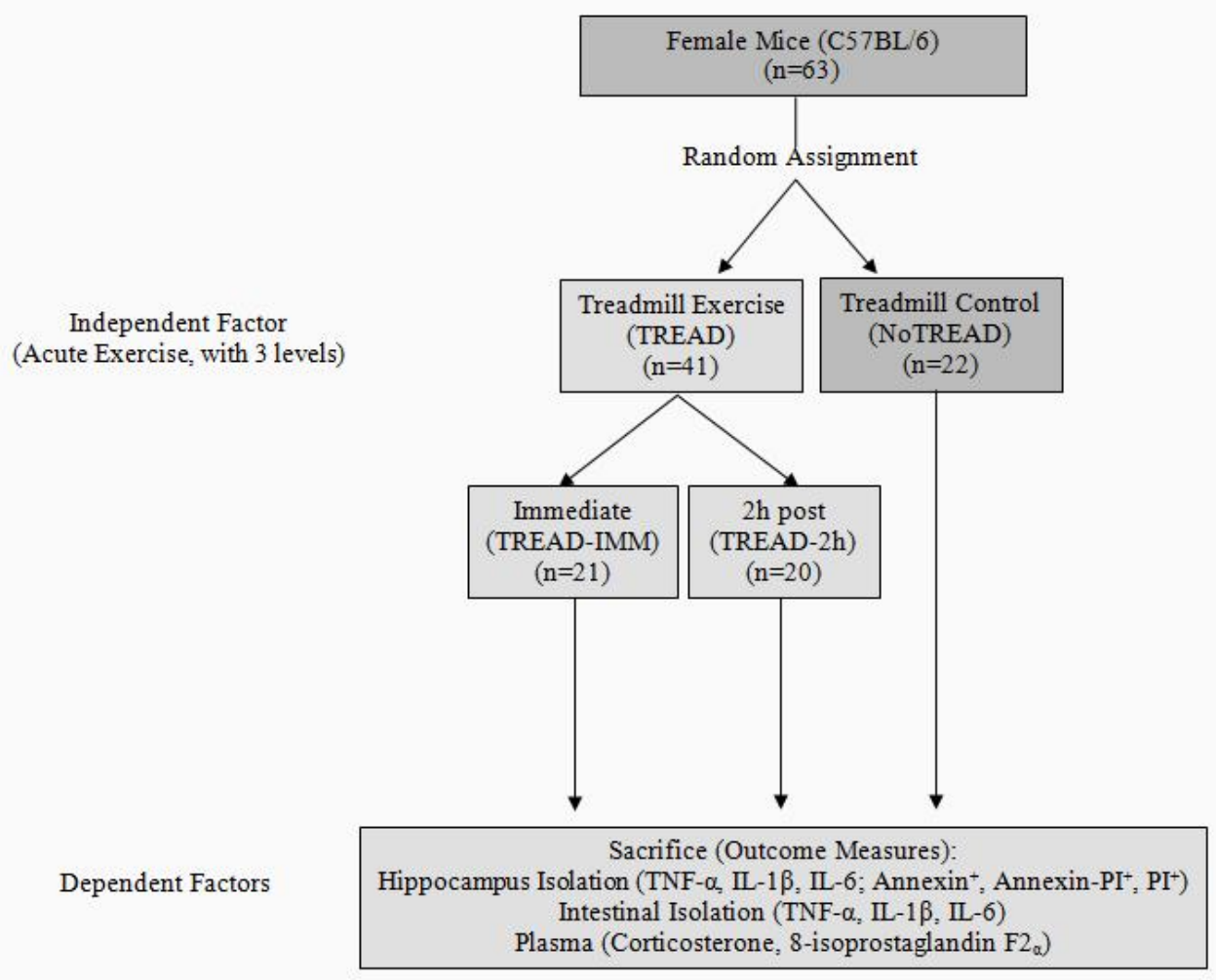
1) Acute exhaustive exercise will be associated with an increase in the expression of both hippocampal and intestinal lymphocyte pro-inflammatory (TNF-α, IL-1β, IL-6) cytokines.
2) Acute exhaustive exercise will be associated with an increase in the number of cells within the hippocampus undergoing apoptosis and necrosis as measured by external expression of Annexin and PI markers.

#### *2.2.1.4 Study Design*

The experiment that was carried out tested the effects of an acute bout of exercise (oxidative stress challenge) on the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and markers of apoptosis ($Annexin^+$, $Annexin\text{-}PI^+$, $PI^+$) in mouse hippocampus. Using an analysis of variance model (one way), the independent factor was acute treadmill exercise, with 3 levels: sacrifice immediately after exercise, sacrifice 2 hours after exercise, and no treadmill exercise. The dependent factors were: 1) hippocampal levels of TNF-α, IL-1β, and IL-6, and 2)

percentages of Annexin$^{+}$, Annexin-PI$^{+}$, and PI$^{+}$ hippocampal cells. Plasma levels of corticosterone and 8-isoprostaglandin F2$_{\alpha}$ were also evaluated as indicators of oxidative stress. The rationale for obtaining sample at more than one time point post oxidative exercise challenge is because of what is known about the transcription kinetics of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6: there is an increase in NF-κB protein concentration in splenic lymphocytes 0.5h to 1.5h after LPS stimulation and this is followed by an elevation in TNF-α and IL-6 mRNA that peaks 2h later. IL-1β mRNA concentration is also increased 2h post exercise and remains so from 2h to 8h post stimulation (Zhou et al., 2003). As such, these time points are appropriate to observe possible changes in the respective cytokine expression. The design for Study #1 is outlined in **Figure 2**.

**Figure 2.** Study #1 Experimental Design



### 2.2.2 Study #2

In Study #2, the impact of long-term voluntary exercise training in female C57BL/6 mice on pro- and anti-inflammatory cytokine expression and phenotypes and apoptotic status of cells within the hippocampus were examined.

#### *2.2.2.1 Research Questions*

1) Does long-term voluntary free wheel running in female C57BL/6 mice affect pro-(TNF-α, IL-1β, IL-12) and anti-(IL-10, IL-1ra) inflammatory, and pleiotropic (IL-6) cytokine expression in the hippocampus?

2) Does long-term voluntary free wheel running in female C57BL/6 mice affect the quantification of phenotypes and apoptotic status of immune cells within the hippocampus?

#### *2.2.2.2 Rationale*

Physical activity may buffer age-related cognitive impairment and dementias by altering the cytokine environment in the CNS (Packer et al., 2010). Seven weeks of forced treadmill training in rats reduced the expression of IL-1β in hippocampus, IL-6 in cerebellum, and IL-1ra in pituitary (Chennaoui et al., 2008). In endurance-trained athletes, increases in IL-6 were found in cerebral blood after 60 min of exercise at 60% $VO_{2max}$. A second bout led to a five-fold greater release of IL-6 (Nybo et al., 2002). IL-6 is pleiotropic acting as a pro- or anti- inflammatory cytokine (Corwin et al, 2000). Rats given 8 weeks of swim training had elevated plasma IL-6 without increases in either TNF-α or IL-1 (Bonyadi et al., 2009). Whereas exercise-induced IL-6 may protect against TNF-α related changes through negative feedback, its direct role in central inflammation has not been characterized. Higher baseline concentrations of hippocampal IL-1β

and TNF-α were found in sedentary Tg2576 mice (an animal model of AD) than in wild-type controls (Nichol et al., 2008). After 3 weeks of freewheel running these cytokine differences between strains were no longer significant. Freewheel running in Tg2576 mice also reduced cognitive deficits to wild-type levels (Parachikova et al., 2008). Twelve weeks of forced treadmill running decreased mRNA and protein levels of TNF-α and IL-1β in healthy male rat forebrains (Ang et al., 2004). Voluntary exercise training improved performance on cognitive tasks related to the hippocampus such as spatial memory and maze performance (Churchill et al., 2002); elevated TNF-α and IL-1β inhibit memory formation (Butler et al., 2004). No studies thus far have examined voluntary training in healthy animals in relation to the hippocampal cytokine environment that has been observed in diseased and aged mouse models. The cytokines chosen included the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-12) implicated in cognitive decline and dementia (Maccioni et al., 2009). These cytokines are produced in the CNS by microglia (Hanisch, 2002). IL-10 and IL-1ra expression in the hippocampus were examined as anti-inflammatory cytokines (Moore et al, 2001) that counter the effects of TNF-α and IL-1β, respectively. IL-10 is also produced by microglia, as is IL-1ra (Hanisch, 2002). The expression of the pleiotropic cytokine IL-6 was assessed as it can exacerbate or ameliorate the effects of pro-inflammatory cytokines depending on the cytokine milieu.

#### *2.2.2.3 Hypotheses*

1) Long-term voluntary wheel running will be associated with a decrease in the expression of hippocampal pro-inflammatory cytokines (TNF-α, IL-1β, IL-12).

2) Long-term voluntary wheel running will be associated with an increase in the expression of hippocampal anti-inflammatory cytokines (IL-10, IL-1ra).

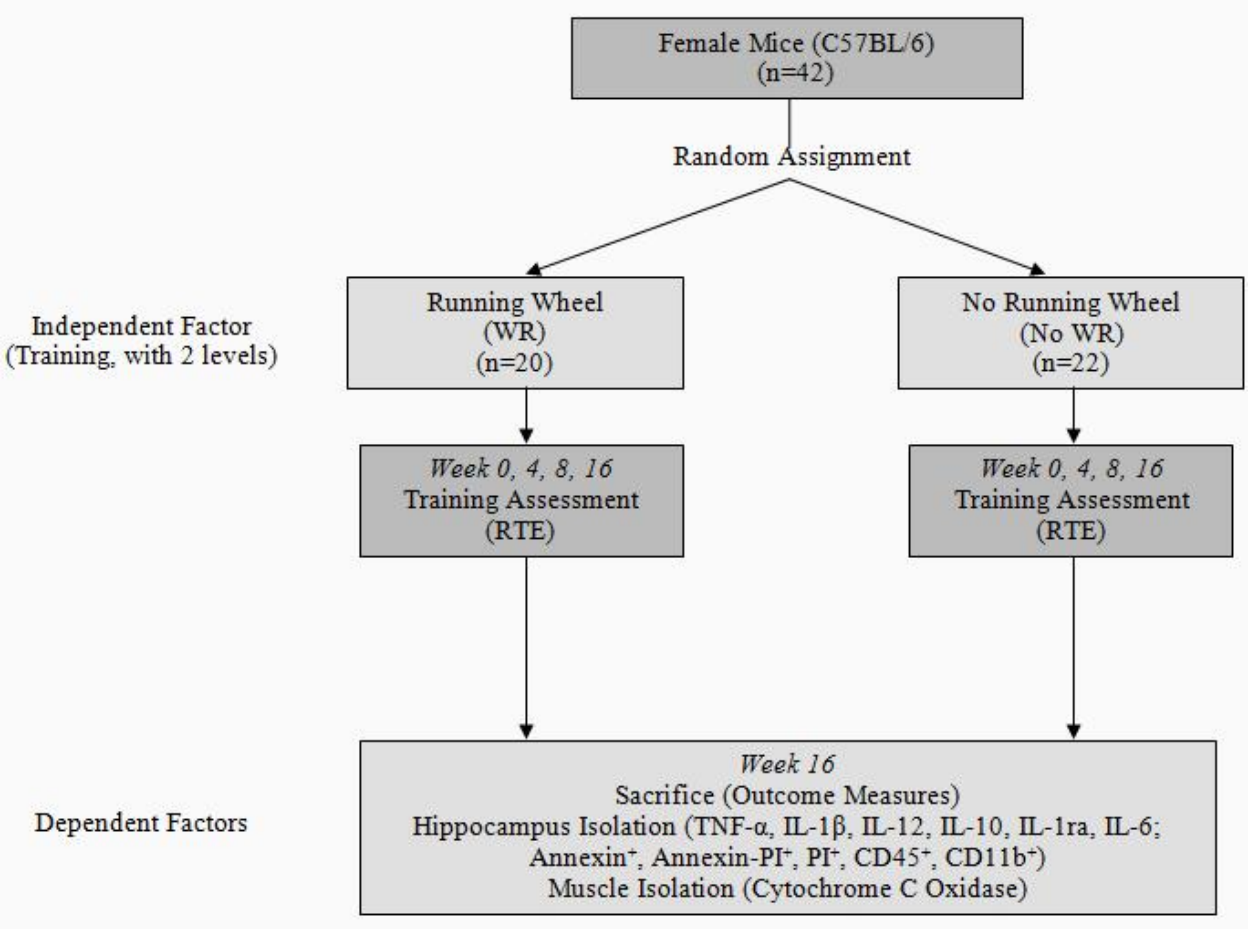
3) Long-term voluntary wheel running will be associated with either an increase or decrease in the expression of hippocampal IL-6. Since this cytokine is pleiotropic, the direction of effect is unclear.

4) Long-term voluntary wheel running will be associated with a decrease in the number of cells within the hippocampus undergoing apoptosis and necrosis as measured by external expression of Annexin and PI markers.

#### *2.2.2.4 Study Design*

The experiment carried out tested the effects of long-term voluntary exercise training on the expression of pro-inflammatory (TNF-α, IL-1β, IL-12), anti-inflammatory (IL-10, IL-1ra), and pleiotropic (IL-6) cytokines in mouse hippocampus. The study also assessed the apoptotic status ($Annexin^+$, $Annexin\text{-}PI^+$, $PI^+$) of mouse hippocampal cells and the percentage of $CD45^+$ and $CD11b^+$ cell subtypes. Using an analysis of variance design, the independent factor was training (wheel running vs. no wheel running) condition and the dependent factors included the outcome measures listed above. The design for Study #2 is outlined in **Figure 3**.

**Figure 3.** Study #2 Experimental Design



# CHAPTER 3:
# STUDY #1: ACUTE EXERCISE AND INFLAMMATORY CYTOKINE AND APOPTOTIC RESPONSES IN THE MOUSE HIPPOCAMPUS AND INTESTINAL LYMPHOCYTES

The work presented in this chapter has been accepted for publication as:

Pervaiz N. and Hoffman-Goetz, L. (2011). Immune cell inflammatory cytokine responses differ between central and systemic compartments in response to acute exercise in mice. *Exercise Immunology Review*; accepted for publication 28 April 2011.

## 3.1 Chapter Overview / Abstract

Exhaustive exercise induces apoptosis and oxidative stress in systemic organs and tissues and is associated with increased levels of pro-inflammatory cytokines. The effects of acute exercise on cytokine expression and apoptosis of immune cells in the central nervous system (CNS) have not been well characterized. We investigated the effects of a single bout of strenuous exercise on the expression of TNF-α, IL-6, and IL-β, as well as the apoptotic status of cells in the hippocampus of healthy mice. To compare central vs. systemic differences, cytokine expression in the intestinal lymphocytes of a subset of mice were also assessed. Female C57BL/6 mice were divided into three groups: sedentary controls (NOTREAD) (n = 22), treadmill exercise with immediate sacrifice (TREAD-Imm) (n = 21), or treadmill exercise with sacrifice after 2 hours (TREAD-2h) (n = 20). TNF-α, IL-6, and IL-1β expression in the hippocampus and intestinal lymphocytes were measured by Western blot analysis. Percentages of hippocampal cells undergoing apoptosis (Annexin$^{+}$) or necrosis (Propidium Iodide$^{+}$) were determined through flow cytometry. Plasma levels of 8-isoprostane and corticosterone were measured using commercially available EIA kits. Acute treadmill exercise lead to significant decreases in TNF-α ($p<0.05$) and increases in IL-6 ($p<0.05$) expression in the hippocampus of healthy mice. No effects of acute exercise on the apoptotic status of hippocampal cells were observed. In intestinal lymphocytes,

the exercise bout lead to significant increases in TNF-α ($p<0.05$), IL-6 ($p<0.05$), and IL-1β ($p<0.05$). Acute exercise was associated with a significant increase in both plasma 8-isoprostane ($p<0.05$) and corticosterone ($p<0.05$) levels. Acute exercise differentially affects the pattern of pro-inflammatory cytokine expression in the hippocampus compared to intestinal lymphocytes and, further, does not induce apoptosis in hippocampal cells.

### 3.2 Introduction

Exercise can have a positive and a negative impact on the immune system depending on its duration and intensity. Acute exercise is associated with reduced immune function and increased risk of infection (Kakanis et al., 2010; Mars et al., 1998; Nieman, 2003). Moreover, exhaustive exercise leads to leukocytosis immediately followed by lymphocytopenia (Pedersen and Hoffman-Goetz, 2000), which may be due in part to exercise-induced DNA fragmentation and apoptosis (Lin et al., 1999; Mooren et al., 2002; Navalta et al., 2010). Many of these effects are thought to be the result of increased oxidative stress in the affected tissues. Oxidative stress is an imbalance between endogenous antioxidants, such as superoxide dismutase and glutathione peroxidase, and reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radical (Sies, 1997). Acute exercise leads to increases in oxygen consumption, which then results in the formation of ROS and consumption of intracellular antioxidant levels, alterations in mitochondrial membrane potential, and DNA damage leading to apoptosis (Mooren et al., 2002; Phaneuf and Leeuwenburgh, 2001). Essential components of apoptotic cell death are caspase-3 and caspase-7 (Adrain et al., 2005; Degterev et al., 2003) both of which are significantly elevated after prolonged exhaustive exercise (Hoffman-Goetz and Spagnuolo, 2007; Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). In addition to changes in oxygen

metabolism, acute exercise alters the cytokine balance systemically. For example, physical activity at intensities greater than 70% $VO_{2max}$ increases the levels of TNF-α (a cytokine that can activate apoptosis) in plasma (Ostrowski et al., 1999; Starkie et al., 2000; Steinacker et al., 2004; Zaldivar et al., 2006) and colonic lymphocytes (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). Similar exercise-induced increases were also observed for the cytokines IL-1β and IL-6 (Ostrowski et al., 1999; Starkie et al., 2000; Zaldivar et al., 2006) suggesting that strenuous exercise (and especially eccentric exercise with muscle damage) results in inflammation.

Few studies, however, have determined if acute exercise affects pro-inflammatory cytokine status in the central nervous system (CNS) and none have examined its effects on apoptosis of immune cells in the brain. Scopel et al. (2006) found that in rats two weeks of treadmill exercise at 60% $VO_{2max}$ worsens existing damage to hippocampal mitochondria induced by in vitro oxygen and glucose depletion. In contrast, Ackigoz et al. (2006) reported that exhaustive treadmill running (25 m/min, 5° slope) in Wistar rats did change superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities or thiobarbituric acid reactive susbstance (TBARS) levels in the hippocampus, prefrontal cortex, or striatum; this exercise protocol was therefore not associated with significant lipid peroxidation in these brain compartments. In an older study, Radák et al. (1995) reported that exhaustive treadmill exercise at 24 m/min and 15% incline did not alter levels of SOD, catalase, GPx or TBARS in the hippocampus and cerebellum of rats. Somani et al. (1996) reported no changes in SOD activity in the cortex, striatum, cerebellum, medulla, and hypothalamus of rats following treadmill exercise at 100% $VO_{2max}$ for 40 min. Together these results imply that the brain is protected from the systemic inflammatory damage related to oxidative stress which occurs with high-intensity aerobic exercise.

However, a recent review by Packer et al. (2010) suggests that acute exercise may still pose an inflammatory "threat" to the CNS. Steensberg et al. (2006) found that cerebrospinal fluid (CSF) levels of HSP72 (an indicator of oxidative stress) were increased 5-fold in healthy men who underwent 2 hours of cycle ergometry; no changes in CSF IL-6 concentrations were noted and TNF-α levels remained undetectable before and after the exercise bout. In contrast, cerebral IL-6 levels (as measured by internal jugular venous to arterial differences) were significantly elevated in men who participated in two successive 60 min bouts of cycle ergometry (Nybo et al., 2002). Animal studies (Carmichael et al., 2005; Carmichael et al., 2006; Carmichael et al., 2010) also indicate that muscle-damaging downhill treadmill exercise in mice leads to elevations in IL-1β in the cortex and cerebellum through activation of perivascular and meningeal macrophages. Thus, the direction of effects of exhaustive exercise on CNS brain inflammatory cytokine responses is unclear and some of this variation may be due to inter-species differences. This is in contrast to the generally pro-inflammatory and apoptotic responses observed in the peripheral compartments after acute exercise. Moreover, the issue of exercise-induced apoptosis in brain immune cells has gone unexplored.

The purpose of this study was to examine the effects of a single bout of acute, strenuous exercise on the expression of classical pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and apoptotic status in the hippocampus of healthy mice. This brain region was chosen because it is involved in cognition, memory, and the stress response (Cotman et al., 2007; Neves et al., 2008). In addition, plasma levels of 8-isoprostaglandin $F2_{\alpha}$ and corticosterone were measured to determine whether the exercise protocol was sufficient to elicit a stress response. A second purpose was to compare these pro-inflammatory cytokine responses centrally vs. peripherally following the acute exercise challenge. We hypothesized that exposure to a strenuous bout of

aerobic exercise would lead to increases in the expression of hippocampal TNF-α, IL-1-β, and IL-6, coupled with increases in the apoptotic status of hippocampus cells, in a manner similar to intestinal lymphocytes (i.e., a peripheral lymphoid compartment). Through this investigation, the relationship between indicators of exercise-induced oxidative stress in the plasma and apoptosis and central inflammatory processes in hippocampal immune cells was explored.

## 3.3 Methods

### 3.3.1 Animals

Female C57BL/6 mice (n = 63) (Harlan Indianapolis, IN, USA), 4-5 months of age, were housed in individual cages at 21 ± 1 °C, on a 12/12 h reversed light/dark cycle. *Ad libitum* access to a standard rodent diet (Lab Rodent Chow, PMI Feeds, Richmond, IN, USA) and tap water were provided. The experimental procedures adhered to the guidelines established by the Canadian Council on Animal Care and were approved by the University Animal Research Ethics Committee.

### 3.3.2 Exercise Protocol

Mice were matched on weight and randomly assigned to one of three treadmill exercise conditions: (1) treadmill running (90 min, 2° slope) with sacrifice immediately after exercise (TREAD-Imm; n = 21), treadmill running (same duration, speed and grade) with sacrifice 2 h after exercise (TREAD-2h; n = 20), and control animals that were exposed to treadmill noise and vibrations for 90 min, without running, before sacrifice (NOTREAD; n = 22). The running protocol consisted of a 10 min warm-up, 30 min at 22m/min, 30 min at 25 m/min, 30 min at 28 m/min, and a 5 min deceleration to 0 m/min on an Omni-Max metabolic treadmill (Omni Tech

Electronics, Columbus, OH, USA). All running took place at the beginning of the dark cycle (between 7 and 9 am). Mice were motivated to run by gentle prodding using a nylon brush and were fasted overnight prior to the start of exercise.

### 3.3.3 Plasma Collection

Mice were sacrificed by sodium pentobarbital overdose (0.6-0.8 cc per mouse, i.p.). After confirmation of a negative toe pinch response, skin was grasped at the mid-ventral position of the body and an incision was made across the chest to expose the rib cage. This was cut to expose the heart, and blood was collected immediately using a heparinized syringe. Blood was centrifuged at 1500 *g* for 6 min and plasma was collected and stored at -80 °C until analysis of corticosterone and 8-isoprostaglandin $F2_{\alpha}$.

### 3.3.4 Hippocampus Removal and Single Cell Suspensions

Excision of mouse hippocampi was performed according to Hassan et al. (1991). All brain dissections took place on an ice-mounted stage. Decapitation was completed immediately following sacrifice. A midline incision was made along the skull, granting access to underlying structures, and the brain was excised and washed in cold PBS (0.5% BSA/PBS), transferred to the dissection stage and bisected at the midline. A clean number-1 paintbrush was inserted into the fissure beneath the dorsal cerebral cortex, and the hippocampi from both hemispheres were visualized, isolated, and placed in 1.5 mL RPMI (1640, 2.5% FCS), pressed through a 70 μm cell strainer, and centrifuged at 1500 RPM for 5 min. Cells were resuspended in 5 mL RPMI at room temperature, layered over 5 mL of Lympholyte M (Cedarlane Laboratories, Hornby, ON, Canada), and centrifuged at 1250 *g* for 20 min. Cells at the interface were recovered, washed,

suspended in 300 μL PBS and counted by microscopy. Cell samples were stored at -80 °C until analysis.

### 3.3.5 Assessments of Apoptosis of Hippocampal Cells

Immediately following the preparation of hippocampal single cell suspensions, 1 x $10^5$ hippocampal cells were incubated for 15 min in the dark with 2.5 μl of Annexin V-FITC (Pharmingen, San Diego, CA, USA), 2.5 μl of Propidium Iodide (PI) (Sigma Chemical, St. Louis, MO, USA), and 100 μl of Annexin binding buffer, in order to obtain percentages of apoptotic and necrotic cells as has been previously described (18).

### 3.3.6 Protein Determination and Western Blot Analysis of Hippocampal Cell and Intestinal Lymphocyte TNF-α, IL-1β, and IL-6

Hippocampal cells were lysed, placed on ice for 45 min, and the lysates centrifuged (10,000 *g*, 15 min) for protein determination by bicinchoninic acid (BCA) assay. Protein supernatant (40 μg) and molecular weight markers (Full Range Rainbow, Amersham Biosciences, Buckinghamshire, UK) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12-15%), transferred to a polyvinyldiene fluoride (PVDF) membrane, and stained with Ponceau S to confirm quality of transfer and equal loading. Membranes were then incubated with primary antibody for 1 h (1:200 in 10% FBS-TBST): TNF-α (sc-1350), IL-1β (sc-71435), or IL-6 (sc-1265) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and subsequent incubation with secondary antibody for 1 h: horseradish peroxidase-conjugated anti-goat (TNF-α) or anti-mouse (IL-1β, IL-6) at 1:2000 in FBS-TBST. Protein quantity was determined using ECL Plus Western blotting detection reagent (Amersham

Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-imaging system (Cambridge, UK).

Intestinal lymphocytes were collected as described (Hoffman-Goetz et al., 2010) from a subset of mice (n = 30) for comparison with hippocampal cells and to be used as internal controls to document whether the acute exercise protocol led to previously observed systemic cytokine changes in TNF-α, IL-1β, and IL-6. Western blot analysis of intestinal lymphocytes was performed as described above for hippocampus.

### 3.3.7 Corticosterone Assessment

Plasma samples were assessed for corticosterone levels using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). Purification requirements of the samples were determined using the cold spike protocol, and concentration of corticosterone was measured at 412 nm using a PowerWave 340 microplate spectrophotometer (Biotek Instruments, Vermont, USA), according to the manufacturer's protocol. All samples were run in duplicate. The intra-assay % CV was 6.3% and the lower detection limit was 30 pg/mL.

### 3.3.8 8-isoprostaglandin $F2_{\alpha}$ Assessment

Plasma 8-isoprostaglandin $F2_{\alpha}$ (8-isoprostane) levels were quantified using a direct EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Samples were hydrolyzed (25 µl 10 N NaOH: 100 µl sample) at 45 °C for 2 h, neutralized with 12 N HCl, centrifuged (5 min, 14,000 *g*), and supernatant incubated with 8-isoprostane antibody for 24 h at 4 °C. Absorbance was measured at 405 nm at room temperature (PowerWave 340 microplate spectrophotometer, Biotek

Instruments, Vermont, USA). All samples were run in duplicate. The intra-assay % CV was 8.6% and the lower limit of detection was 2.7 pg/ml.

### 3.3.9 Statistical Analysis

Cytokine concentrations and measures of apoptosis and necrosis were analyzed using one-way ANOVA with acute treadmill exercise challenge (3 levels: NOTREAD, TREAD-IMM, TREAD-2h) as the independent factor and cytokine protein expression, % $Annexin^{+}$, and % $PI^{+}$ as the dependent factors (SPSS Version 18; Chicago, IL, USA). Corticosterone and 8-isoprostane levels were analyzed using one-way ANOVA with acute treadmill exercise challenge (2 levels: NOTREAD, TREAD-IMM) as the independent factor and corticosterone and 8-isoprostane concentrations as the dependent factor (SPSS Version 18; Chicago, IL, USA). Post hoc analysis was determined with Tukey's HSD test and all ANOVAs results were checked for homogeneity of variance with h Levene's test). Significant difference from chance alone was accepted if $p < 0.05$; all values are expressed as group means ± 1 SEM for respective units.

## 3.4 Results

### 3.4.1 Body Mass

At sacrifice, the mean body mass of mice was 26.7 ± 0.8 g (NOTREAD: 27.7 ± 0.7 g; TREAD-Imm 26.1 ± 0.8 g; TREAD-2h: 26.2 ± 0.8 g) and these groups did not differ ($F$ (2, 62) = 1.309, n.s.).

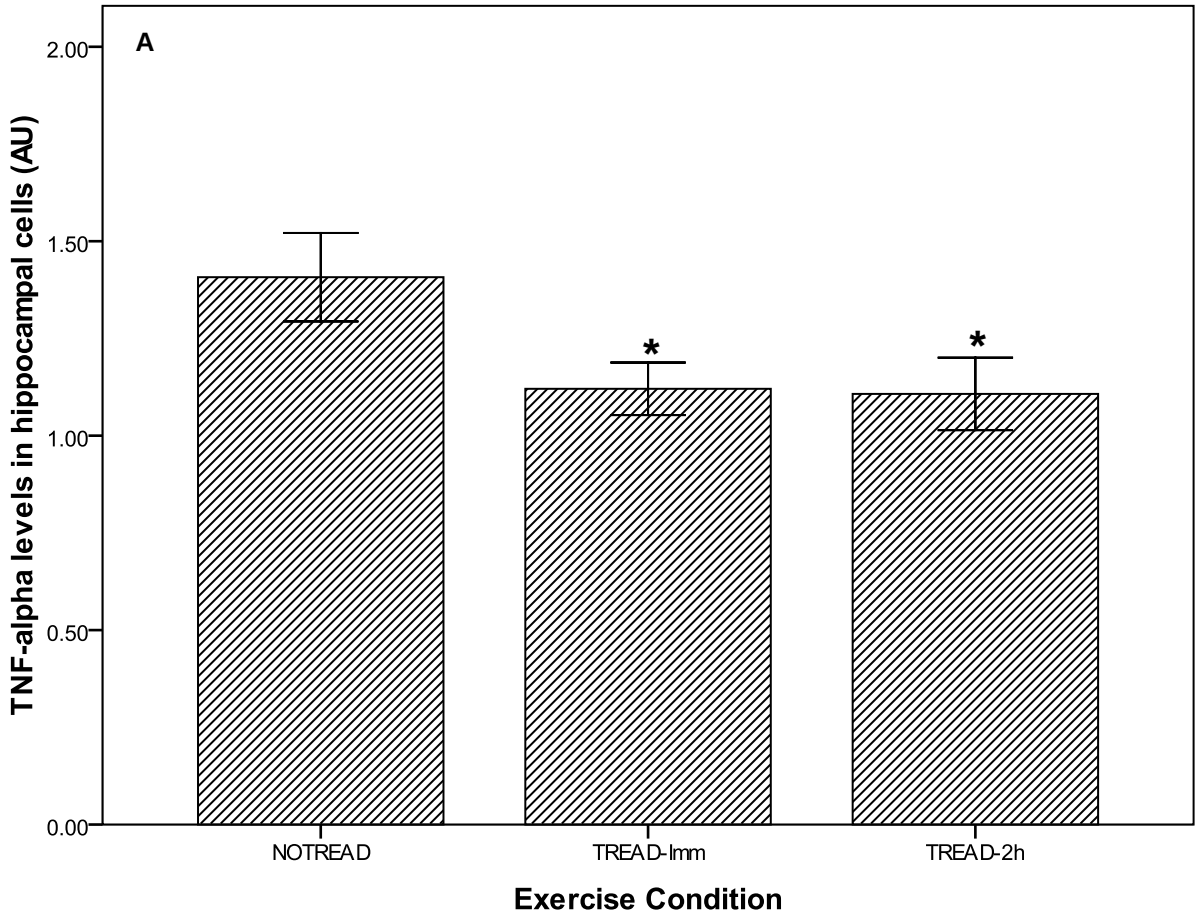
### 3.4.2 Apoptosis

No differences were observed with respect to the percentage of Annexin$^+$ ($F$ (2, 62) = 0.231, n.s.) or PI$^+$ ($F$ (2, 62) = 0.696, n.s.) hippocampal cells between the NOTREAD (Annexin$^+$: 4.0 ± 0.3 %; PI$^+$: 3.6 ± 0.3 %), TREAD-IMM (Annexin$^+$: 4.2 ± 0.3 %; PI$^+$: 4.0 ± 0.3 %), and TREAD-2h (Annexin$^+$: 3.9 ± 0.3 %; PI$^+$: 3.8 ± 0.3 %) mice.

### 3.4.3 Hippocampal Cytokines

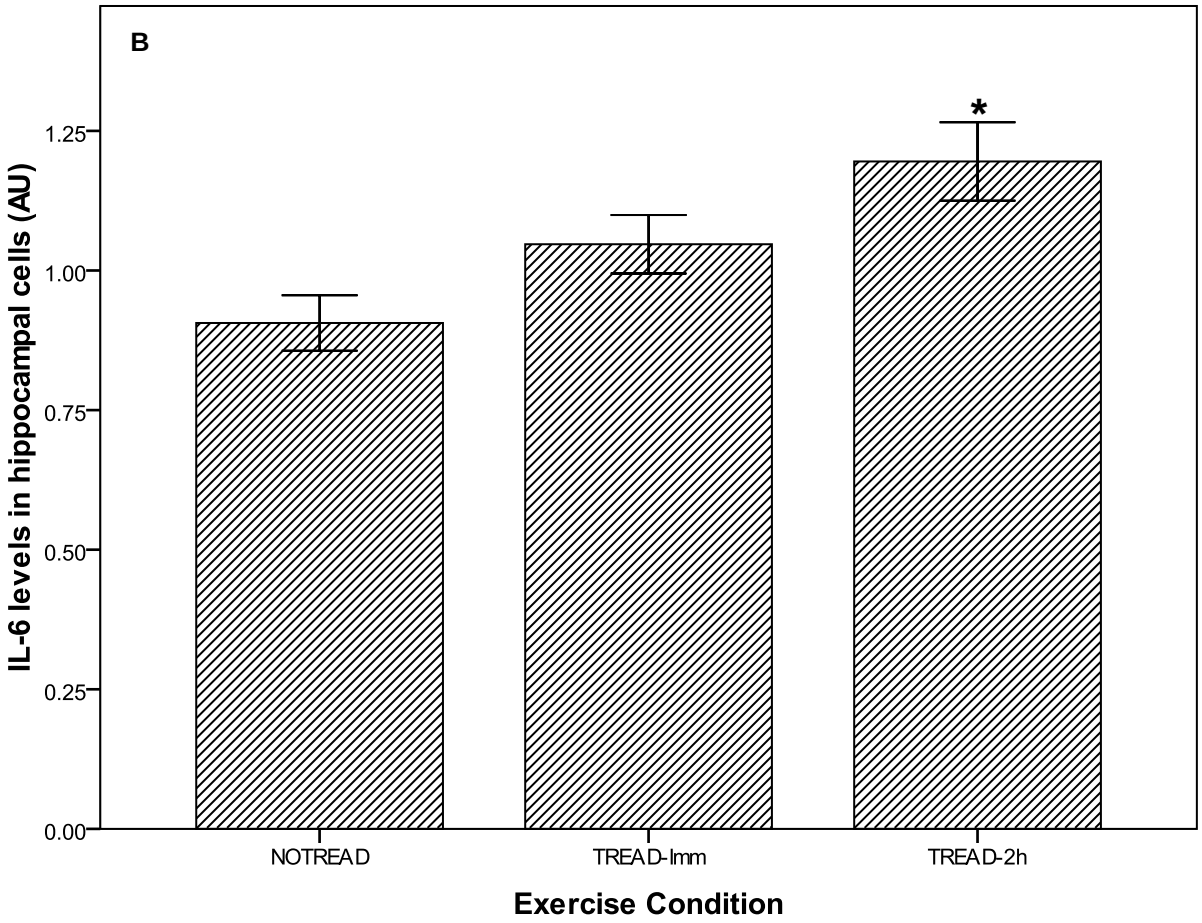
**Figure 4** shows the effects of acute treadmill exercise on the expression of pro-inflammatory cytokines (in Arbitrary Units [AU]) in the hippocampus. There was a significant effect of acute treadmill exercise on TNF-α expression ($F$ (2, 58) = 3.31, $p < 0.05$) and expression of this cytokine was lower in TREAD-IMM (1.1 ± 0.1 AU) and TREAD-2h (1.1 ± 0.1 AU) compared to NOTREAD (1.4 ± 0.1 AU) mice. Acute exercise significantly affected IL-6 expression in mouse hippocampus ($F$ (2, 58) = 6.23, $p < 0.05$) with this cytokine being higher in TREAD-2h (1.2 ± 0.06 AU) compared to NOTREAD (0.9 ± 0.06 AU) and TREAD-Imm (1.0 ± 0.06 AU) animals. Expression of IL-1β in mouse hippocampus did not differ as a function of acute treadmill exercise ($F$ (2, 58) = 0.23, n.s.).

**Figure 4A.** Cytokine expression in hippocampal cells of mice



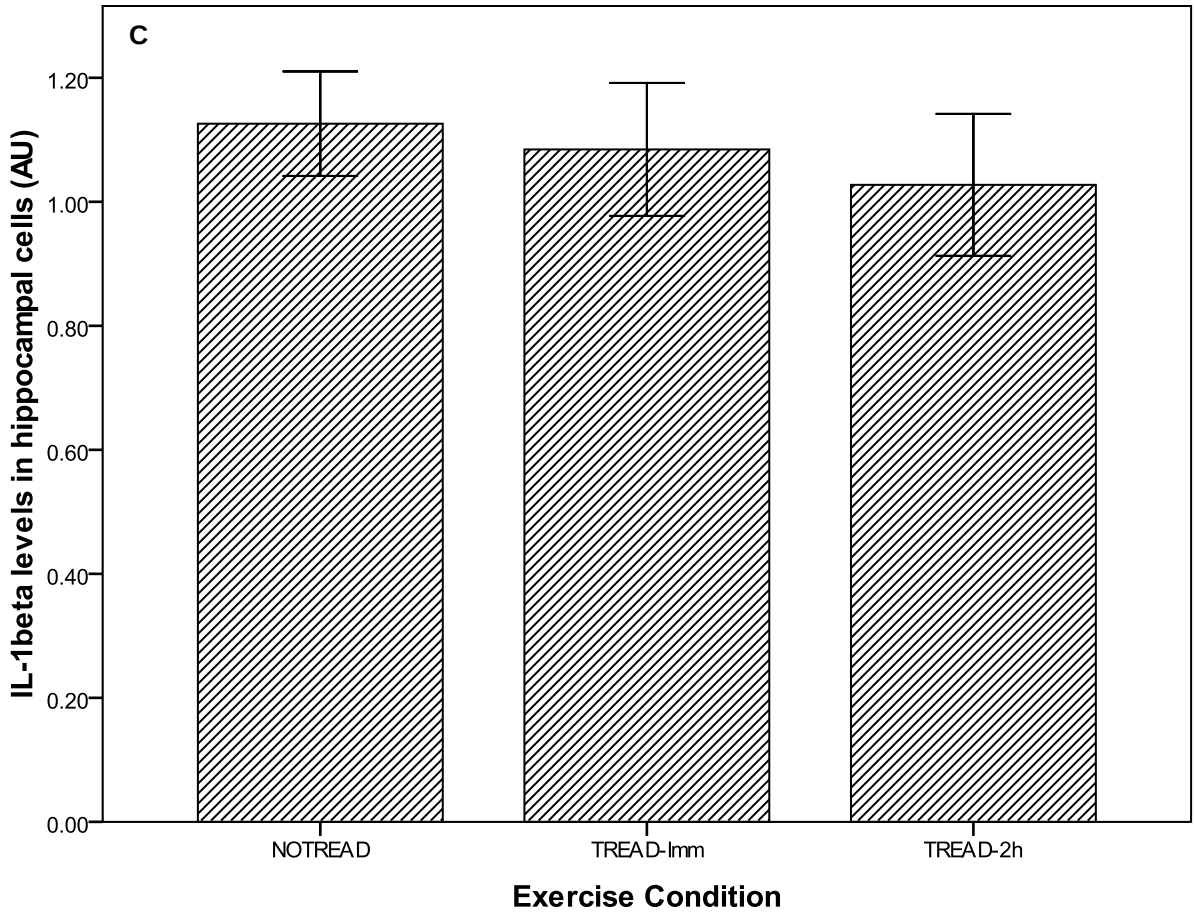

Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in hippocampal cells of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel A*: TNF-α expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

**Figure 4B.** Cytokine expression in hippocampal cells of mice




Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in hippocampal cells of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel B*: IL-6 expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

**Figure 4C.** Cytokine expression in hippocampal cells of mice



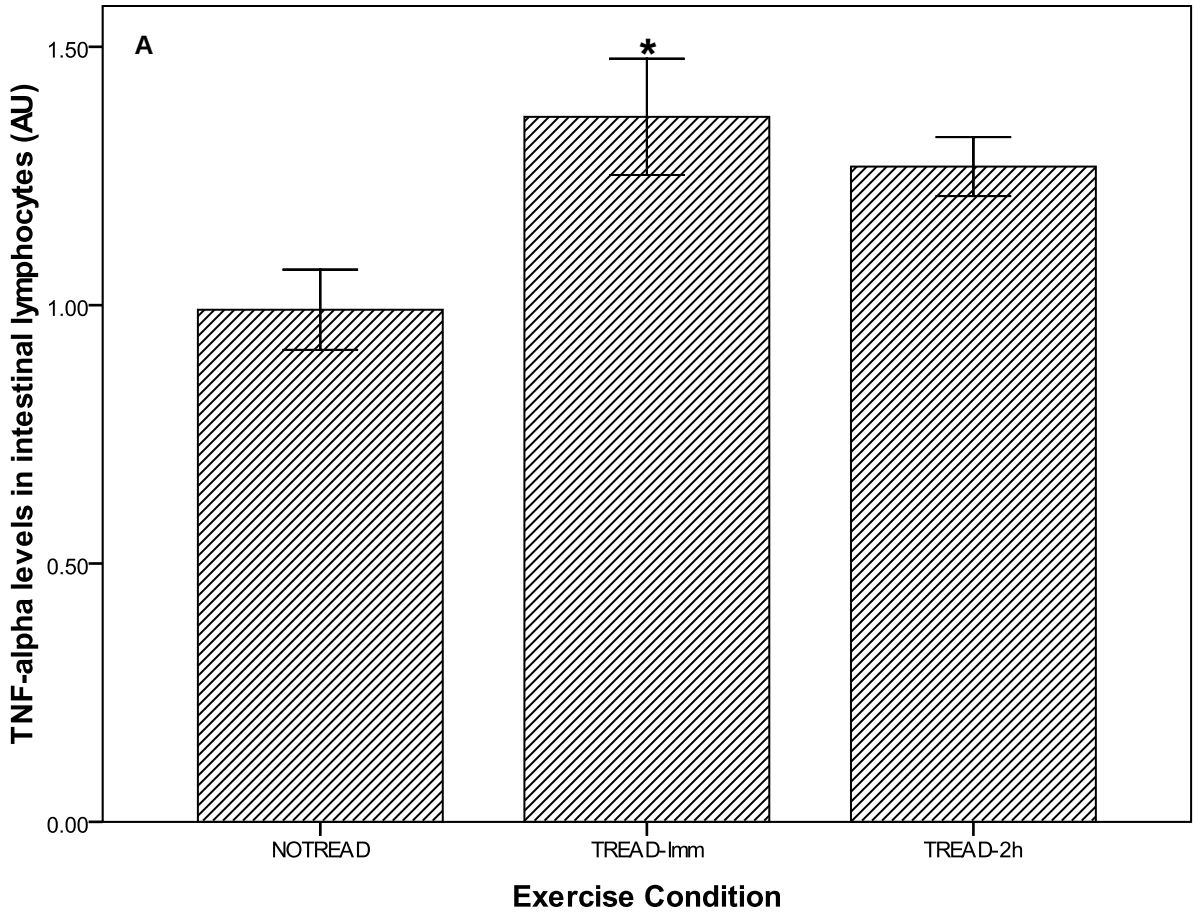

Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in hippocampal cells of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel C*: IL-1β expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

### 3.4.4 Intestinal Lymphocyte Cytokines

**Figure 5** shows the effects of acute treadmill exercise on the expression of pro-inflammatory cytokines in intestinal lymphocytes. There was a significant effect of acute treadmill exercise on TNF-α expression ($F$ (2, 28) = 5.21, $p < 0.05$) due to higher expression in TREAD-IMM (1.4 ± 0.1 AU) compared to the NOTREAD (1.0 ± 0.1 AU) mice. TNF-α expression in intestinal lymphocytes was also elevated in TREAD-2h (1.3 ± 0.1 AU) compared to NOTREAD animals, but this difference only approached significance ($p = 0.06$). A significant effect of acute exercise on intestinal IL-6 was found ($F$ (2, 28) = 6.60, $p < 0.05$). A small and non-significant decrease in IL-6 expression occurred in the intestinal lymphocytes from TREAD-IMM (0.9 ± 0.1 AU) mice compared to NOTREAD (1.1 ± 0.1 AU) mice. The TREAD-2h (1.3 ± 0.1 AU) mice, however, had significantly higher expression of IL-6 in intestinal lymphocytes to the NOTREAD animals. Intestinal lymphocyte IL-1β expression was affected by acute exercise ($F$ (2, 29) = 5.13, $p < 0.05$) with this cytokine elevated in the TREAD-IMM (1.2 ± 0.1 AU) and TREAD-2h (1.3 ± 0.1 AU) compared to the NOTREAD (0.9 ± 0.1) mice.

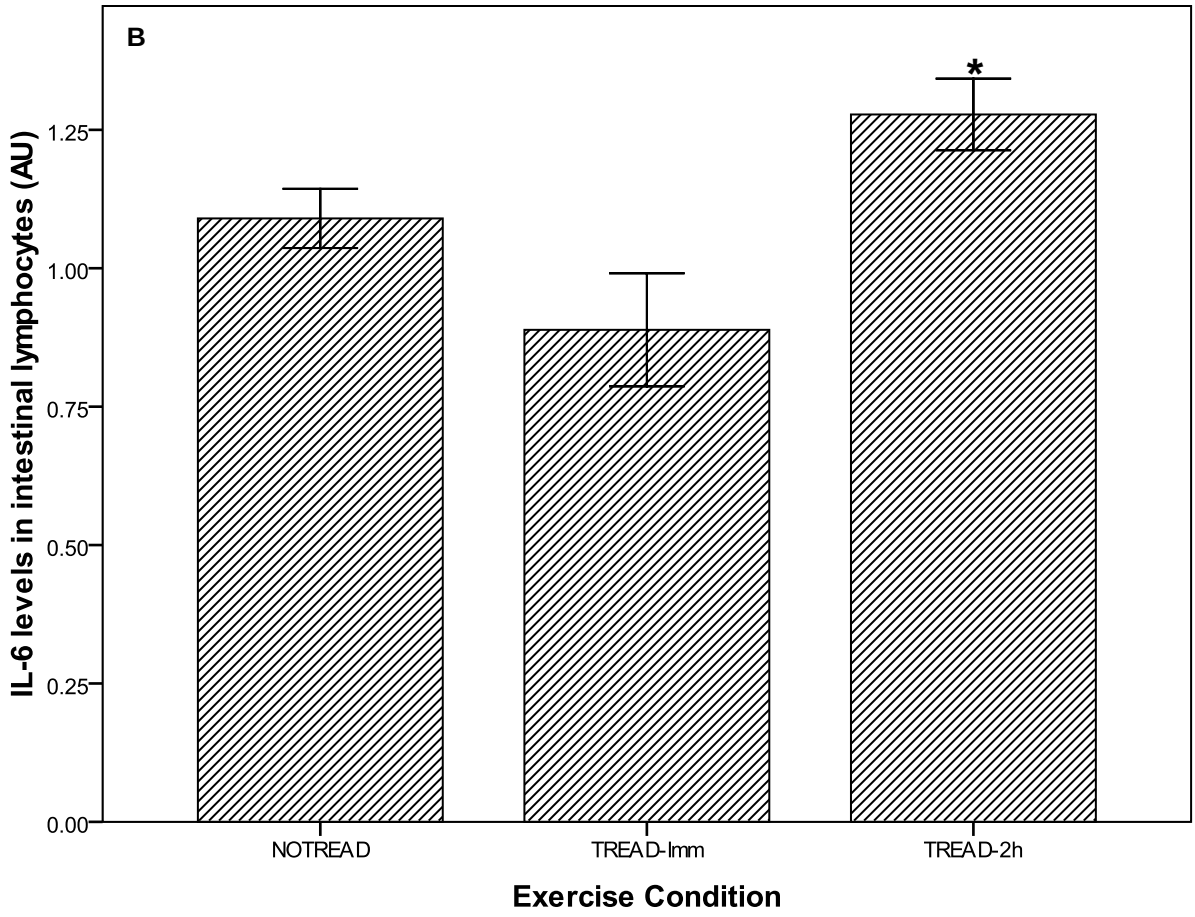
Representative immunoblots for TNF-α expression in hippocampal cells and intestinal lymphocytes for NOTREAD, TREAD-Imm, and TREAD-2h mice are shown in **Figure 6**. Note that only the smaller (17 kDa) cleaved form of the cytokine was analyzed and presented in Figure 4 Panel A and Figure 5 Panel A. The larger pro-form (28 kDa) was not analyzed.

**Figure 5A.** Cytokine expression in intestinal lymphocytes of mice



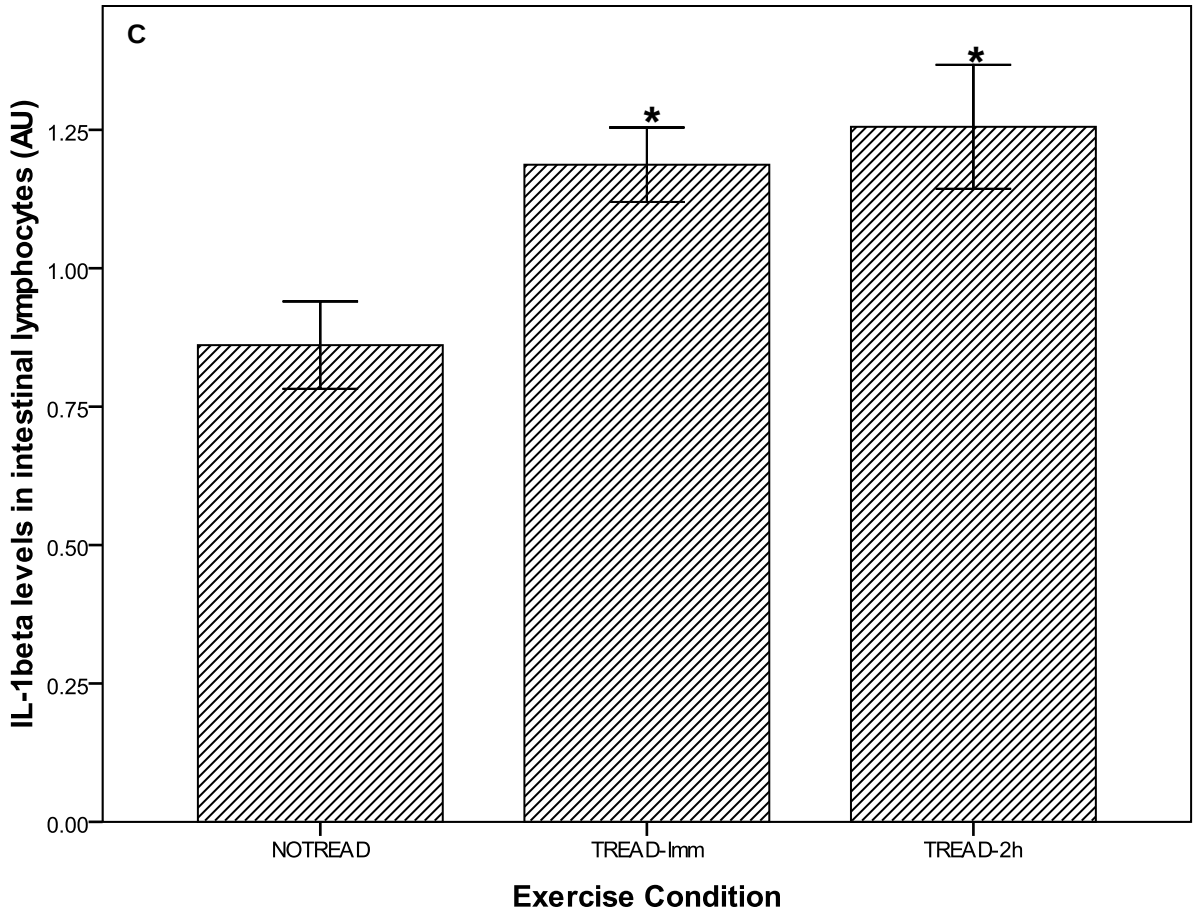

Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in intestinal lymphocytes of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel A*: TNF-α expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

**Figure 5B.** Cytokine expression in intestinal lymphocytes of mice



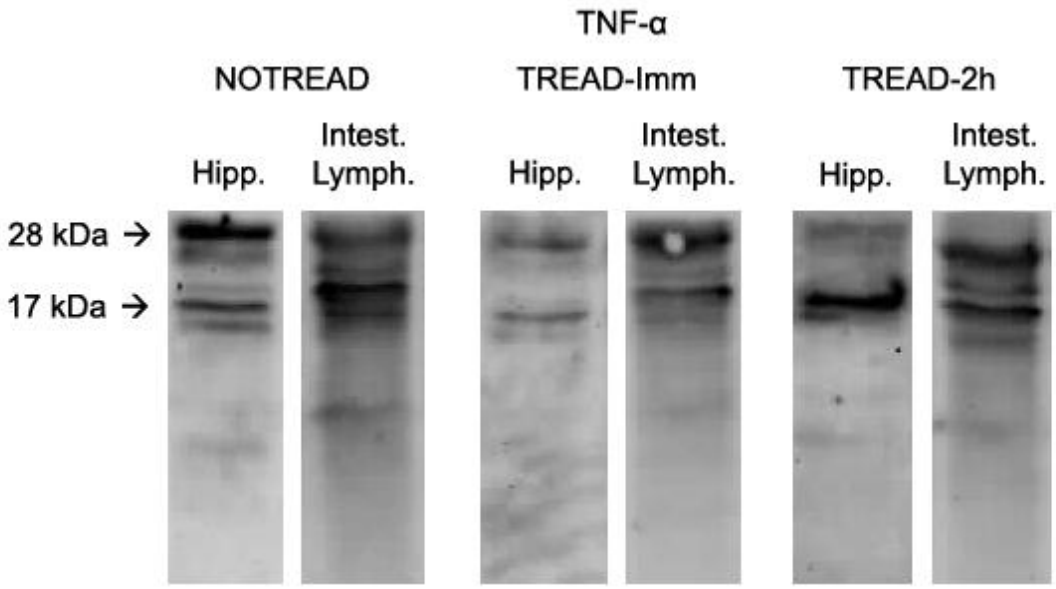

Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in intestinal lymphocytes of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel B*: IL-6 expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

**Figure 5C.** Cytokine expression in intestinal lymphocytes of mice




Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in intestinal lymphocytes of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel C*: IL-1β expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

**Figure 6.** Immunoblots

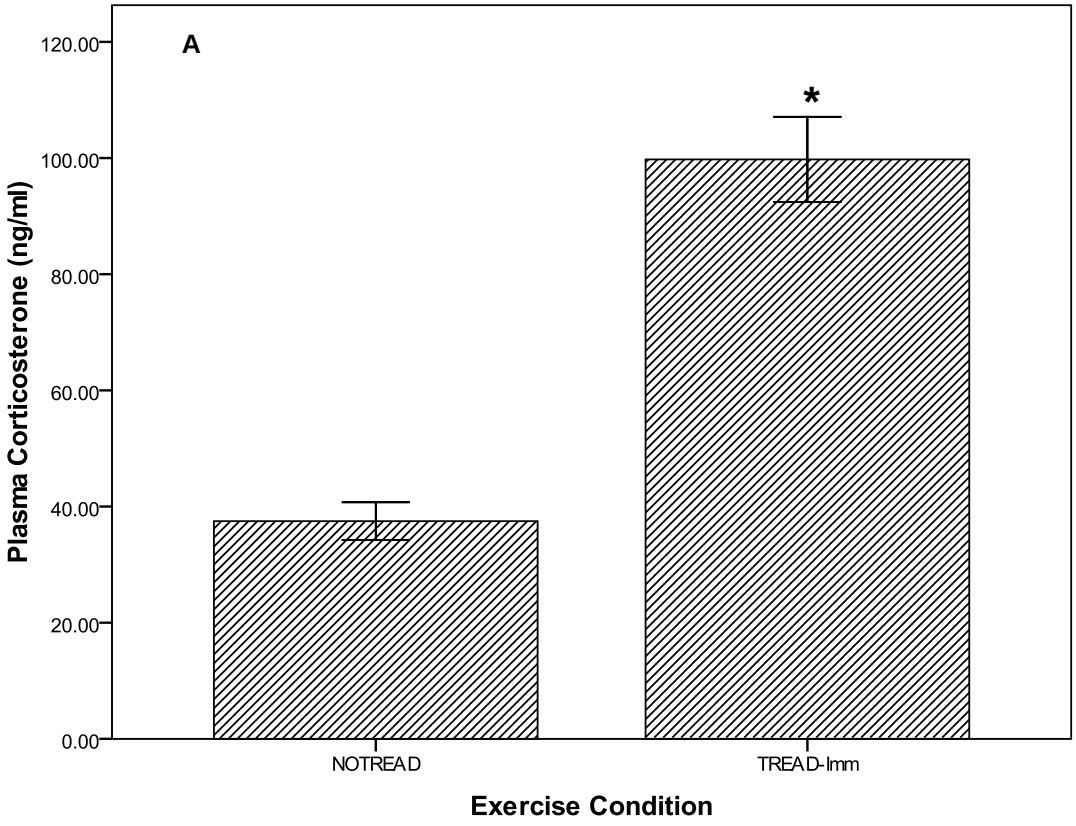



Representative immunoblots for TNF-α in hippocampal cells and intestinal lymphocytes of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) vs. sedentary controls (NOTREAD). Arrows indicate molecular weight (kDa) for the two forms of TNF-α: a larger pro-form at 28 kDa and a smaller cleaved form at ~17 kDa. Only the smaller molecular weight cytokine was analyzed in the experiments.

### 3.4.5 Cortisterone and 8-isoprostaglandin $F2_{\alpha}$ (8-isoprostane)

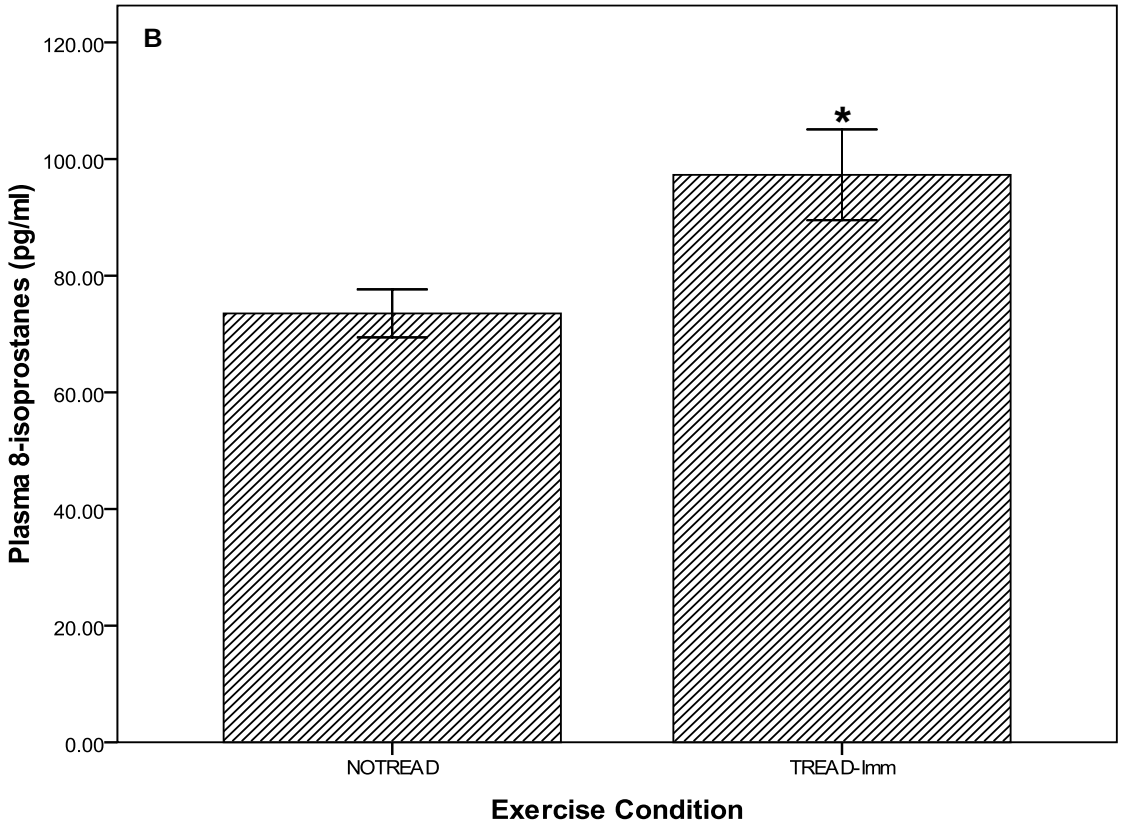
**Figure 7** shows the plasma corticosterone (Panel A) and 8-isoprostane (Panel B) responses to acute treadmill exercise. The treadmill running led to a significant increase in plasma corticosterone concentration ($F$ (1, 39) = 60.25, $p < 0.05$) in TREAD-IMM (99.8 ± 5.7 ng/ml) compared to NOTREAD (37.5 ± 5.7 ng/ml) mice. Acute treadmill exercise also was associated with a significant increase in plasma 8-isoprostane levels ($F$ (1, 32) = 7.53, $p < 0.05$) in TREAD-IMM (97.3 ± 6.2 pg/ml) compared to NOTREAD (73.5 ± 6.0 pg/ml) mice. Plasma corticosterone and 8-isoprostane levels were not assessed in the TREAD-2h group as previous studies (Hoffman-Goetz and Quadrilatero, 2003; Hoffman-Goetz et al., 2010) have shown that differences in these measures are no longer significant after a 2 hour rest period.

**Figure 7A.** Plasma Corticosterone and 8-isoprostanes




Plasma corticosterone and 8-isoprostane concentrations in mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel A*: Plasma corticosterone concentrations [ng/ml]. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

**Figure 7B.** Plasma Corticosterone and 8-isoprostanes




Plasma corticosterone and 8-isoprostane concentrations in mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel B*: Plasma 8-isoprostane concentrations [pg/ml]. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

### 3.5 Discussion

We determined the effects of a single bout of strenuous aerobic exercise on cellular apoptosis and necrosis, and on the expression of pro-inflammatory cytokines in the hippocampi of healthy mice. A secondary objective was to evaluate intestinal lymphocyte pro-inflammatory cytokine expression in a subset of the experimental groups, along with physiological markers of stress, to determine that the exercise protocol was sufficient to elicit inflammatory cytokine changes as we previously reported (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010).

Although several studies have investigated the phenomenon of acute exercise-induced apoptosis in systemic lymphoid compartments, no previous studies, to our knowledge, have explored such effects in the healthy brain. Our novel results suggest that the hippocampus may be protected against the loss of cells incurred as a result of intense physical activity. This perspective is suggested by related findings in the literature. For example, Kim et al. (2010) investigated the hippocampi obtained from rats undergoing 10 days of treadmill exercise following induced traumatic brain injury (TBI). TBI was found to impair short-term memory, increase DNA fragmentation, elevate caspase-3 and Bax expression, and decrease Bcl-2 protein expression in the hippocampus. However, in the exercised animals, there was less memory impairment, DNA fragmentation, and caspase-3 and Bax expression, indicating that physical activity reduces the apoptosis associated with central trauma. Although this study examined repeated bouts of treadmill running over a 10 day period, each "session" of forced exercise did not exacerbate traumatic damage. Um et al. (2008) found that long-term treadmill running inhibits the apoptotic cascade in the brain by reducing cytochrome c, caspase-9, and caspase-3 protein levels, while inducing the expression of superoxide dismutase-1, catalase, and Bcl-2 to combat the effects of oxidative stress. Radák et al. (2001) examined the effects of a prolonged

acute exercise bout on markers of oxidative damage in the hippocampus of rats following a period of stress; immobilization increased lipid peroxidation, carbonylated protein concentration, DNA damage, and reduced glutamine synthetase activity. A single bout of acute exercise was able to restore levels to those observed in control animals. We suggest that intense aerobic exercise (at least at the intensity and duration used in this study) may not be sufficient to generate apoptotic conditions in the brain, and instead initiates the generation of conditions that may even be anti-apoptotic. Nevertheless, the literature in this area is limited (Packer et al., 2010), and caution must be used when comparing these studies: some utilized training (Um et al., 2008), others repeated acute exercise (Kim et al., 2010), and still others a single-bout of acute exercise (Radák et al., 2001).

In contrast to our initial hypothesis, high-intensity exercise decreases the expression of TNF-α and increases the expression of IL-6 in the hippocampus. IL-6 has both pro- and anti-inflammatory functions, depending on the surrounding cytokine milieu, and elevated plasma IL-6 inhibits circulating levels of TNF-α both directly and through up-regulation of the soluble TNF receptor and IL-1ra (Pedersen and Febbraio, 2005; Pedersen, 2009; Starkie et al., 2003). Thus, the higher IL-6 and lower TNF-α expression observed in our study may be a "mechanism" not only where acute exercise leads to decreased immunity to infection, but also preserves cognitive capacity, immediately after the physical stressor. TNF-α has a largely anti-pathogenic activity and is found throughout many areas of the central nervous system. It is responsible for MHC I and II expression in the glia and is highly involved in nitric oxide production in the CNS as a means of eliminating infectious agents. In contrast, IL-6 is primarily localized within the hippocampus and prefrontal cortex, promotes neuron survival factor, protects against excitotoxic brain damage, and has stress modulating effects on cognition (Wilson et al., 2002). Acute

exercise-induced increases in central IL-6 expression may also be responsible for greater cognitive task performance during, and immediately after, exercise bouts of varying intensity (Barella et al., 2010; Lambourne and Tomporowski, 2010).

We also found that the pattern of cytokine expression in the CNS differs from that of intestinal (systemic) tissues after an acute exercise challenge. In the hippocampus there were decreases in TNF-α, increases in IL-6, and no change in IL-1β expression after an acute exercise challenge in mice. In the intestine, however, all of the pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) showed increased expression. The intestinal lymphocyte cytokine pattern confirms earlier research that acute exercise leads to increases in TNF-α, IL-6, and IL-1β expression in intestinal lymphocytes, blood, and muscle (Hoffman-Goetz et al., 2010; Liburt et al., 2010; Nemet et al., 2002). Acute exercise-induced increase in intestinal TNF-α expression is accompanied by elevations in pro-apoptotic proteins and lymphocytosis, which are thought to be a result of the oxidant stress generated by the stressor (Hoffman-Goetz et al., 2010). Administration of an anti-oxidant prevented exercise-induced lymphocyte apoptosis (Quadrilatero and Hoffman-Goetz, 2004), establishing the fact that acute exercise can have damaging effects in the intestine (and likely other systemic immune compartments) as a result of oxidative stress. The mechanism of oxidative stress in the periphery is also indicated by the lack of apoptotic responses in intestinal lymphocytes following injection of corticosterone at concentrations observed with intense treadmill exercise (Quadrilatero and Hoffman-Goetz, 2005).

Acute high-intensity treadmill exercise leads to systemic elevations in markers of oxidative stress, including plasma 8-isoprostanes and corticosterone concentrations (Hoffman-Goetz et al., 2010; Huang et al., 2010; Steensberg et al., 2006). Glucocorticoids, in particular, are modulators of cytokine activity (Pace and Miller, 2009) and cortisol secretion is correlated with

IL-6 release and TNF-α suppression in blood obtained from major depression patients after an endotoxin challenge (Vedder et al., 2007). Audet et al. (2010) examined these phenomena in male mice, utilizing an acute psychosocial stressor (pairing of submissive and dominant mice) to evaluate corticosterone and cytokine responses. Mice with high plasma corticosterone responses to stress also had elevated expression of IL-6 (but not IL-1β) and of mRNA for IL-6 and IL-1β in the hippocampus. The authors suggested that IL-1β protein expression increases at a later time-point not considered in their study. In addition, hippocampal TNF-α mRNA in response to stress was unchanged, but pre-frontal cortex TNF-α mRNA production was lower in corticosterone high responders. Circulating levels of corticosterone are thus increased under immunological or psychological stress (Degterev et al., 2003) and in response to intense exercise (Brown et al., 2007); this is coupled with increases in brain IL-6 in rats in response to chronic mild stress (Mormede et al., 2002).

In addition, glucocorticoid administration inhibits plasma TNF-α increases that occur following an endotoxin challenge in healthy humans (Barber et al., 1993; Beishuizen and Thijs, 2003). LPS-induced serum corticosterone levels are positively correlated with brain IL-6 and IL-1β concentrations 2 hours after endotoxin challenge (Chen et al., 2005). Central TNF-α levels were found to be elevated 16 hours after LPS stimulation whereas plasma (peripheral) corticosterone, IL-6, and IL-1β levels had already returned to baseline concentrations. Moreover, corticosterone administered intraperitoneally to adrenalectomized rats crosses the blood-brain-barrier with uptake and retention of the hormone in the hippocampus (McEwen et al., 1969). These studies suggest that systemic glucocorticoids (whether from endogenous or exogenous sources), affect central and peripheral cytokine expression. Thus, it may be the case that

exercise-induced changes in central pro-inflammatory cytokine synthesis or balance are influenced by corticosterone rather than oxidative responses to the exercise.

This study is not without limitations. Firstly, we did not separate cell subsets in the hippocampus in order to determine whether or not there were differential effects of acute exercise depending on cell type. Instead, our hippocampal single cell suspensions consisted of a mixture of microglia and other non-immune cells, making it difficult to interpret the source of cytokines observed, as well as impact of apoptotic changes in specific cell populations. Another limitation is that only a single bout of exercise was given prior to sacrifice. Human studies have shown that successive exercise bouts can lead to major increases in central IL-6 levels (Nybo et al., 2002) and this must also be addressed in future animal investigations. It is unclear whether repeated exercise will lead to similar cytokine changes in the mouse hippocampus. Furthermore, this study was cross sectional, as we only measured changes immediately and two hours after the exercise bout. It cannot be determined from our results whether the differences in cytokine concentrations observed in the hippocampus and intestine were transient or more long-lasting. Studies will be needed with additional post-exercise time points to provide this clarification and to assess the kinetics of central vs. systemic cytokine expression in immune cells. As such, future experiments should include timed resting controls to address potential temporal effects. In addition, we report on levels of corticosterone in the plasma, but did not measure brain glucocorticoid expression. If corticosterone is affecting the concentration of central pro-inflammatory cytokines, it is essential to determine if this is a result of systemic or central sources of this stress hormone. Furthermore, it needs to be clarified whether corticosterone is actually responsible for the observed cytokine changes. Future investigations may involve repeating the exercise protocol with adrenalectomized mice, or with administration of cortisol

receptor antagonists, to test this hypothesis. Studies must also include testing of other stress hormones, such as catecholamines, and additional markers of oxidative stress, including dichlorofluorescein diacetate. We did not measure apoptotic status (i.e., Annexin V positive) in intestinal lymphocytes because of limited tissue availability. However, we have shown elsewhere that aerobic exercise leads to a loss of intestinal lymphocytes and accompanying increases in apoptotic cells (Hoffman-Goetz and Quadrilatero, 2003). Our experiments were conducted only with female C57BL/6 mice, which did not allow for the determination of any gender-specific effects in the hippocampus or intestine due to acute exercise. Females were chosen in order to 1) allow comparison with earlier studies from our lab (Hoffman-Goetz and Quadrilatero, 2003; Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010), and 2) because they are better runners and show less bout-length attrition than males of this strain (De Bono et al., 2006).

In conclusion, a single bout of intense aerobic treadmill running in healthy female C57BL/6 mice does not affect the percentage of apoptotic hippocampal cells, but alters the expression of pro-inflammatory cytokines by decreasing TNF-α and increasing IL-6 in the hippocampus immediately and 2 hours after cessation of exercise. These changes in the brain do not mirror the cytokine changes observed in intestinal lymphocytes. In the intestine, the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) is increased after intense exercise. We suggest that the oxidative stress that accompanies acute exercise is not sufficient to generate damaging (apoptotic) effects in the central nervous system, as this compartment may be protected to preserve cognitive function during physical activity. We also tentatively propose that exercise-induced elevations in circulating corticosterone levels may be one mechanism to explain the pattern of hippocampal cytokine expression. Glucocorticoids are known to increase and reduce central levels of IL-6 and TNF-α, respectively. Future studies on physical activity and

the central expression of specific pro- (e.g., caspases, Bax) and anti- (e.g., Bcl-2) apoptotic proteins and other pro-inflammatory cytokines will be necessary. Whether reducing or blocking glucocorticoid release (e.g., adrenalectomy) affects brain pro-inflammatory cytokine response to acute exercise stress remains to be determined.

# CHAPTER 4:
# STUDY #2: EXERCISE TRAINING AND PRO- AND ANTI-INFLAMMATORY CYTOKINE AND APOPTOTIC RESPONSES IN THE MOUSE HIPPOCAMPUS

The work presented in this chapter has been accepted for publication (Appendix F) as:

Pervaiz N. and Hoffman-Goetz, L. (2011). Freewheel training alters mouse hippocampal cytokines. *International Journal of Sports Medicine*; DOI: 10.1055/s-0031-1279780.

## 4.1 Chapter Overview / Abstract

The effects of 16 weeks of voluntary wheel running in healthy female mice on hippocampal expression of pro-(TNF-α, IL-1β, IL-12) and anti-(IL-10, IL-1ra) inflammatory and pleiotropic (IL-6) cytokines and apoptotic status of specific cell subsets ($CD45^+$, $CD11b^+$) were studied. Mice were assigned to wheel running (WR; n = 20) or a control condition (No WR; n = 22) and sacrificed after the 16 weeks. Data collected included measures of training status (running volume, body weight, run-to-exhaustion time, and skeletal muscle cytochrome c oxidase activity), flow cytometric analysis of cell phenotypes and apoptosis ($CD45^+$, $CD11b^+$, $Annexin^+$, $Annexin^+/PI^+$, $PI^+$), and cytokine concentrations in cell lysates. WR mice had measurable training effects and significantly lower TNF-α ($p<0.05$) and higher IL-6 ($p<0.05$), IL-1ra ($p<0.05$) and IL-12 ($p<0.05$) expression in the hippocampus compared to controls. IL-1β, IL-10, and the percent of apoptotic and dead cells did not change due to training. Taken together, and in relation to the complex interactions between cytokines, the results suggest a possible mechanism whereby exercise training buffers from dementia and cognitive decline through changes in the central cytokine milieu in the hippocampus.

## 4.2 Introduction

Alzheimer's disease (AD) and dementias constitute a significant public health burden. By 2050, it is estimated that one in 85 people may be living with AD (Brookmeyer et al., 2007). Dementias comprise a complex spectrum of diseases with common traits including amyloid protein growth, neurodegradation, neurofibrillary plaque and tangle formation, and inflammation in the central nervous system (CNS) (Pope et al., 2003). The pathogenesis of dementias may be influenced by pro- and anti- inflammatory immune mechanisms (Packer et al., 2010; Maccioni et al., 2009). For example, TNF-α levels are increased in the frontal, temporal, and parietal cortices (Grammas and Ovase, 2001) and serum IL-1β levels are elevated (Alvarez et al., 1996) in AD patients. Neurofibrillary plaques and tangles and risk of cognitive decline (Krabbe et al., 2009) are correlated with levels of IL-1β and TNF-α. Lipopolysaccharide (LPS) stimulation of microglia (CNS phagocytes) increases TNF-α and IL-1β release, generation of reactive oxygen species, and oxidative stress that damages cells and tissues (Schwab and McGeer, 2008). Age-related amyloid-β formation induces a response similar to LPS, resulting in a pro-inflammatory environment in the brain, a reduction in microglial phagocytic capacity, and the inability to clear abnormal protein aggregates (Koenigsknecht-Talboo and Landreth, 2005).

Most dementias have long asymptomatic periods. A modest delay in onset could result in reductions in the social and economic burdens that accompany disease (Larson, 2010). Exercise is one factor identified in risk reduction for dementias (Coley et al., 2008). Laurin et al. (2001) reported that exercise lowered risks for "cognitive-impairment-not-dementia" in adults compared to those with no physical activity. Training improved executive control tasks and decreased reaction time in older adults (Kramer et al., 1999), promoted neuronal growth, survival and differentiation, and improved cognitive performance (Cotman et al., 2007).

Physical activity may buffer age-related cognitive impairment and dementias by altering the cytokine environment in the CNS (Packer et al., 2010). Seven weeks of forced treadmill training in rats reduced the expression of IL-1β in hippocampus, IL-6 in cerebellum, and IL-1ra in pituitary (Chennaoui et al., 2008). In endurance-trained athletes, increases in IL-6 were found in cerebral blood after 60 min of exercise at 60% $VO_{2max}$. A second bout led to a five-fold greater release of IL-6 (Nybo et al., 2002). IL-6 is pleiotropic acting as a pro- or anti- inflammatory cytokine (Corwin, 2000). IL-6 increases after 3h of exercise at 75% $VO_{2max}$ were associated with lower circulating levels of endotoxin-induced TNF-α in healthy males (Starkie et al., 2003). Rats given 8 weeks of swim training had elevated plasma IL-6 without increases in either TNF-α or IL-1 (Bonyadi et al., 2009). Whereas exercise-induced IL-6 may protect against TNF-α related changes through negative feedback, its direct role in central inflammation has not been characterized. Higher baseline concentrations of hippocampal IL-1β and TNF-α were found in sedentary Tg2576 mice (an animal model of AD) than in wild-type controls (Nichol et al., 2008). After 3 weeks of freewheel running these cytokine differences were no longer significant. Freewheel running in Tg2576 mice also reduced cognitive deficits to wild-type levels (Parachikova et al., 2008). Twelve weeks of forced treadmill running decreased mRNA and protein levels of TNF-α and IL-1β in healthy male rat forebrains (Ang and Gomez-Pinilla, 2007). Voluntary exercise training improved performance on cognitive tasks related to the hippocampus such as spatial memory and maze performance (Churchill et al., 2002); elevated TNF-α and IL-1β inhibit memory formation (Butler et al., 2004).

The purpose of this study was to describe the effects of long-term voluntary exercise in healthy mice on the expression of cytokines in the hippocampus, a brain structure involved in learning and memory. No studies thus far have examined voluntary training in healthy animals in

relation to the hippocampal cytokine environment that has been observed in diseased and aged mouse models. The cytokines chosen included the classic pro-inflammatory cytokines (TNF-α, IL-1β, and IL-12) implicated in cognitive decline and dementia (Maccioni et al., 2009). These cytokines are produced in the CNS by microglia (Hanisch, 2002). IL-10 and IL-1ra expression in the hippocampus were examined as classic anti-inflammatory cytokines (Moore et al., 2001) that counter the effects of TNF-α and IL-1β, respectively. IL-10, normally a T cell cytokine, is also produced by microglia, as is IL-1ra (Hanisch, 2002). The expression of the pleiotropic cytokine IL-6 was assessed as it can exacerbate or ameliorate the effects of pro-inflammatory cytokines depending on the cytokine milieu. We hypothesized that freewheel training in mice would decrease hippocampal expression of TNF-α, IL-1β, and IL-12, increase expression of IL-10 and IL-1ra, and either increase or decrease IL-6 expression (no directional effect was hypothesized due to pleiotropy) relative to controls.

## 4.3 Methods

### 4.3.1 Animals

Female C57BL/6 mice (n = 42) (Harlan, Indianapolis, IN, USA), 3 – 4 weeks of age, were housed individually at 21 ± 1 °C, on a 12/12 h reversed light/dark cycle, and provided *ad libitum* access to a standard rodent diet (Lab Rodent Chow, PMI Feeds, Richmond, IN, USA) and tap water. The experimental procedures were approved by the university animal research ethics board and were conducted in accordance with the ethical standards of the International Journal of Sports Medicine (Harris and Atkinson, 2009).

### 4.3.2 Training Protocol

Mice were matched by weight and randomized to individual cages with 24h access to running wheels (WR; n = 20) or standard cages without running wheels (No WR; n = 22) for 16 weeks. Each wheel (23.0 cm in diameter) was equipped with a magnetic switch, and the number of completed revolutions per 15 min interval during the dark cycle was captured automatically through a computer monitoring system (Mini-Mitter, Sunriver, OR, USA). Data were converted to distance run (km) and summed by month.

### 4.3.3 Assessment of Training Status

To verify that freewheel running for 16 weeks produced measureable physiological effects, several biomarkers of training status were assessed. These included run-to-exhaustion time, cytochrome c oxidase activity in skeletal muscle, and body weight. Total running volume over the course of 16 weeks was also monitored.

#### *4.3.3.1 Run-to-Exhaustion (RTE)*

As described elsewhere (Hoffman-Goetz et al., 2009), a run-to-exhaustion treadmill protocol was administered to mice in WR and No WR groups at 0, 8, and 16 weeks to determine training effects of freewheel running. Mice ran during the dark cycle and treadmill speed was gradually increased over the first 10 min to 28m/min, after which the animals ran until volitional fatigue (Campisi et al., 2002). The final (16 week) RTE was performed 96h before the end of training to ensure that there was no “acute exercise” carry-over effect and in-cage wheels were locked 24h before sacrifice.

#### *4.3.3.2 Skeletal Muscle Cytochrome C Oxidase (CO) Activity*

After 16 weeks of freewheel running, mouse skeletal muscle oxidative enzymes were measured. Mice were sacrificed by overdose of sodium pentobarbital (0.6-0.8 cc per mouse, i.p.). Soleus (SOL) and plantaris (PLANT) were isolated, flash frozen in liquid nitrogen, and stored at -80 °C. Samples were divided into 5-10 mg segments, homogenized in buffer [glycerol (50%), sodium phosphate buffer (20 mM), 2-mercaptoethanol (5 mM), ethylenediaminetetraacetic acid (EDTA, 0.5 mM), BSA (10%)], and sonicated [3 mm tip, 20 s (2 s on, 5 s off) at 60 Hz; Vibra Cell, Sonics and Materials, Danbury, CT, USA]. Soleus and plantaris homogenates were diluted in buffer to a ratio of 1:50 and 10 μL of reduced cytochrome c were added to 970 μL of phosphate buffer (37 °C). Cytochrome c oxidase enzyme activity was measured spectrophotometrically at 550 nm as the decrease in cytochrome c absorbance. Lowry assay (Lowry et al., 1951) was used to determine protein content.

### 4.3.4 Determination of Cytokine and Apoptotic Protein Expression in Hippocampal Cells

#### *4.3.4.1 Hippocampus Removal and Preparation of Single Cell Suspensions*

Mouse hippocampi were excised according to Hassan et al. (1991). Brain dissections took place on a stage over ice. Following decapitation, the skin above the skull was incised along midline allowing access to underlying structures. The brain was excised, washed in cold PBS (0.5% BSA/PBS), transferred to the dissection stage, bisected at midline, a clean number-1 paintbrush inserted into the fissure beneath the dorsal cerebral cortex and the underlying hippocampi visualized. Hippocampi isolated from both hemispheres were placed in 1.5 mL RPMI (1640, 2.5% FCS), pressed through a 70 μm cell strainer, centrifuged at 1500 RPM for 5 min, cells suspended in 5 mL RPMI at RT, layered over 5 mL of Lympholyte M (Cedarlane

Laboratories, Hornby, ON, Canada), centrifuged at 1250 *g* for 20 min and cells at the interface recovered, washed, and suspended in 300 μL PBS. Cells were counted by microscopy and standardized by mL of buffer.

#### *4.3.4.2 Assessments of Phenotypes and Apoptosis of Hippocampal Cells*

Because expression of cytokines could reflect 1) changes in the percent of microglia cells (which produce cytokines) or 2) death of hippocampal cells, we determined the phenotypes and cell viability of hippocampal cells from trained and untrained mice. Cell phenotypes were determined by flow cytometry using FITC-conjugated monoclonal antibodies (Cedarlane Laboratories, Hornby, ON, Canada) for leukocyte (anti-$CD45^+$, clone: YW62.3; common leukocyte antigen) and macrophage (anti-$CD11b^+$, clone: M1/70.15, rat IgG2b) numbers and percentages. 5 x $10^5$ cells were stained with antibody, incubated in the dark at 4 °C for 45 min, washed with 500 μL PBS, centrifuged at 450 *g* for 10 min and suspended in 500 μL PBS for flow cytometric analysis (Epic XL Flow Cytometer, Beckman Coulter, Hialeah, FL, USA).

1 x $10^5$ hippocampal cells were incubated with 2.5 μL of Annexin V-FITC (Pharmingen, San Diego, CA, USA), 2.5 μL of PI (Sigma Chemical, St. Louis, MO, USA), and 100 μL of Annexin binding buffer in the dark for 15 min to measure apoptosis and necrosis (Hoffman-Goetz and Spagnuolo, 2007).

#### *4.3.4.3 Western Blot Analysis of TNF-α, IL-1β, IL-6, IL-12, IL-10, and IL-1ra*

Hippocampal cells were lysed [300 mM NaCl, 50 mM Tris-Cl (PH 7.6), 0.5% Trition X-100, 0.1 mM phenylmethylsulfonyl fluoride], placed on ice for 45 min, after which the lysates were centrifuged (10,000 *g*, 15 min) for protein determination by BCA assay. Protein supernatant

(40 μg) and molecular weight markers (Full Range Rainbow, Amersham Biosciences, Buckinghamshire, UK) were electrophoresed on a 12-15% SDS-PAGE gel, transferred to a PVDF membrane, and stained with Ponceau S to confirm quality of transfer and equal loading. The membranes were incubated with primary antibody for 1 h (1:200 in 10% FBS-TBST): TNF-α (clone: N-19; goat anti-human polyclonal IgG, MW = 17 kDa), IL-1β (clone: Fx02; mouse anti-rat monoclonal IgG, MW = 17 kDa), IL-6 (clone: M-19; goat anti-mouse IgG, MW = 28 kDa), IL-12 (clone: EQ-7; rat anti-mouse monoclonal IgG, MW = 70 kDa), IL-10 (clone: M-18; goat anti-mouse IgG, MW = 15 kDa), or IL-1ra (clone: M-20; goat anti-mouse IgG, MW = 25 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with secondary antibody for 1h: horseradish peroxidise-conjugated anti-goat (TNF-α, IL-10), anti-mouse, or anti-rabbit IgG at 1:2000 in FBS-TBST. ECL Plus Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-imaging System (Cambridge, UK) were used to determine proteins.

### 4.3.5 Experimental Design and Statistical Analysis

All variables including initial and end body weights, RTE, skeletal muscle enzymes, phenotypes, apoptotic markers, and cytokines were analyzed using one-way ANOVA with training condition (WR or No WR) as the independent factor (SPSS Version 18; Chicago, IL, USA). Running volumes (distances) by month were analyzed as a repeated measures ANOVA. Levene's test was used to check for homogeneity of variance. Significant differences were accepted if $p < 0.05$. Values are group means ± 1 SEM for respective units (e.g., μmol/min/g; arbitrary [densitometric] units; g).

## 4.4 Results

### 4.4.1 Physiological Indicators of Training and Exercise

**Table 1** shows the distance run (volume) in km, RTE in min, body weight in g, and cytochrome c oxidase activity (μMol/min/g protein) for the mice with access to running wheels for 16 weeks versus those with no in-cage wheels. Running volume increased consistently and significantly from week 4 through week 12, after which it stabilized through week 16. WR and No WR mice did not differ in initial body weights. After training, WR mice were significantly lighter than No WR mice. WR mice did not differ from No WR mice at week 0 RTE assessment but had significantly longer RTE at weeks 8 and 16 compared with No WR animals. WR mice also had significantly higher CO activity in SOL and PLANT compared to No WR mice.

### 4.4.2 Training Status and Mouse Hippocampi

#### *4.4.2.1 Phenotypes and Apoptosis*

The percentage of early apoptotic ($Annexin^+$), late apoptotic ($Annexin^+/PI^+$), and necrotic ($PI^+$) hippocampal cells did not differ between the WR and No WR groups (**Table 2**). There were also no differences between WR and No WR mice in the percentages of hippocampal $CD45^+$ leukocytes or $CD11b^+$ macrophages or the estimated number of these cells per mL (of buffer)

(**Table 2**).

**Table 1**

| Group | Running volume in km (SEM) | | | | RTE in min (SEM) | | | Body weight in g (SEM) | | Cytochrome c oxidase (µMol/min/g protein) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Weeks 1-4 | Weeks 4-8 | Weeks 8-12 | Weeks 12-16 | Week 0 | Week 8 | Week 16 | Week 0 | Week 16 | Soleus | Plantaris |
| **WR** | 149.8 ± 10.8 | 171.2 ± 9.1 | 192.4 ± 8.1 | 192.1 ± 9.0 | 41.8 ± 4.5 | 69.8 ± 4.6 | 69.1 ± 4.9 | 18.7 ± 0.4 | 25.6 ± 0.4 | 18.1 ± 0.6 | 16.6 ± 0.5 |
| **No WR** | -- | -- | -- | -- | 37.7 ± 4.3 | 36.1 ± 4.4 | 30.2 ± 4.7 | 18.8 ± 0.3 | 26.7 ± 0.4 | 12.5 ± 0.5 | 9.2 ± 0.5 |
| **F (df)** | 3.96 (1, 3) | | | | 0.43 (1, 41) | 29.95 (1, 41) | 32.90 (1, 41) | 0.09 (1, 41) | 5.18 (1, 41) | 51.40 (1, 35) | 122.19 (1, 35) |
| **p** | 0.01 | | | | 0.52 | < 0.0001 | < 0.0001 | 0.76 | 0.03 | < 0.0001 | < 0.0001 |

**Table 1.** Wheel running volume (km) over 16 weeks, run-to-exhaustion (RTE) times (min), body weight (g), and skeletal muscle enzyme activity (cytochrome c oxidase: µMol/min/g protein) in C57BL/6 mice given 16 weeks of freewheel running. Values are means ± one standard error. WR = wheel running; No WR = no wheel running.

**Table 2**

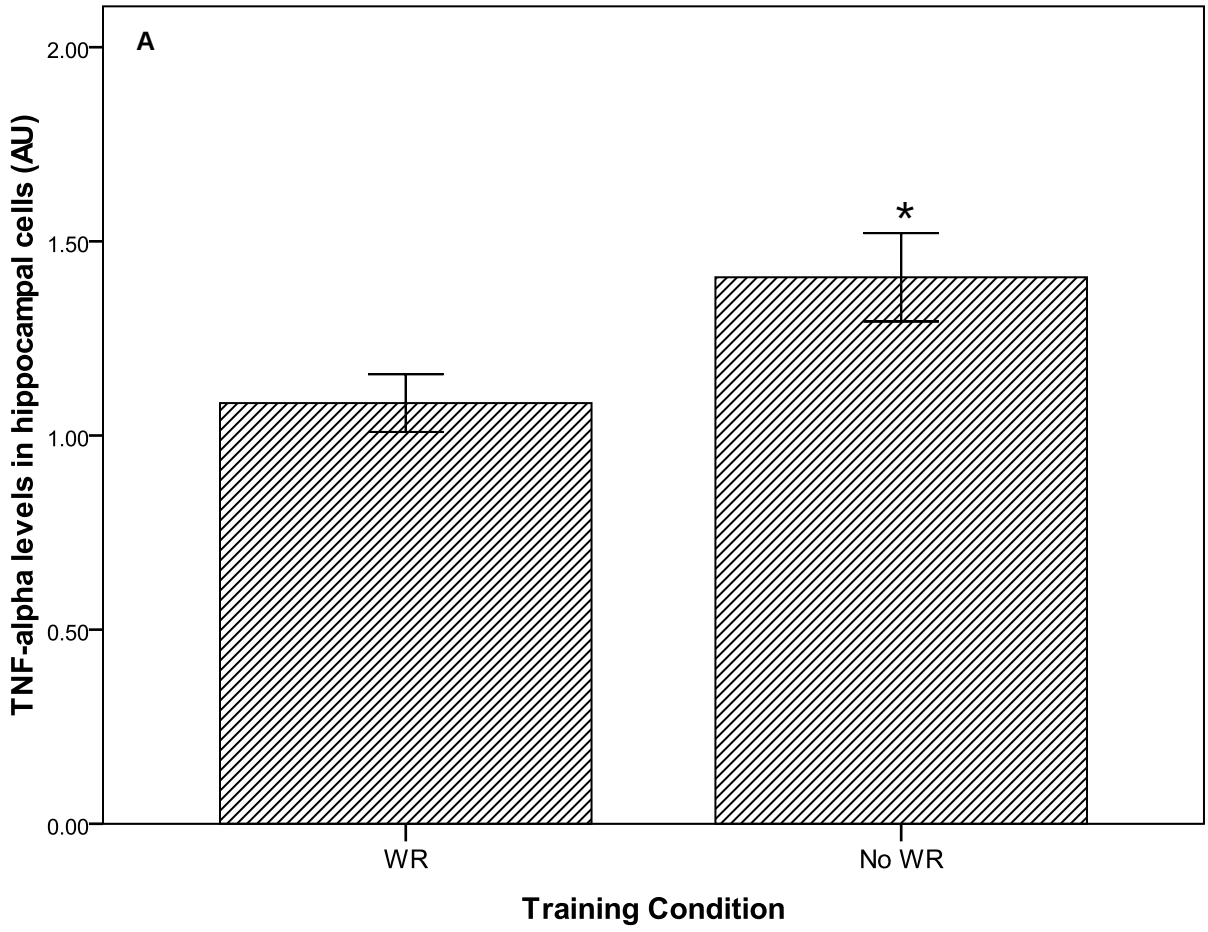
| Surface Marker | Training Group | Mean % (SEM) | F (df) | p |
|---|---|---|---|---|
| **$CD45^+$** | WR | 38.6 ± 2.5 | 2.54 (1, 41) | 0.12 |
| | No WR | 44.1 ± 2.4 | | |
| **$CD11b^+$** | WR | 25.3 ± 1.8 | 2.91 (1, 41) | 0.10 |
| | No WR | 29.5 ± 1.7 | | |
| **$Annexin^+$** | WR | 4.2 ± 0.3 | 0.16 (1, 41) | 0.70 |
| | No WR | 4.0 ± 0.3 | | |
| **$Annexin^+/PI^+$** | WR | 77.9 ± 2.1 | 0.00 (1, 41) | 0.99 |
| | No WR | 77.9 ± 2.0 | | |
| **$PI^+$** | WR | 3.7 ± 0.3 | 0.12 (1, 41) | 0.74 |
| | No WR | 3.6 ± 0.3 | | |
| **Surface Marker** | **Training Group** | **Mean Cell Count / ml (SEM)** | **F (df)** | **p** |
| **$CD45^+$** | WR | $2.1 \times 10^3 \pm 0.4 \times 10^3$ | 3.62 (1, 41) | 0.06 |
| | No WR | $3.0 \times 10^3 \pm 0.3 \times 10^3$ | | |
| **$CD11b^+$** | WR | $2.1 \times 10^3 \pm 0.3 \times 10^3$ | 0.85 (1, 41) | 0.36 |
| | No WR | $2.5 \times 10^3 \pm 0.2 \times 10^3$ | | |

Percent and number of cell phenotypes (per ml) of $CD45^+$ and $CD11b^+$ cells and percent early apoptotic ($Annexin^+$), late apoptotic ($Annexin^+/PI^+$), and necrotic ($PI^+$) cells in hippocampus of C57BL/6 mice given 16 weeks of freewheel running. Values are means ± one standard error. WR = wheel running; No WR = no wheel running.

#### *4.4.2.2 Pro- and Anti-inflammatory Cytokines*

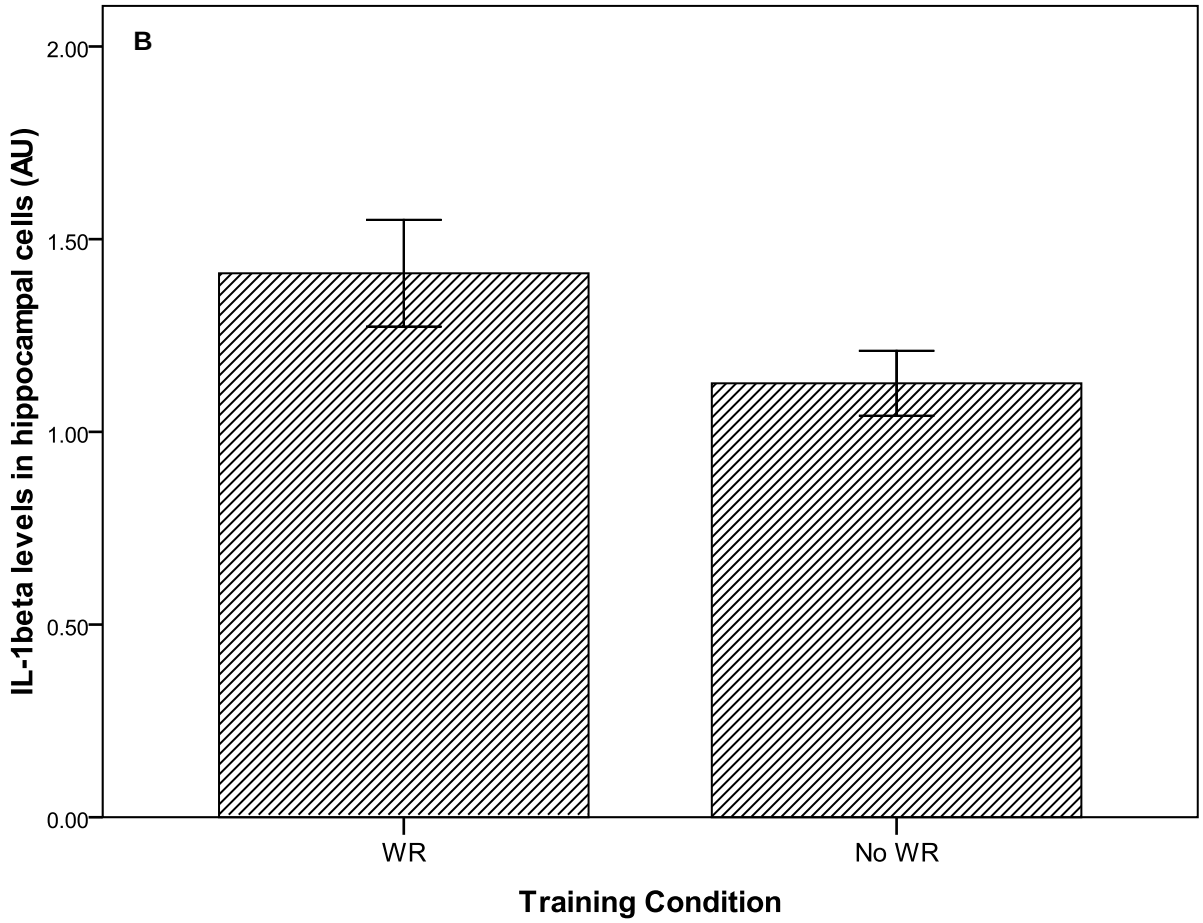
**Figure 8** shows the effect of 16 weeks of freewheel running in mice on the expression of pro- and anti-inflammatory cytokines (in Arbitrary Units [AU]) in the hippocampus. Training was associated with significantly lower expression of TNF-α [$F(1, 39) = 5.68$, $p < 0.05$] in WR compared to No WR mice. Higher expression of IL-6 [$F(1, 39) = 7.02$, $p < 0.05$], IL-12 [$F(1, 39) = 7.05$, $p < 0.05$], and IL-1ra [$F(1, 39) = 7.42$, $p < 0.05$] was also observed in WR compared to No WR mice. In contrast, 16 weeks of freewheel running did not change the expression of IL-1β [$F(1, 39) = 3.09$, $p > 0.05$] or IL-10 [$F(1, 39) = 0.26$, $p > 0.05$] in mouse hippocampus.

**Figure 8A.** Cytokine expression in hippocampal cells of mice



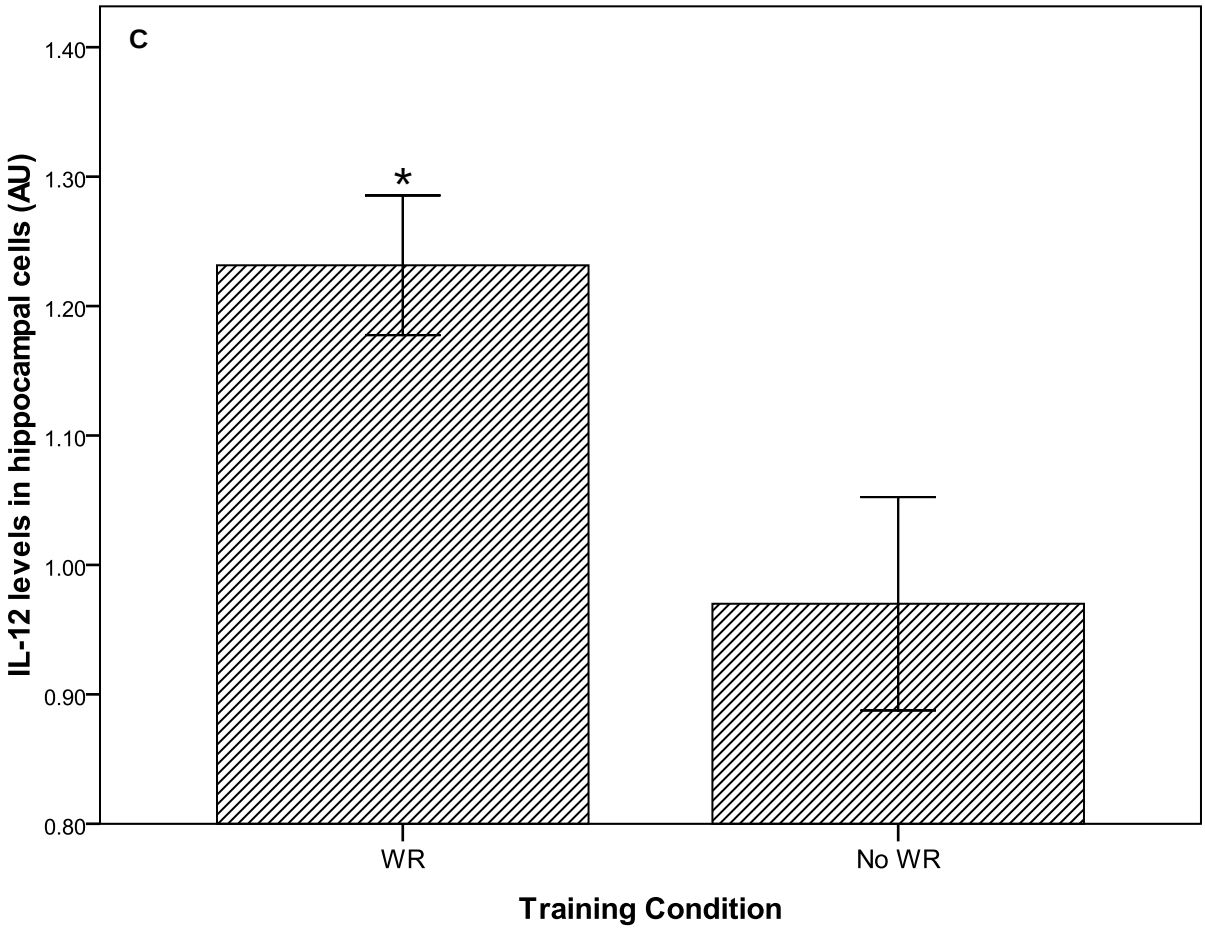

Pro-(TNF-α, IL-1β, IL-12) and anti-(IL-6, IL-1ra, IL-10) inflammatory cytokine expression [in arbitrary units (AU)] in hippocampal cells of C57BL/6 mice given 16 weeks of freewheel running versus no in-cage wheels. *Panel A*: TNF-α expression. Values are means ± one standard error. Significance indicated by an asterisk (*). WR = Wheel running; No WR = no wheel running.

**Figure 8B.** Cytokine expression in hippocampal cells of mice



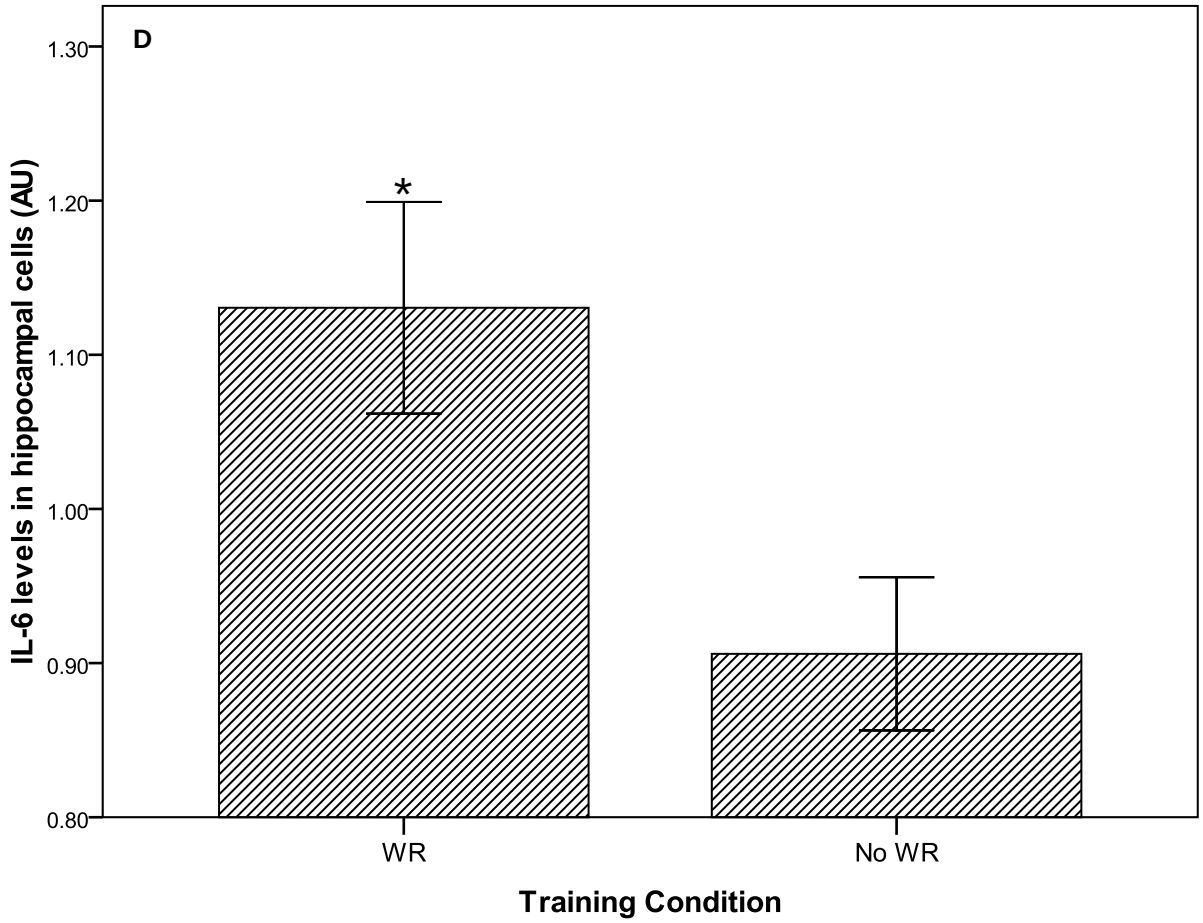

Pro-(TNF-α, IL-1β, IL-12) and anti-(IL-6, IL-1ra, IL-10) inflammatory cytokine expression [in arbitrary units (AU)] in hippocampal cells of C57BL/6 mice given 16 weeks of freewheel running versus no in-cage wheels. *Panel B*: IL-1β expression. Values are means ± one standard error. Significance indicated by an asterisk (*). WR = Wheel running; No WR = no wheel running.

**Figure 8C.** Cytokine expression in hippocampal cells of mice



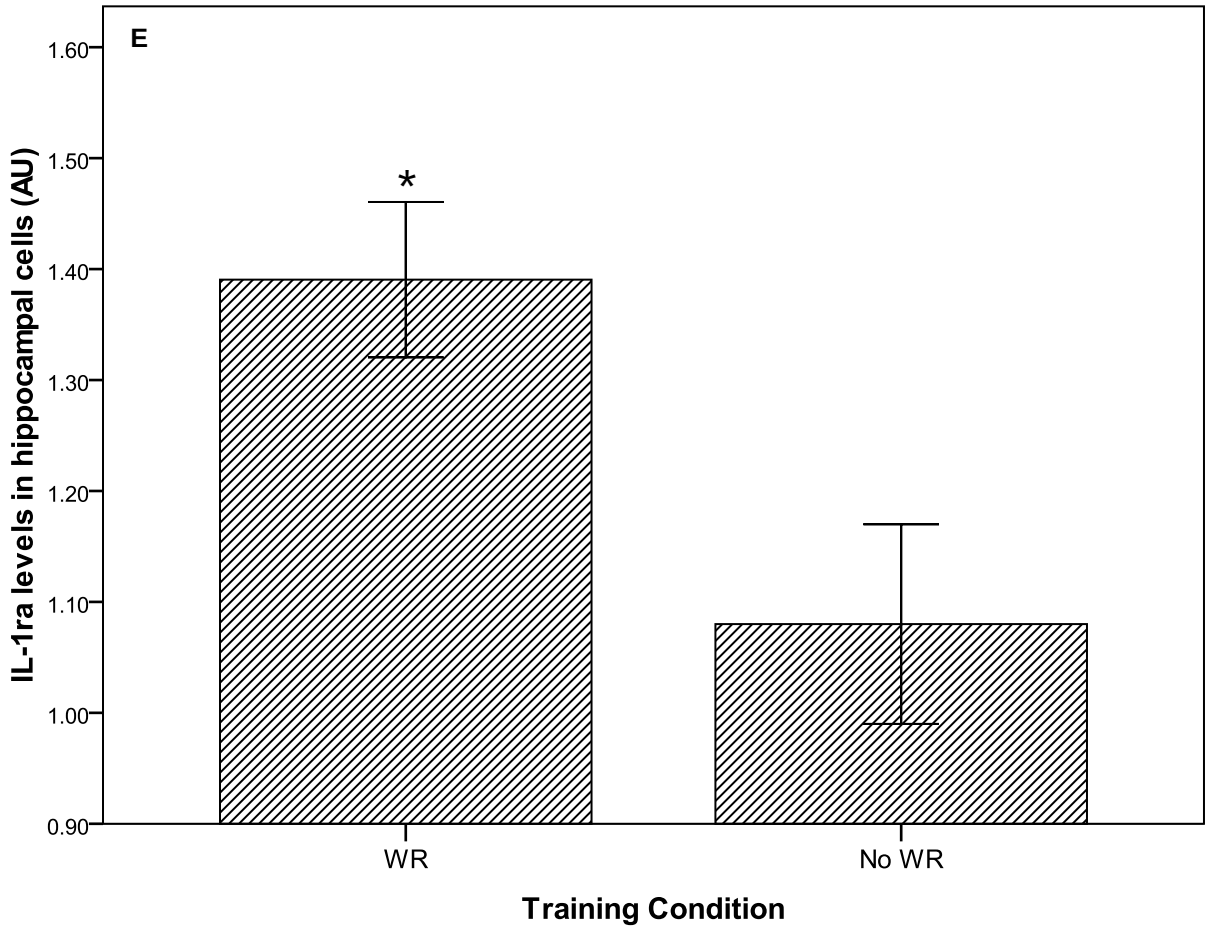

Pro-(TNF-α, IL-1β, IL-12) and anti-(IL-6, IL-1ra, IL-10) inflammatory cytokine expression [in arbitrary units (AU)] in hippocampal cells of C57BL/6 mice given 16 weeks of freewheel running versus no in-cage wheels. *Panel C*: IL-12 expression. Values are means ± one standard error. Significance indicated by an asterisk (*). WR = Wheel running; No WR = no wheel running.

**Figure 8D.** Cytokine expression in hippocampal cells of mice



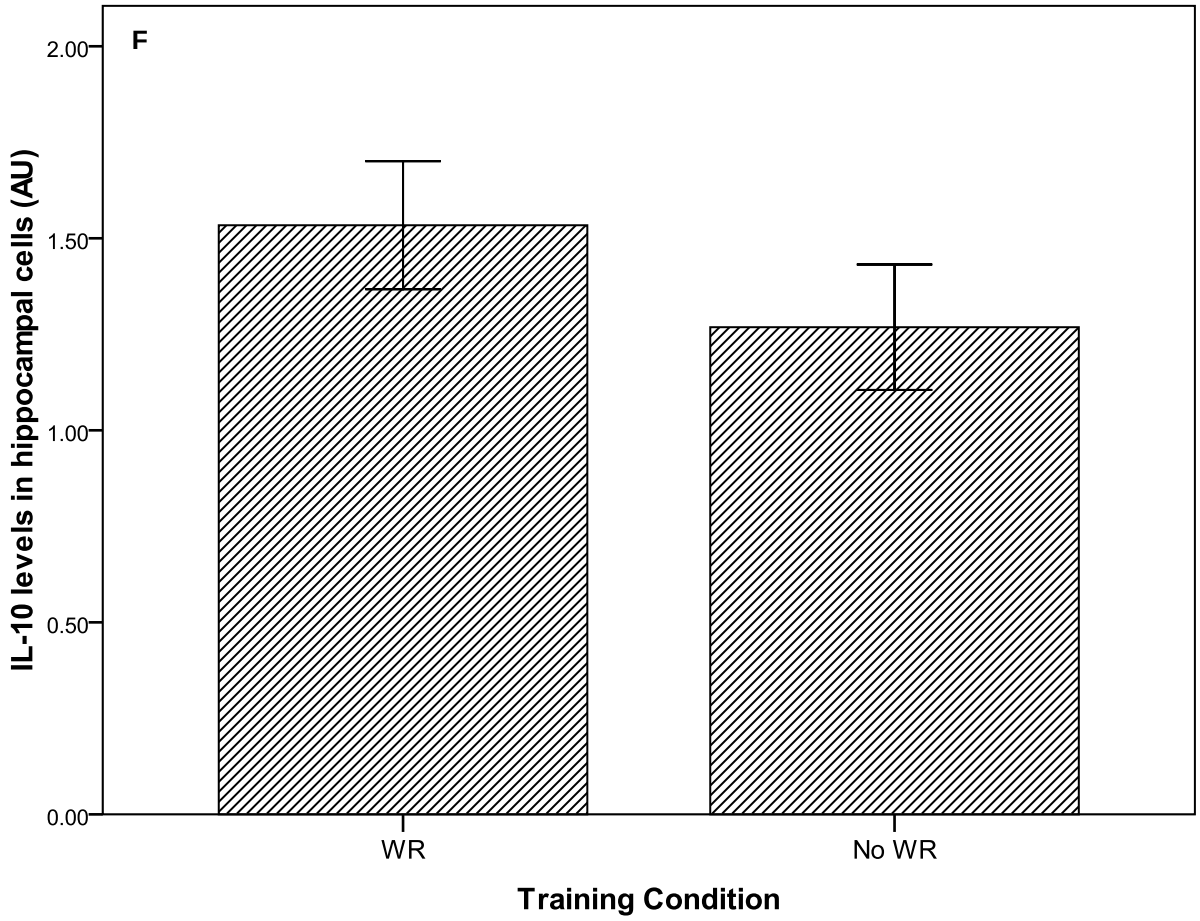

Pro-(TNF-α, IL-1β, IL-12) and anti-(IL-6, IL-1ra, IL-10) inflammatory cytokine expression [in arbitrary units (AU)] in hippocampal cells of C57BL/6 mice given 16 weeks of freewheel running versus no in-cage wheels. *Panel D*: IL-6 expression. *Panel* Values are means ± one standard error. Significance indicated by an asterisk (*). WR = Wheel running; No WR = no wheel running.

**Figure 8E.** Cytokine expression in hippocampal cells of mice




Pro-(TNF-α, IL-1β, IL-12) and anti-(IL-6, IL-1ra, IL-10) inflammatory cytokine expression [in arbitrary units (AU)] in hippocampal cells of C57BL/6 mice given 16 weeks of freewheel running versus no in-cage wheels. *Panel E*: IL-1ra expression. Values are means ± one standard error. Significance indicated by an asterisk (*). WR = Wheel running; No WR = no wheel running.

**Figure 8F.** Cytokine expression in hippocampal cells of mice




Pro-(TNF-α, IL-1β, IL-12) and anti-(IL-6, IL-1ra, IL-10) inflammatory cytokine expression [in arbitrary units (AU)] in hippocampal cells of C57BL/6 mice given 16 weeks of freewheel running versus no in-cage wheels. *Panel F*: IL-1ra expression. Values are means ± one standard error. Significance indicated by an asterisk (*). WR = Wheel running; No WR = no wheel running.

## 4.5 Discussion

We determined the effects of long-term freewheel running in healthy mice on the expression in the hippocampus of TNF-α, IL-1β, IL-12, IL-10, IL-1ra, and IL-6. Enumeration of the percent apoptotic $CD45^+$ leukocytes and $CD11b^+$ microglia in the hippocampus was also measured. The underlying rationale was to assess potential benefits that long-term voluntary physical activity could have on cytokine-mediated inflammation in the hippocampus and overall "brain health."

The study findings are novel: 16 weeks of freewheel running in mice significantly decreased the expression of the pro-inflammatory cytokine TNF-α, accompanied by significant increases in the expression of the pleiotropic cytokine IL-6, the anti-inflammatory cytokine IL-1ra, and the pro-inflammatory cytokine IL-12 in the hippocampus. To our knowledge, no previous studies have specifically assessed long-term voluntary exercise training on the expression of a panel of pro- and anti- inflammatory cytokines in the hippocampus of healthy mice. Furthermore, none have examined the effects of exercise training on hippocampal cell apoptosis. The results suggest that freewheel training may influence the hippocampus cytokine environments found with aging and disease models by inducing a decrease in TNF-α and an increase in IL-6 expression centrally.

Pro-inflammatory TNF-α is involved in the etiology of dementias and AD; increased levels centrally amplify IL-1β effects in the formation of abnormal amyloid proteins in neurofibrillary plaques and tangles (Maccioni et al., 2009). Moreover, elevation in TNF-α in various brain regions of AD patients has been documented (Perry et al., 2001) and is associated with cognitive decline (Krabbe et al., 2009). TNF-α-deficient mice had accelerated reflex development, faster maturation of the hippocampus in young mice, region-specific increases in

nerve growth factor, and improved performance on spatial learning tasks (Golan et al., 2004). Clark et al. (2010) suggest central TNF-α suppresses adult neurogenesis and reduces net neuron numbers with aging. Regulation of hippocampal TNF-α expression through exercise training may provide insights into interventions to potentially decrease age and disease-related cognitive deficits and limit neuronal damage. Lower TNF-α expression observed in trained mice was accompanied by higher IL-6 expression. Increases in circulating endogenous IL-6 and infusion of recombinant IL-6 inhibit endotoxin-induced increases in blood TNF-α levels in humans (Pedersen and Febbraio, 2005). In addition to "blocking" TNF-α, exercise-induced IL-6 reduces inflammatory "burden" by up-regulating soluble TNF receptor and IL-1ra (Pedersen, 2009). While these effects were demonstrated in plasma and muscle, our results suggest an increase in hippocampal IL-1ra expression with training. It is possible that increased IL-6 expression with freewheel running contributes to down-regulation of TNF-α and up-regulation of IL-1ra in the hippocampus of healthy mice. Additional studies are needed to determine if training increases hippocampal soluble TNF receptor expression and if this is preceded by an IL-6 increase.

The greater expression of IL-12 with long-term voluntary exercise training has interesting implications. IL-12 is a pro-inflammatory cytokine that guides the generation of the Th1 response and strengthens $CD8^+$ T-cell cytoxicity (Akimoto et al., 2000; Egilmez and Kilinc, 2010). Rentzos et al. (2006) found reduced levels of IL-12 in the cerebrospinal fluid of patients with AD; they suggested that decreases in IL-12 alters the neurotrophin profile and leads to impaired neuroprotection. Training-induced increases in IL-12 may indicate that the hippocampus is "primed" against infection, uncontrolled cell division, and protein production in dementia-related plaques.

No differences in hippocampal expression of IL-1β or IL-10 occurred due to training. There are few reports on exercise and brain IL-1β levels and the findings are ambiguous. Forced treadmill training reduced IL-1β levels in rat hippocampus but it was unclear whether this was due to training or carryover from exercise prior to sacrifice (Chennaoui et al., 2008). Training-induced decreases in hippocampal IL-1β levels occurred in Tg2576 but not healthy control mice (Nichol et al., 2008). Hence, training may confer protection in a disease-like state when IL-1β is already elevated, but may not reduce "baseline" levels in healthy animals.

There were no changes in IL-10 expression in healthy mouse hippocampal cells. Exercise-induced IL-6 synthesis furthers the expression of IL-10 in muscle (Pedersen, 2006) and levels of this cytokine increase in white adipose tissue with training (Lira et al., 2009). However, peripheral increases in IL-10 have only been shown to be therapeutically effective in the CNS when the blood-brain-barrier is disrupted due to infection or trauma (Kastin et al., 2003). Moreover, *in situ* production of IL-10 by microglia is negligible unless there is close contact with infiltrating T lymphocytes (Yong and Marks, 2010).

We considered the results with respect to each cytokine individually. However, the biology suggests that a more complex scenario of cytokine interaction occurs rather than a simple anti- vs pro- inflammatory dichotomy. A meta-analysis of cytokine expression in AD by Swardfager et al. (2010) found that TNF-α, IL-1β, IL-6, and IL-12 levels were significantly elevated in peripheral blood of AD patients compared to healthy controls and these changes were propagated across the blood brain barrier. Excessive elevations in IL-6 are induced by TNF-α and IL-1β activation creating an environment in which IL-6 acts in a pro-inflammatory manner (Clark et al., 2010). Without the presence of the former two cytokines, the role of excess IL-6 in the brain is unclear. We found that voluntary exercise training reduced central TNF-α, increased

IL-6, and had no impact on IL-1β expression in healthy mice. If TNF-α is a cytokine that "regulates" the function of other cytokines, training may confer protection against disease initiation by disrupting the activity of this key cytokine. Exercise training is also comparable to calorie restriction in terms of effects on synapse function (Valdez et al., 2010). Calorie restriction prevents the age-related TNF-α increase in mouse serum (Spaulding et al., 1997) and up-regulation of IL-10 in diabetic rats (Ugochuku and Figgers, 2007). Energy restriction leads to enhanced learning and decreased memory deterioration in rodents (Clark et al., 2010) similar to the effects of exercise training (Churchill et al., 2002).

The impact of training on apoptosis of mouse hippocampal cell subsets was analyzed. Voluntary exercise training did not affect cell counts or percentages of $CD45^+$ leukocytes or $CD11b^+$ microglia undergoing early or late apoptosis. Furthermore, no differences the in numbers of these cell types provides support for the absence of apoptotic changes between the experimental groups. Other cell subsets populating the hippocampus include astrocytes, but were not assessed as they do not affect cytokine levels in the absence of infection or injury (Ang and Gomez-Pinilla, 2007). Lymphocyte subsets, such as CD3 positive cells, are not normally present in healthy hippocampus (Fischer and Reichmann, 2001) and hence were not assessed in this study. Our data do not provide mechanisms for the differences in apoptosis between tissue compartments with training. However, the absence of a training effect on the hippocampal cell subsets may reflect two phenomena. First, $CD45^+$ leukocytes and macrophages only infiltrate the hippocampus in large numbers in response to glial cell chemokine secretion after injury (Babcock et al., 2003). Second, no change in $CD11b^+$ microglia percentages may indicate the source of cytokines, such as IL-6, is not hippocampal microglia but external to this brain compartment or even to the CNS. Microglia secrete a repertoire of cytokines, including IL-6, and

this synthesis can be either constitutive or inducible (Hanisch, 2002). Microglial IL-6 production occurs after injury or infection in the CNS with recruitment of astrocytes for repair (Raivich et al., 1999). In our study, there was no insult to the brains of mice due to voluntary wheel running. Thus, it is possible that the IL-6 measured in the hippocampus was not of microglia origin. IL-6, a myokine with endocrine functions (Pedersen, 2009), can cross the blood-brain barrier (Banks et al., 1995). The main and earliest source of exercise-related IL-6 is likely contracting skeletal muscle (Pedersen, 2009). Nevertheless, our results do not allow identification of the actual source (CNS, muscle, other tissue) of IL-6 and the cytokines determined in this study.

This work has limitations. Changes in cytokines were observed but without numerical or apoptotic changes in the hippocampal cell subsets that produce them. We speculate that differences in cytokine levels, and notably IL-6, may be attributable to changes in skeletal muscle cytokine levels, which can cross the blood-brain-barrier and influence CNS function. However, we did not assess expression of skeletal muscle IL-6, which would have allowed for confirmation of training-induced changes in that tissue. Hippocampal expression of cytokines was assessed in healthy mice, and long-term freewheel running was associated with a "favourable" decrease in TNF-α and increase in IL-1ra cytokine expression. The use of a healthy model does not inform how training affects disease progression, but provides insight into possible immune mechanisms for prevention of AD and cognitive decline. Another limitation is that hippocampal cytokine expression was not determined in relation to age or behavioural sequelae (e.g., memory and learning tasks). However, others have shown that training improves cognitive ability, memory, and maze performance in aged Tg2576 (AD-like) mice. We also did not assess transcription factors in the hippocampus after exercise training. NFκB, for instance,

regulates the synthesis of TNF-α and IL-6 (Brown et al., 2008) and measurement may clarify the source of cytokines in the hippocampus after training.

In summary, freewheel running for 16 weeks in healthy young female C57BL/6 mice is associated with decreased expression of the pro-inflammatory cytokine TNF-α, and increased expression of the pleiotropic cytokine IL-6, the anti-inflammatory cytokine IL-1ra, and pro-inflammatory cytokine IL-12 in hippocampus. These training-induced cytokine changes suggest a complex biology rather than a simple pro vs. anti-inflammatory dichotomy and a potential "cytokine/immune mechanism" in brain health. Training did not alter the percentage of $CD45^+$ or $CD11b^+$ hippocampal cell subsets nor their apoptotic status. Whether the observed cytokine changes in hippocampus are due to increased expression in other tissues such as skeletal muscle and uptake into the CNS or diffuse from other brain regions is an area of future research.

# CHAPTER 5:
# GENERAL DISCUSSION

## 5.1 Key Findings

Exercise chronicity (acute vs. chronic) emerged as an important variable across both studies, with effects on cytokine concentrations and on plasma levels of stress hormones. Stress characteristics of the exercise (forced vs. voluntary) were also important in the cytokine results. Another important variable identified was tissue compartment, as the results of Study #1 indicate potential similarities and differences in cytokine concentrations and apoptotic status, respectively, between central and systemic tissues.

### 5.1.1 Exercise Chronicity: Acute Exercise vs. Training

Exercise chronicity emerged as an important factor when both of the studies presented in this thesis were considered together. Chronicity refers to the duration and frequency of an event, and, in the context of the experiments presented, refers to the differences in exercise regimens prescribed in both studies. Study #1 assessed the impact of a single bout of acute exercise on hippocampal cytokine concentrations, cellular apoptotic status, and plasma levels of stress hormones. Study #2 evaluated the effects of long-term voluntary exercise training on the aforementioned outcome measures and the phenotypes of cells in the hippocampus. In Study #1, acute exercise in mice was associated with higher hippocampal protein expression of the pro-inflammatory cytokine TNF-α and the pleiotropic cytokine IL-6 compared to sedentary controls. In Study #2, long-term training in mice was associated with increased hippocampal protein expression of these two cytokines, and also increased protein levels of the pro-inflammatory IL-12 and anti-inflammatory IL-1ra cytokines. Neither study indicated effects of exercise (acute or chronic) on apoptotic status in mouse hippocampi; differences in percentages of $Annexin^{+}$,

Annexin$^+$/PI$^+$, or PI$^+$ cells were not observed. Likewise, the voluntary training modality used in Study #2 did not impact the percentages of CD45$^+$ or CD11b$^+$ cells within the mouse hippocampi. In Study #1, a bout of exhaustive acute exercise was found to be associated with higher plasma concentrations of the stress hormones 8-isoprostaglandin F2$_\alpha$ (8-isoprostanes) and corticosterone immediately after exercise. In Study #2, training was associated with increased activity of cytochrome c oxidase in skeletal muscle (soleus, plantaris) in comparison to untrained controls.

Physical activity has different effects on immunological parameters depending on the type, duration, intensity, and number of exercise exposures. Modalities (i.e., treadmill running vs. freewheel running) can also vary within exercise type (i.e., acute exercise vs. training). For instance, a single exposure to acute exhaustive treadmill exercise differs from repeated exposures, as well as from treadmill training. In addition, animal studies often utilize forced training (treadmill running or swimming) protocols, which differ from voluntary exercise training (freewheel running), although the direction of their effects on immune parameters are often similar. Most studies utilizing animal models of neurodegeneration are of the voluntary, wheel running type and results indicate improvements in measures of cognitive function. Such improvements, however, have also been observed in forced exercise regimens (Ang et al., 2006). Yuede et al. (2009) examined the differences between forced and voluntary training on plaque deposition, hippocampal volume, and behaviour in Tg2576 mice. Animals in the voluntary training group were placed in a cage with access to a running wheel for one hour each day, five days per week, for 16 weeks. Mice in the forced training group were placed on a motor driven treadmill for one hour each day, five days per week, for 16 weeks at the average velocity of the voluntary group (determined by converting average daily distance into velocity). The latter group

was motivated by a mild foot shock upon contact with the back of the treadmill. It was determined that wheel running led to an increase in exploratory behaviour and fewer amyloid plaques than treadmill running. Analysis of thioflavin S stained tissue sections from the hippocampus and cortex revealed that both groups had less plaque deposition than sedentary controls. Training, irrespective of modality, was also associated with increased hippocampal volumes compared to the untrained animals. Amyloid-β (Aβ-40 and Aβ-42) protein concentrations, however, did not change as a result of either training modality. This may indicate that both voluntary and forced training are beneficial in terms of improving memory and cognition, and this, in part, may be due to plaque reduction.

Aβ induces inflammation in the brain via increased levels of the cytokines IL-1β and TNF-α. In vivo administration of antagonists to these cytokines eliminates the leukocyte extravasation and vascular damage associated with Aβ accumulation in mice (Sutton et al., 1999). In the series of experiments presented in this thesis, both forced acute exercise and long-term voluntary training were associated with lower expression of hippocampal TNF-α protein and no observable changes in IL-1β levels in healthy mice. In addition, training resulted in elevated levels of hippocampal IL-1ra. It is possible that the protection against cognitive decline, plaque formation, and decreased hippocampal volume observed by Yuede et al. (2009) is a result of reductions in key pro-inflammatory cytokines, which would otherwise work synergistically with both forms of Aβ to exacerbate inflammation in the central nervous system. Nichol et al. (2008) found that Tg2576 mice given three weeks of freewheel running had reductions in hippocampal TNF-α and IL-1β protein concentrations, with levels approaching those of controls. Interestingly, Tg2576 mice given three weeks of freewheel running also displayed improvements in radial arm water maze performance, indicating enhanced learning and memory compared to

non-exercised controls (Parachikova et al., 2008). Training is associated with decreased concentrations of hippocampal pro-inflammatory cytokines (TNF-α and IL-1β most notably), amyloid-β levels, and plaque deposition. These changes are coupled with increased hippocampal volume and cognitive performance suggesting that physical activity is neuroprotective and prevents central inflammatory damage.

It is also important to consider the role of IL-6 in the two experiments comprising this thesis. IL-6 is a pleiotropic cytokine, indicating that it can act in both a pro- and an anti-inflammatory manner (Corwin, 2000; de Gonzalo-Calvo et al., 2010). Mice subjected to an acute bout of strenuous exercise had increased levels of hippocampal IL-6 compared to sedentary animals. Similarly, voluntary training led to an increase in hippocampal IL-6 concentrations. IL-6 acts in a *pro-inflammatory* manner when stimulated by TNF-α and IL-1 (Dinarello, 1996; de Gonzalo-Calvo et al., 2010). However, it also acts in an *anti-inflammatory* manner through negative feedback of IL-1 and TNF-α production (Corwin, 2000). It is reasonable to suggest that physical activity leads to increases in IL-6, followed by reductions in central levels of TNF-α arising from upregulation of soluble TNF receptor and IL-1ra (Pedersen and Febbraio 2005; Starkie et al., 2003). Tilg et al. (1994) administered IL-6 intravenously for five consecutive days (120-hour continuous infusion) to patients enrolled in phase I and II trials performed by the Cytokine Working Group. A short-lived elevation in plasma IL-1ra was observed after IL-6 infusion. The authors concluded that IL-6 administration stimulated an increase in glucocorticoid release and limited the levels of circulating TNF-α (Parant et al., 1991). They hypothesized that the spike and rapid decrease in IL-1ra levels occurred in a similar fashion, as IL-6-induced IL-1ra synthesis in peripheral blood monocytes is completely abrogated by dexamethasone administration (a synthetic glucocorticoid). Exercise-induced IL-6 synthesis furthers the

expression of IL-10 in muscle and in white adipose tissue (Pedersen, 2006; Lira et al., 2009). In Study #2, training was not associated with any significant change in IL-10 levels in the hippocampus. Peripheral increases in IL-10 have only been shown to be therapeutically effective in the CNS when the blood-brain-barrier is disrupted, and *in situ* production of IL-10 by microglia is negligible unless there is close contact with infiltrating T lymphocytes. Within the brain, IL-6 localizes to the hippocampus and prefrontal cortex where it protects against excitotoxic brain damage and has stress modulating effects on cognition (Wilson et al., 2002). Thus, in both thesis experiments, the case can be made that IL-6 elevation and TNF-α reduction could contribute to the increases in cognitive performance observed in other exercise studies (e.g., Parachikova et al., 2008; Barella et al., 2010; Lambourne et al., 2010). It would be important to design future studies to consider these cytokines, physiological measures of brain damage and inflammation, and behavioural measures of cognition in the context of the hippocampus during physical activity.

Stress hormones, such as glucocorticoids, are also important factors to consider in comparison of acute and chronic exercise effects on pro-inflammatory cytokine expression. In Chapter 3, it was observed that a single bout of exhaustive exercise was associated with significant elevations in measures of oxidative stress (plasma 8-isoprostanes and corticosterone). Chronic exposure to glucocorticoids (GC) can lead to hippocampal damage (Sapolsky, 1987). The hippocampus is the primary target site for glucocorticoids, with this tissue having one of the highest concentrations of GC receptors in the brain (Conrad, 2005; McEwen et al., 1969). Cortisol levels increase with immunological stress and in response to intense exercise, which can then lead to increases in brain IL-6 (Mormede et al., 2002) and suppression of plasma TNF-α (Beishuizen and Thijs, 2003). However, results from the training study described herein did not

show changes in plasma corticosterone levels in the blood of mice (Appendix D). Agha et al. (2010) differentiated between forced exercise training and voluntary exercise training effects in mice on plasma levels of corticosterone. Male wild-type CD-1 mice were assigned to 8 weeks of treadmill running, wheel running, or a sedentary condition. At the end of 8 weeks, mice were exposed to one 30 minute bout of acute treadmill exercise and sacrificed immediately or one hour post exercise. Training modality (wheel running or treadmill running) did not affect corticosterone levels after the acute exercise exposure and all groups had significantly elevated corticosterone concentrations immediately after the acute treadmill bout. These effects were "washed out" after one hour. Such results suggest that the exercise training type does not affect plasma corticosterone concentration, and that after one hour of rest, concentration returns to pre-exercise values.

Thus, exposure to corticosterone, and subsequent suppression of TNF-α and elevation of IL-6 observed with acute exercise, may be transient; corticosterone exposure may offer a means of protection against inflammatory damage during a "fight or flight" response (acute exercise), but these effects are likely short-term. Maier and Watkins (1998) argued that the fight or flight response results in decreased immunity to infection, but requires the organism to increase sensory ability to detect predators (often at a distance), motor capacity to flee, and sensorimotor integration to direct movement away from the detected threat. Extrapolating from the results of Study #1, acute exercise may lead to a decrease in central immunity to infection through a reduction in TNF-α and the preservation of cognitive capacity required to escape a perceived threat. Although 2 hours after acute treadmill exercise, expression of hippocampal TNF-α protein was lower than in sedentary mice, the effects at later time points (e.g., 6, 12, or 24 hours) after exercise remain unknown. Future studies will be needed to document additional post-exercise

time points in order to determine whether the decrease in TNF-α and increase in IL-6 observed with physical activity in these experiments is a lasting effect, and if such effects have biological (cognitive) relevance.

### 5.1.2 Stress Characteristics of Exercise

Stress characteristics of exercise reflect whether the delivery/regimen is forced or voluntary. The use of treadmill-based exercise protocols increases the level of plasma corticosterone in rodents. Ke et al. (2010) compared serum corticosterone levels in Sprague-Dawley rats assigned to seven days of voluntary wheel running, forced treadmill running, or a control group after induction of stroke using a middle cerebral artery occlusion/reperfusion model. Corticosterone concentration was significantly higher in the forced exercise group compared to the control group. Forced exercise stimulated a 210.8% increase in corticosterone relative to controls (i.e., 227.5 ng/ml vs. 73.2 ng/ml), while voluntary exercise led to only a 5.95% (i.e., 77.5 ng/ml) increase in this hormone. Similar results were obtained by Hayes et al. (2008), who assigned Sprague-Dawley rats to 3 weeks of voluntary wheel running, forced treadmill running, or sedentary conditions. Serum corticosterone was significantly elevated in forced runners (277.2 ng/ml) compared to both the voluntary (164.6 ng/ml) and sedentary (173.2 ng/ml) groups. In that study, forced exercise stimulated a 60% increase in corticosterone relative to controls, and voluntary exercise was without effect. In the forced treadmill exercise described in Chapter 3, plasma corticosterone was significantly higher after cessation of the acute exercise bout compared to sedentary controls. The present study also found that voluntary training did not significantly affect baseline corticosterone levels compared to untrained mice (see Appendix D for results). A 166.1% increase in corticosterone was observed with forced treadmill exercise

(NOTREAD vs. TREAD-Imm), while only a 1.2% increase was noted with voluntary training (WR vs. No WR). Forced exercise is clearly associated with increased physiological stress response, and, as mentioned in the previous section, may play a role in maintenance of cognitive function during periods of strenuous physical activity.

### 5.1.3 Tissue Compartment: Hippocampal Cells vs. Intestinal Lymphocytes

The impact of physical activity on the apoptotic status of cells within the hippocampus of healthy mice was assessed. In both acute exercise and training experiments described in this thesis, no differences in the percentage of apoptotic or necrotic hippocampal cells were observed. Previous work demonstrated that long-term voluntary training reduces apoptosis of submandibular and intestinal lymphocytes (Boudreau and Hoffman-Goetz, 2006; Hoffman-Goetz et al., 2010). In contrast, acute exhaustive exercise increased lymphocyte apoptosis systemically (Hoffman-Goetz and Quadrilatero, 2003). That neither acute exercise nor training had any effect on apoptosis in the hippocampus (at least at the time points measured) suggests that this compartment may be protected against stress-induced cell loss. Increases in leukocyte and macrophage numbers in the hippocampus tend to be followed by apoptosis, but this only occurs after injury or infection (Babcock et al., 2003). Mice were not exposed to any infectious or injurious agents in the experiments described in this thesis research. One possibility to explain the lack of apoptotic response of hippocampal cells following acute exercise is that of maintenance of cognitive function in situations where predatory escape is necessary (Maier and Watkins, 1998). Wild mice run in short and fast bursts rather than prolonged bouts (De Bono et al., 2006). Although this may leave the "body" susceptible to infection or injury, it would also allow the brain to remain essentially "unharmed."

Another factor to consider is that of ischemia-reperfusion. There is a significant redistribution of blood flow during exercise; blood flow is increased to active muscles whereas splanchnic circulation (in organs such as the intestine) is greatly decreased (Laughlin et al., 1982). An ischemic decrease in oxygenated blood leads to the accumulation of hypoxanthine and xanthine oxidase, the latter being an enzyme involved in purine catabolism. Upon cessation of exercise, there is significant reperfusion of splanchnic tissues and, consequently, reintroduction of molecular oxygen into tissues. Molecular oxygen reacts with hypoxanthine and xanthine oxidase to produce an oxygen free radical "burst", significantly increasing superoxide anion and hydrogen peroxide, which induce apoptosis in the surrounding cells (Mallick et al., 2004). In the CNS, however, exercise does not normally lead to ischemia-reperfusion injury. Instead, treadmill exercise increases perfusion and decreases vascular resistance in the brain (Delp et al., 2001). A recent study by Zhang et al. (2011) also indicated that moderate intensity exercise (brisk walking) increases cerebral blood flow in women aged 62-79 years.

Why should there be less apoptosis and maintenance of cerebral blood flow? Arora et al. (2009) presented an evolutionary argument for decreased apoptosis in the brain: less apoptosis in the CNS would allow for enhanced neuronal connections to be established. However, the price for increased cognition may be increased risk of uncontrolled cell growth (cancer) and other conditions associated with reduced apoptotic function.

Mouse systemic and central responses to acute exercise also differed. Intestinal lymphocytes were collected from a subset of mice in order to compare cytokine effects in systemic versus central tissue. Acute treadmill exercise led to an increase in TNF-α, IL-1β, and IL-6 in intestinal lymphocytes as has been previously observed (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). Acute exercise was associated with a decreased TNF-α protein

expression in the hippocampus and had no effect on IL-1β protein levels. Systemic tissues may be protected against infection and injury (by increases in pro-inflammatory agents) that may occur in a predatory escape situation, while the brain is relatively clear of these cytokines in order to maintain cognitive function (Maier and Watkins, 1998).

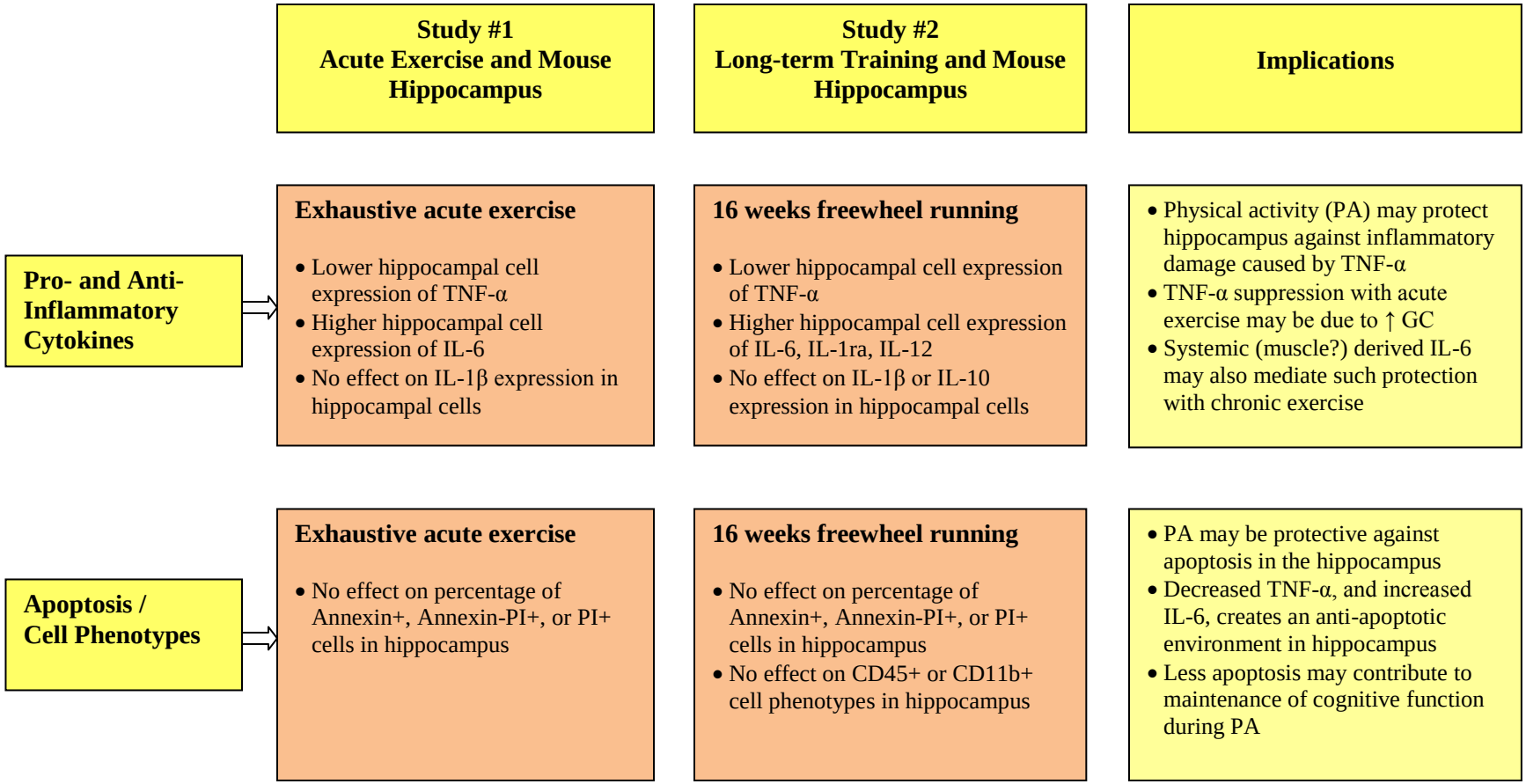
## 5.2 Research Implications and Recommendations

The results of the two experiments described in this thesis show that both acute exercise and long-term exercise training in mice reduce hippocampal cell expression of the pro-inflammatory cytokine TNF-α and increase hippocampal cell expression of IL-6. Lower TNF-α expression in acute/forced exercise may be due to IL-6-induced release of glucocorticoids (corticosterone) which suppresses or dampens TNF-α activity. Increased IL-6, which is able to cross the blood-brain-barrier (Banks et al., 1995), with training may also decrease TNF-α expression by upregulating the secretion of soluble TNF receptor (Pedersen, 2009; Tilg et al., 1994). Neither of the exercise modalities led to changes in the percentage of hippocampal cells undergoing apoptosis. The hippocampal cytokine effects (and lack of apoptosis effects) observed are in contrast to the acute exercise-induced pro-inflammatory cytokine synthesis and lymphocyte apoptosis in the intestine. Taken together, the results suggest that the hippocampus may be protected against exercise-induced inflammatory damage during periods of stress (psychological or physiological). The cytokine changes support the hypothesis that physical activity is neuroprotective, and may reduce the risk of cognitive decline in transgenic mice. A summary diagram of key findings from this thesis research and their implications are shown in Figure 9. A major recommendation is that future experiments include behavioural assessments (e.g., water maze performance) to allow assessment of correlations between exercise-induced microglial cytokine changes and cognitive performance.

With respect to human populations, studies must be designed to determine whether similar cytokine and apoptotic changes occur as observed in animal models. Investigators should consider acute exercise protocols and training interventions with assessment of cerebrospinal fluid cytokine levels in healthy individuals, those with mild dementia and early stage

Alzheimer's disease in future studies. Such investigations will enable researchers and clinicians to determine the exercise "prescription" (type, duration) or "dose" necessary to reduce cognitive decline.

**Figure 9.** Key Findings about Effect of Exercise on Cytokines and Apoptosis in Mouse Hippocampus



## 5.3 Limitations

There are limitations to these research findings, some of which have been described in Chapters 3 and 4. There are, however, additional caveats regarding the experimental methods. Hippocampal cells were isolated for analysis of apoptosis by flow cytometry whereas cytokine concentrations were assessed by Western blotting. It can be argued that the latter is less sensitive than flow cytometry and that quantification of cytokine expression may have been more accurate had flow cytometry been used (Bachelet et al., 1998). Western blotting was used for analysis of cytokine levels to maintain consistency with previous work (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010) and to allow comparisons between hippocampal cells and intestinal lymphocytes. Furthermore, this work only assessed protein expression of cytokines as access to polymerase chain reaction (PCR) equipment was not available. Using PCR would have allowed for determination of mRNA expression and subsequent correlation with cytokine protein levels by Western blotting. These methods would be useful for transcription and translation inferences. Nevertheless, future studies are needed in which multiple techniques are compared for hippocampal and intestinal cell cytokine responses.

Another limitation of the findings is that only plasma corticosterone was used as a marker of stress. Other stress hormones known to change with exercise (e.g., epinephrine and norepinephrine) were not evaluated. Corticosterone was used as the main measure of stress as plasma levels of this hormone have been implicated in lymphocyte apoptosis in response to strenuous exercise (Hoffman-Goetz and Quadrilatero, 2003). Using corticosterone allowed for comparison with previous studies (Hoffman-Goetz et al., 2010) and assessment of plasma corticosterone levels in relation to hippocampal apoptosis. Catecholamines and other stress hormones, however, should be considered to further explore connections between stress,

cytokine expression, and apoptosis in the hippocampus in relation to cognitive decline. Similar comments can be made regarding the use of plasma 8-isoprostanes versus other markers of oxidative damage in hippocampal cells. The reason that this marker was chosen is that it is a common measure of oxidative stress (Huang et al., 2010), and is known to increase in plasma after acute exercise (Steensberg et al., 2002). Measuring plasma 8-isoprostanes allowed for the confirmation that the acute exercise protocol used in Study #1 was sufficient to induce systemic oxidative stress.

The research in this thesis focused exclusively on mice and this, as with any animal model study, limits the extent to which the results can be applied to humans. Moreover, only female mice were used. However, females are better runners than males of the C57BL/6 strain and have greater distances run and less bout-length attrition (De Bono et al., 2006). Female C57BL/6 mice also run for longer periods of times at higher speeds than males (Koteja et al., 1999). Finally, female C57BL/6 mice have been used extensively in studies of exercise and lymphocyte apoptosis and intestinal lymphocyte cytokines; hence, there is a large database on the "expected" cytokine response in this gender and strain.

## 5.4 Concluding Comment

In conclusion, a single bout of intense aerobic treadmill running in healthy female C57BL/6 mice did not affect the percentage of apoptotic hippocampal cells, but decreased the expression of the pro-inflammatory cytokine TNF-α and increased IL-6 in the hippocampus immediately and 2 hours after exercise. These changes in the brain did not mirror the cytokine changes observed in intestinal lymphocytes. In the intestine, the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) was increased after intense exercise. It is proposed that the exercise-associated oxidative stress is not sufficient to generate damaging (apoptotic) effects in the central nervous system, and this compartment may be protected to preserve cognitive function during "fight or flight". It is also suggested that exercise-induced elevation in corticosterone may be one mechanism to explain the pattern of hippocampal cytokine expression. Glucocorticoids can increase or reduce central levels of IL-6 and TNF-α, respectively. Future studies on physical activity and CNS expression of specific pro- (e.g., caspases, Bax) and anti- (e.g., Bcl-2) apoptotic proteins and other pro-inflammatory cytokines will be necessary. Whether reducing or blocking glucocorticoid release (e.g., adrenalectomy) affects brain pro-inflammatory cytokine response to acute exercise stress remains to be determined.

Freewheel running for 16 weeks in healthy young female C57BL/6 mice was associated with decreased expression of the pro-inflammatory cytokine TNF-α, and increased expression of the pleiotropic cytokine IL-6, the anti-inflammatory cytokine IL-1ra, and pro-inflammatory cytokine IL-12 in hippocampus. It is proposed that training-induced IL-6 may reduce hippocampal TNF-α through an increase in soluble TNF receptor secretion. These training-induced cytokine changes suggest a complex biology rather than a simple pro vs. anti-

inflammatory dichotomy and a potential "cytokine/immune mechanism" in brain health. Training did not alter the percentage of $CD45^+$ or $CD11b^+$ hippocampal cell subsets nor their apoptotic status. Whether the observed cytokine changes in the hippocampus are due to increased expression in other tissues such as skeletal muscle and uptake into the CNS or diffuse from other brain regions is an area of future research.

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# APPENDIX A:

## Sample Size Calculation

**Sample Size Calculation**

Sample sizes were calculated based on 1-β = 80%, α=0.05 and a two-sample t test using results from work previously published (Hoffman-Goetz et al., 2009). The standard deviations and assumed treatment effects (difference between WR and NWR) for TNF-α is given below.

| Sample Size | | | |
|---|---|---|---|
| Measure | Standard Deviation | Difference* | Sample Size |
| TNF-α | 0.325 | 0.3 | 20 |

* in densitometric units

The sample size calculations were based on the formula provided by Case and Ambrosius (2007) of $n = 2(z_{1-\alpha} + z_{1-\beta})^2\sigma^2 / (\mu_1 - \mu_2)^2$ . Thus, given 3 time points for oxidative stress challenge/acute exercise conditions (NoTREAD, TREAD-IMM, TREAD-2h), the sample size required was a minimum 20 mice for each time point and for each training condition (WR, No WR). A total of 120 mice were acquired for this thesis research, in order to account for any accidental loss of animals, and to allow for the performance of practice dissections. Analysis of some outcome measures occurred with fewer than 20 mice, due to sample loss or insufficient quantity of antibodies.

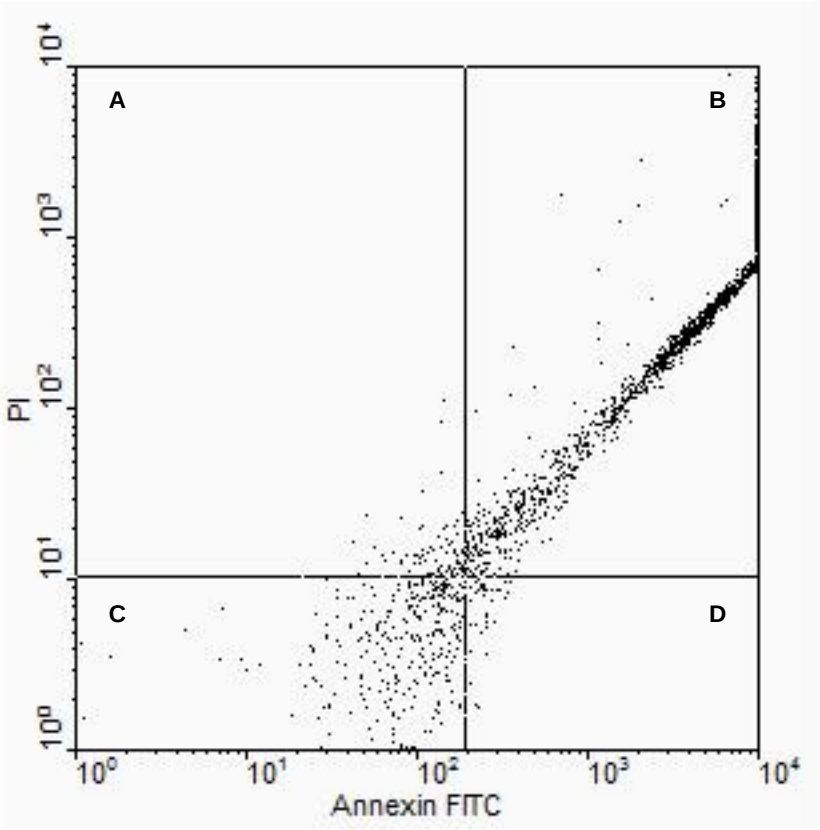
# APPENDIX B:

# Flow Cytometry

Flow cytometry was utilized for the quantification of hippocampal cells undergoing early or late phase apoptosis, or necrosis. The published manuscripts provided in Study #1 and Study #2 do not provide the full methodology used in this technique. Full details are provided below, and an example Flow Cytometry Histogram is given on the following page.

**Flow Cytometry**

Analysis of apoptotic status was performed by flow cytometry (Epics XL Flow Cytometer, Beckman Coulter, Hialeah, FLA, USA) equipped with a 488nm excitation argon laser and emission detection filters at 525nm (green fluorescence) and 575nm (red fluorescence). Apoptosis was determined using Annexin-V, which binds with high affinity to phosphatidylserine externalized on the cell membrane during early apoptosis (Vermes et al, 1995) and propidium iodide (PI), a stain that breaches cells in the late stage of apoptosis and necrosis. This technique has been used extensively in this lab (Hoffman-Goetz and Quadrilatero, 2003; Spagnuolo and Hoffman-Goetz, 2008). 1 x $10^5$ hippocampal cells were incubated for 15 min in the dark with 2.5 μl of Annexin V-FITC (Pharmingen, San Diego, CA, USA), 2.5 μl of Propidium Iodide (PI) (Sigma Chemical, St. Louis, MO, USA), and 100 μl of Annexin binding buffer, in the dark, at room temperature. After incubation the cells were washed, centrifuged, and resuspended in 400μl of binding buffer and analyzed at an excitation wavelength of 488 nm.

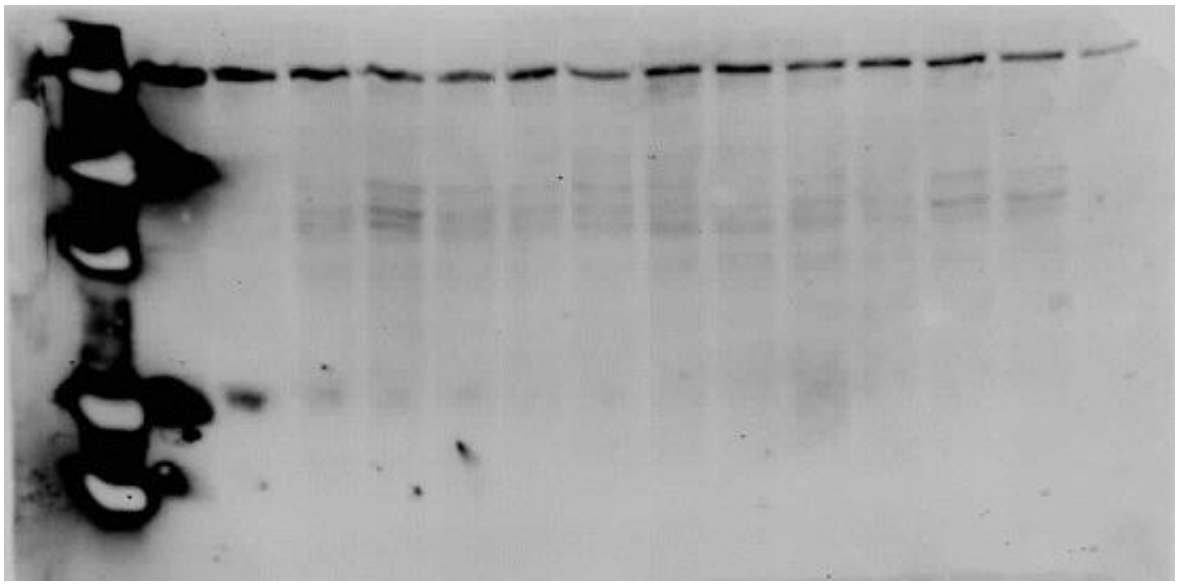



Representative histogram from flow analyses of apoptotic and necrotic hippocampal cells. Hippocampal lymphocytes incubated with Annexin V-FITC and PI. *Quadrant A:* Cells that are $Annexin^{-}PI^{+}$. *Quadrant B*: Cells that are $Annexin^{+}PI^{+}$. *Quadrant C*: Cells that are $Annexin^{-}PI^{-}$. *Quadrant D*: Cells that are $Annexin^{+}PI^{-}$.

# APPENDIX C:

# Other Cytokines

An additional cytokine evaluated in Study #2, but not included in analysis, was the pro-inflammatory cytokine IFN-γ. This protein was excluded from the data, as the Western blot images for this cytokine appeared to be contaminated (spill-over between wells), and the images were of poor quality which prevented adequate resolution for analysis. An example image is provided below.

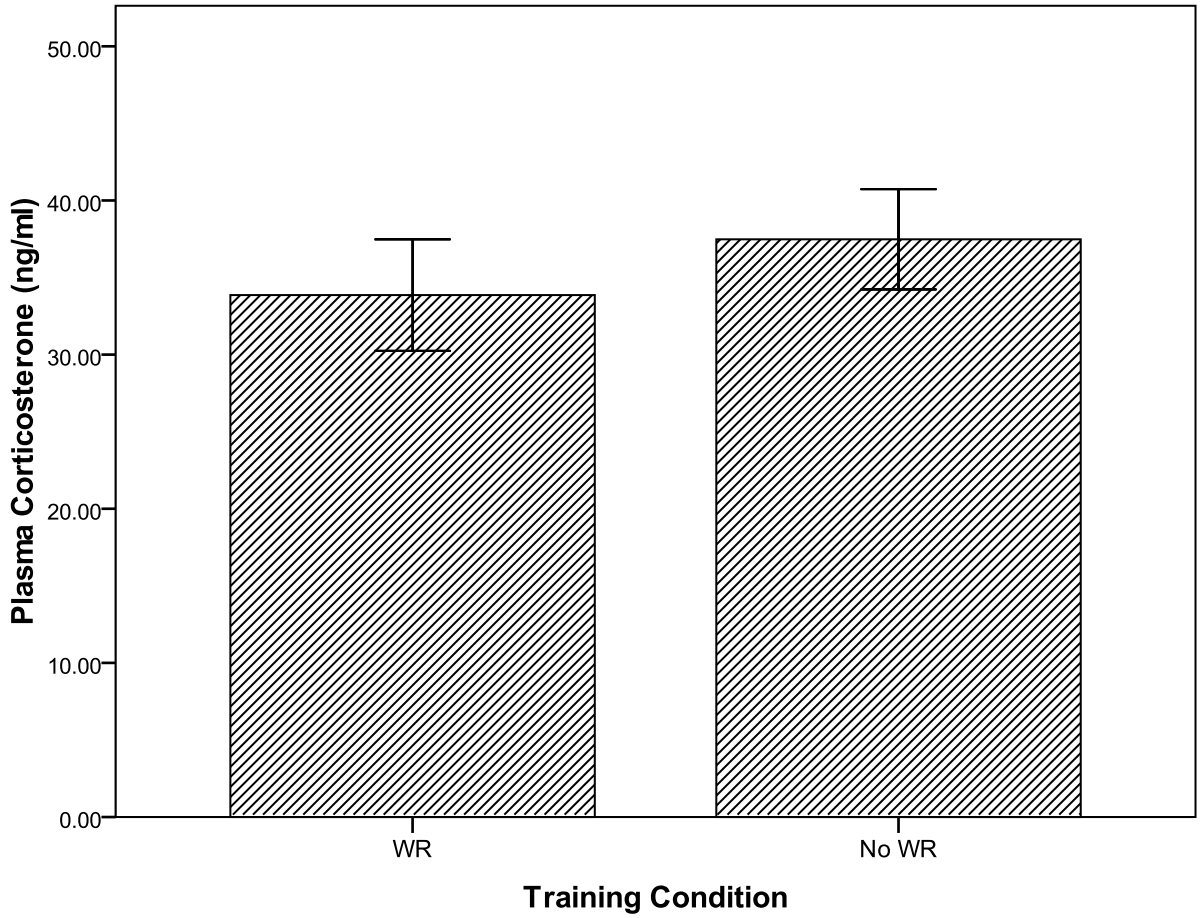


# APPENDIX D:

# Additional Results

Study #2 did not discuss plasma corticosterone levels in trained or untrained mice (WR vs. No WR), although these data was collected for all animals. The results of training and plasma corticosterone concentrations are provided in the table below.

| **Group** | **Plasma corticosterone (ng/mL) (Mean ± SEM)** | **F (df)** | **p** |
|---|---|---|---|
| WR | 33.9 ± 3.4 | 0.55 (1, 39) | 0.46 |
| No WR | 33.5 ± 3.4 | | |



## APPENDIX E:

## Statistical Analysis Data

Examples of statistical analysis output performed using SPSS 17 is provided below.

**Study #1:** Plasma 8-isoprostanes Data

## Univariate Analysis of Variance

**Between-Subjects Factors**

| | | Value Label | N |
|---|---|---|---|
| Exercise | 1.00 | Sed. | 17 |
| | 2.00 | Imm. | 16 |

**Descriptive Statistics**

Dependent Variable:Plasma8iso

| Exercise | Mean | Std. Deviation | N |
|---|---|---|---|
| Sed. | 73.5322 | 16.97590 | 17 |
| Imm. | 97.2889 | 31.14509 | 16 |
| Total | 85.0506 | 27.27925 | 33 |

**Levene's Test of Equality of Error Variances[a]**

Dependent Variable:Plasma8iso

| F | df1 | df2 | Sig. |
|---|---|---|---|
| .572 | 1 | 31 | .455 |

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

**Levene's Test of Equality of Error Variances[a]**

Dependent Variable:Plasma8iso

| F | df1 | df2 | Sig. |
|---|---|---|---|
| .572 | 1 | 31 | .455 |

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Exercise

**Tests of Between-Subjects Effects**

Dependent Variable:Plasma8iso

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 4651.890[a] | 1 | 4651.890 | 7.526 | .010 |
| Intercept | 240512.728 | 1 | 240512.728 | 389.115 | .000 |
| Exercise | 4651.890 | 1 | 4651.890 | 7.526 | .010 |
| Error | 19161.153 | 31 | 618.102 | | |
| Total | 262522.027 | 33 | | | |
| Corrected Total | 23813.043 | 32 | | | |

a. R Squared = .195 (Adjusted R Squared = .169)

**Parameter Estimates**

Dependent Variable:Plasma8iso

| Parameter | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | B | Std. Error | t | Sig. | Lower Bound | Upper Bound |
| Intercept | 97.289 | 6.215 | 15.653 | .000 | 84.613 | 109.965 |
| [Exercise=1.00] | -23.757 | 8.660 | -2.743 | .010 | -41.418 | -6.095 |
| [Exercise=2.00] | 0[a] | . | . | . | . | . |

a. This parameter is set to zero because it is redundant.

## Estimated Marginal Means

### 1. Grand Mean

Dependent Variable:Plasma8iso

| | | 95% Confidence Interval | |
|---|---|---|---|
| Mean | Std. Error | Lower Bound | Upper Bound |
| 85.411 | 4.330 | 76.580 | 94.241 |

### 2. Exercise

Dependent Variable:Plasma8iso

| Exercise | | | 95% Confidence Interval | |
|---|---|---|---|---|
| | Mean | Std. Error | Lower Bound | Upper Bound |
| Sed. | 73.532 | 6.030 | 61.234 | 85.830 |
| Imm. | 97.289 | 6.215 | 84.613 | 109.965 |

**Study #2:** Soleus Cytochrome C Oxidase Data

## Univariate Analysis of Variance

**Between-Subjects Factors**

| | | Value Label | N |
|---|---|---|---|
| Group | 1.00 | WR | 17 |
| | 2.00 | No WR | 19 |

**Descriptive Statistics**

Dependent Variable:CytoCSoleus

| Group | Mean | Std. Deviation | N |
|---|---|---|---|
| WR | 18.0769 | 1.84690 | 17 |
| No WR | 12.4823 | 2.69958 | 19 |
| Total | 15.1242 | 3.65115 | 36 |

**Levene's Test of Equality of Error Variances[a]**

Dependent Variable:CytoCSoleus

| F | df1 | df2 | Sig. |
|---|---|---|---|
| 1.302 | 1 | 34 | .262 |

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Group

**Tests of Between-Subjects Effects**

Dependent Variable:CytoCSoleus

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 280.825[a] | 1 | 280.825 | 51.401 | .000 |
| Intercept | 8378.839 | 1 | 8378.839 | 1533.625 | .000 |
| Group | 280.825 | 1 | 280.825 | 51.401 | .000 |
| Error | 185.756 | 34 | 5.463 | | |
| Total | 8701.265 | 36 | | | |
| Corrected Total | 466.582 | 35 | | | |

a. R Squared = .602 (Adjusted R Squared = .590)

**Parameter Estimates**

Dependent Variable:CytoCSoleus

| Parameter | | | | | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | B | Std. Error | t | Sig. | Lower Bound | Upper Bound |
| Intercept | 12.482 | .536 | 23.278 | .000 | 11.393 | 13.572 |
| [Group=1.00] | 5.595 | .780 | 7.169 | .000 | 4.009 | 7.180 |
| [Group=2.00] | 0[a] | . | . | . | . | . |

a. This parameter is set to zero because it is redundant.

## Estimated Marginal Means

### 1. Grand Mean

Dependent Variable:CytoCSoleus

| | | 95% Confidence Interval | |
|---|---|---|---|
| Mean | Std. Error | Lower Bound | Upper Bound |
| 15.280 | .390 | 14.487 | 16.073 |

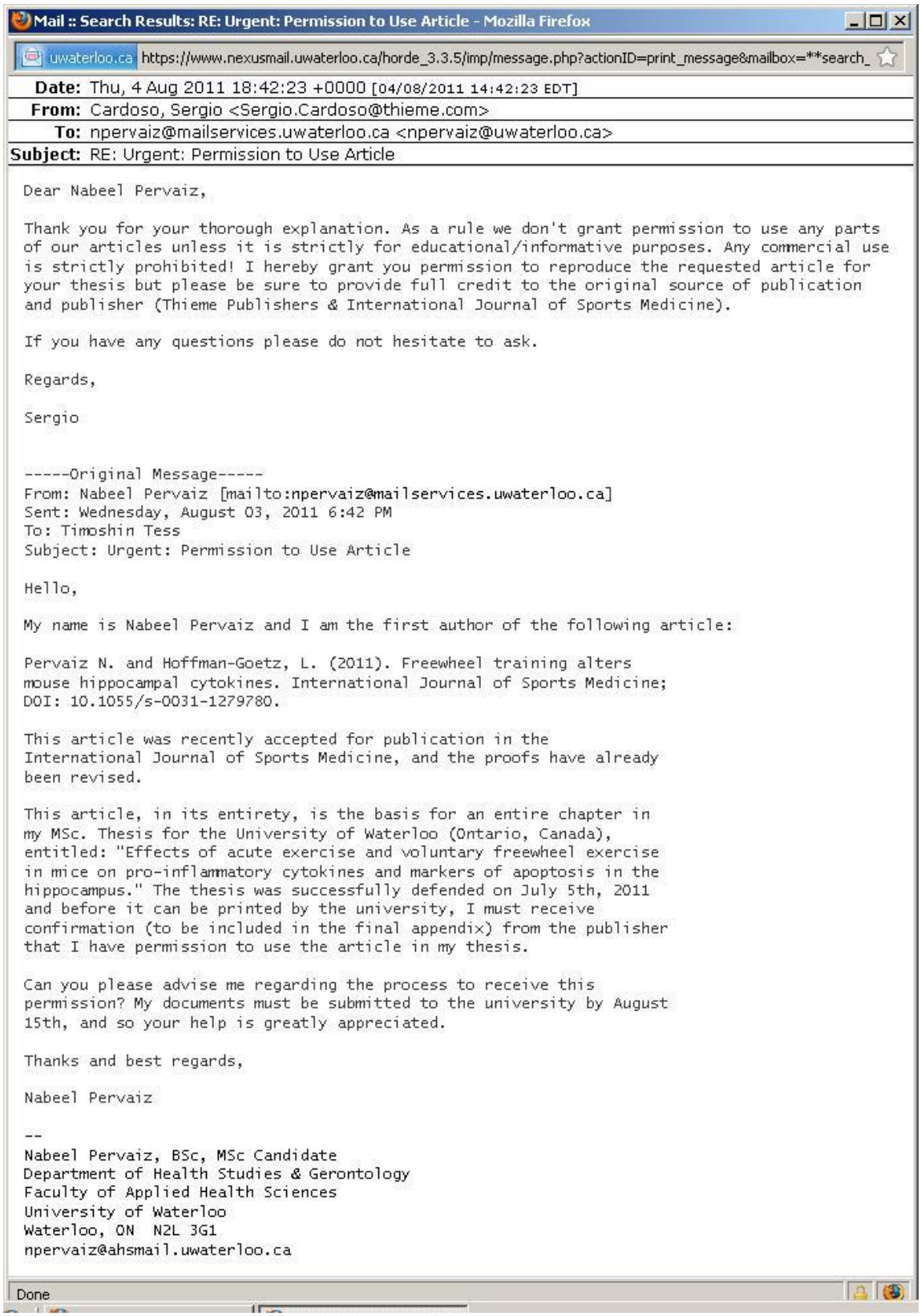
### 2. Group

Dependent Variable:CytoCSoleus

| | | | 95% Confidence Interval | |
|---|---|---|---|---|
| Group | Mean | Std. Error | Lower Bound | Upper Bound |
| WR | 18.077 | .567 | 16.925 | 19.229 |
| No WR | 12.482 | .536 | 11.393 | 13.572 |

## APPENDIX F:

## Permission to Print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Date: Thu, 4 Aug 2011 18:42:23 +0000 [04/08/2011 14:42:23 EDT]
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To: npervaiz@mailservices.uwaterloo.ca <npervaiz@uwaterloo.ca>
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Regards,
Sergio
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Sent: Wednesday, August 03, 2011 6:42 PM
To: Timoshin Tess
Subject: Urgent: Permission to Use Article
Hello,
My name is Nabeel Pervaiz and I am the first author of the following article:
Pervaiz N. and Hoffman-Goetz, L. (2011). Freewheel training alters mouse hippocampal cytokines. International Journal of Sports Medicine; DOI: 10.1055/s-0031-1279780.
This article was recently accepted for publication in the International Journal of Sports Medicine, and the proofs have already been revised.
This article, in its entirety, is the basis for an entire chapter in my MSc. Thesis for the University of Waterloo (Ontario, Canada), entitled: "Effects of acute exercise and voluntary freewheel exercise in mice on pro-inflammatory cytokines and markers of apoptosis in the hippocampus." The thesis was successfully defended on July 5th, 2011 and before it can be printed by the university, I must receive confirmation (to be included in the final appendix) from the publisher that I have permission to use the article in my thesis.
Can you please advise me regarding the process to receive this permission? My documents must be submitted to the university by August 15th, and so your help is greatly appreciated.
Thanks and best regards,
Nabeel Pervaiz
--
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Done