

BOCCHINE, ANTHONY J., M.S. Investigation into Hippocampal-Dependent Memory:
The Effects of Exercise and Nutrition. (2016)
Directed by Dr. Jennifer L. Etnier. 86 pp.

The purpose of this research study was to evaluate the combined effect of chronic exercise training and nutrition on a hippocampal-dependent cognitive task in rodents. Male adult Sprague-Dawley rats ($n=48$) were randomly assigned to a 5- or 6-week forced exercise protocol (trained) or to a sedentary control condition (untrained). The exercise (during last hour of light cycle) duration and intensity were increased over a 4-wk period up to 60 min at 30 m/min and a 6° grade. The weekly exercise consisted of 5 days of exercise followed by 2 days of rest. Rats were on a regular feeding schedule consisting of a 30 min meal (20% daily calories) provided 60 min into the dark period and ad libidum access to chow the last 5 hrs of the dark cycle throughout (remainder of daily calories).

During the last week of exercise, rats' memory was assessed 60 min after a low (15% casein) or high (50% casein) protein meal in a Barnes maze (a task requiring that rodents detect an escape hatch in a fixed location) on 4 consecutive days. BCA biochemical assays were conducted in order to determine protein concentrations from hippocampal brain samples. The BCA assays produced a standard curve that can be used to determine the protein content of the hippocampal samples and a protocol that can be used to homogenize these samples for a more specific assay. Results for path latency in the Barnes maze indicated a main effect for days ($p<0.05$), but no effect of training. There was an observed trend that animals fed a low protein meal prior to the memory task performed worse than high protein groups. Future studies could investigate different

intensities of exercise and volumes of training, in addition to considering the combined effects of exercise and diet on hippocampal-dependent memory.

INVESTIGATION INTO HIPPOCAMPAL-DEPENDENT MEMORY:
THE EFFECTS OF EXERCISE
AND NUTRITION
by
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A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2016

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CHAPTER I

INTRODUCTION

The human brain is composed of billions of neurons that are susceptible to degeneration as one grows older (Erickson & Kramer, 2009; Kramer & Erickson, 2007). As average lifespans are getting longer and adults are living to be much older, this opens the door for a myriad of biological pathologies which threaten brain health (Erickson & Kramer, 2009; Kramer & Erickson, 2007). Neurodegenerative diseases are clinical conditions characterized by the death of neurons and ultimately loss of white matter in brain regions essential to cognitive function (Nagamatsu et al., 2014). Importantly, there is evidence that lifestyle behaviors are associated with a person's risk for developing neurodegenerative conditions (Erickson & Kramer, 2009). For this reason, research is focusing on modifiable habits such as physical activity that have been shown to prevent the cascade of pathological mechanisms which can lead to neurodegeneration (Boyle et al., 2015; Erickson & Kramer, 2009; Hillman, Erickson, & Kramer, 2008). It is becoming increasingly clear that all humans do not experience the effects of senescence at the same rate. There are promising results from cross-sectional studies and randomized control trials which indicate that regular physical activity benefits cognitive function and other aspects of brain health (Bherer, Erickson, & Liu-Ambrose, 2013; Colcombe et al., 2006; Kramer, Erickson, & Colcombe, 2006).

The idea that regular physical exercise can protect brain cells from degeneration even as an individual ages is expanding new lines of research. The current knowledge on the topic highlights such benefits of regular exercise to include improved cognitive performance, enhanced neuroplasticity, and larger hippocampal volume (Colcombe et al., 2006; Erickson et al., 2011). The enhancements in neuroplasticity observed through regular exercise in humans occur in multiple brain regions (Kramer & Erickson, 2007; Voss et al., 2010). The concept of neuroplasticity means that neurons are changeable and allows for branching of new connections to other areas of the brain (Voss, Vivar, Kramer, & van Praag, 2013). It has been proposed that regular exercise is involved with inducing beneficial mechanisms related to neuroplasticity and consequently would be neuroprotective against degeneration (Erickson & Kramer, 2009). A brain area of interest where this positive relationship of exercise and neural plasticity can be observed is the hippocampus, a brain structure that is largely involved with cognitive function (Erickson & Kramer, 2009; Erickson et al., 2011). The majority of what is known about aerobic exercise and cognitive function in humans has been elucidated by research studies that look at the internal architecture of the brain, where the primary structure consists of white matter tracts and connections (Kramer & Erickson, 2007; Voss, Heo, et al., 2013). These white matter connections allow neuron to neuron communication and feedback between the different brain structures (Chaddock-Heyman et al., 2014; Kramer & Erickson, 2007; Voss, Heo, et al., 2013).

There is a positive correlation between regular exercise and white matter volume within specific regions of the brain that are known to function in cognitive processes

(Erickson, 2013). A greater amount of white matter within a brain structure is associated with enhanced connectivity, which allows for faster processing speed and more efficient function (Erickson et al., 2011). This relationship between chronic exercise and cognitive function has been examined in humans through experimental measurement of the white matter volume in the hippocampus using neuroimaging techniques such as functional magnetic resonance imaging (fMRI) (Erickson & Kramer, 2009; Erickson et al., 2011).

Statement of Problem

Current research is aiming to address the biological plausibility of exercise as a means for improving cognitive performance and to understand by what means it enhances neuroplasticity of the hippocampus in humans. The majority of studies that have examined spatial learning and memory processes in humans have used structural and fMRI to measure changes in neural organization as well as white matter volume, which is indicative of neural integrity and growth (Erickson et al., 2011; Szabo et al., 2011). A common limitation in human research is the inability to measure neural factors *in vivo*; this requires more invasive approaches using chronic exercise and an animal model to investigate the hippocampus at the cellular and molecular levels (Voss, Vivar, et al., 2013).

Chronic exercise research in rodents has begun to clarify mechanisms related to how physical activity affects the structure of the hippocampus and its function (Vaynman & Gomez-Pinilla, 2006; Voss, Erickson, et al., 2013). Studies that used behavioral measures such as the Morris water maze and the Barnes maze as a spatial memory testing paradigm found improved performance in rodents who were regularly exercised (Gomes

da Silva et al., 2012; Voss, Vivar, et al., 2013). There is also evidence that neurogenesis and larger volumes of white matter are detectable via immunostaining in the hippocampus of rodents who were exercised compared to sedentary rodents (Gomes da Silva et al., 2012). The current literature shows new neurons can grow through neurogenesis in the central nervous system (CNS), it also indicates that the majority of new neurons are migrating from various subfields of the hippocampus (Gomes da Silva et al., 2012; van Praag, 2008). It is supported that the hippocampus is neuroplastic and that underlying cellular and molecular mechanisms of neuroplasticity are propagated in response to chronic exercise (van Praag, 2009; Vaynman & Gomez-Pinilla, 2006). The biological plausibility of using chronic exercise to improve hippocampal-dependent memory and neuroplasticity was established with animal studies that measured synaptic plasticity, long-term potentiation (LTP), and the expression of neurotrophic factors (Bizon et al., 2009; O'Callaghan, Ohle, & Kelly, 2007; Voss, Vivar, et al., 2013). Investigation has focused on neurotrophic factors that are characteristically expressed after training in rodents including BDNF, insulin growth factor (IGF), and tyrosine kinase B (TrkB) (Voss, Erickson, et al., 2013). The majority of experimental studies have investigated BDNF, finding that it signals and binds to the BDNF-TrkB mediated pathway, which enhances the growth and survival of neurites, or precursors to neuron stem cells (Aguiar Jr et al., 2011; Erickson, Miller, & Roecklein, 2012; Voss, Erickson, et al., 2013). There is a need to investigate other mediating factors involved in the effects of chronic exercise on hippocampal-dependent memory and its neuroplasticity. This

curiosity has led to further research into cell signaling factors, such as cytokines, which are known to be involved in regulating different cellular processes.

Cytokines are a broad category of small pleiotropic proteins, which function in cell signaling via binding of membrane-bound receptors located on target cells (Petersen & Pedersen, 2005). Cytokines can be classified through the type of receptor to which they bind and are released by a secretory cell to influence the behavior of their target cells (Pedersen & Fischer, 2007; Petersen & Pedersen, 2006). Cytokines are produced primarily in the immune system, however recent studies in rodents support the secretion of cytokines through metabolic, neural, and endocrine cells (Pedersen & Fischer, 2007). The contraction of skeletal muscle during exercise elicits many physiological changes, including the release of various cytokines into the circulation (Petersen & Pedersen, 2005). Factors such as cytokines that are secreted into the blood circulation in this manner have been termed “exercise-induced” factors because they are directly stimulated by skeletal muscle contraction during physical exercise (Pedersen & Fischer, 2007; Petersen & Pedersen, 2005, 2006).

One cytokine that has been demonstrated to cross the blood brain barrier (BBB), interleukin-6 (IL-6), has recently drawn interest in the exercise and cognition research. Through work done by Pederson et al. (2002), the release of the cytokine IL-6 during exercise was observed to target multiple organs (e.g., the liver, adipose tissue, and heart) as a hormone. It is now supported that IL-6 expression is upregulated in the CNS following chronic exercise, where it can be secreted by neuron support cells known as astrocytes (Kalashnyk, Lykhamus, Oliynyk, Komisarenko, & Skok, 2014; Nybo, Nielsen,

Pedersen, Moller, & Secher, 2002). The skeletal muscle is suggested to function as a peptide-releasing endocrine organ, making it possible that contracting skeletal muscle may influence brain metabolism as a result of exercise (Pedersen & Febbraio, 2005; Pedersen & Fischer, 2007). Such regulation could be mediated via muscle-derived IL-6 or other candidate exercise-induced factors (Pedersen & Febbraio, 2005).

The investigation of the effects of cytokines on neural cells is rather new, accordingly there is little information about how they affect cells of the CNS (Rasmussen et al., 2011). A cytokine is neurotrophic if it is secreted by a neuronal cell in either the periphery or the CNS and then proceeds to bind to receptors embedded in membranes of neural cells specifically (Pedersen & Febbraio, 2005; Rasmussen et al., 2011). The notion that cytokines work in the CNS came through work with astrocyte cell cultures and rodent models which simulate brain injury and neurodegeneration (Leibinger et al., 2013; Nakanishi et al., 2007). IL-6 is a neurotrophic cytokine that responds to various forms of brain inflammation and mediates recruitment of cells/proteins involved in the immune response to migrate to the point of injury for repair of damaged tissue (Leibinger et al., 2013; Pan, Yu, Hsueh, Zhang, & Kastin, 2008). IL-6 can signal as a pro-inflammatory factor during inflammation or bodily injury, and this response is dependent on the activation of other signaling pathways, such as tumor necrosis factor (TNF) or nuclear factor kappa light chain enhancer of B cell (NFkB) (Wu et al., 2008). It is thought that IL-6 can serve the antagonistic function when acting as a factor produced by skeletal muscle (Petersen & Pedersen, 2005). Thus, IL-6 could serve a different function when acting as an exercise-induced factor produced by skeletal muscle and secreted into the

circulation as compared to its innate pro-inflammatory response (Petersen & Pedersen, 2005). During exercise it has been shown that IL-6 is produced by muscle fibers via a TNF-independent pathway, which makes this signaling event different than the innate inflammatory response (Pedersen & Febbraio, 2005; Petersen & Pedersen, 2006). This is important to note because if IL-6 plays a role in signaling neurons in the hippocampus to undergo neurogenesis, the result of the signaling cascade in response to exercise might be different than the inflammatory response associated with apoptosis.

Rasmussen and colleagues (2011) found that IL-6 is released in the brain both during and after exercise. Their findings suggested that since IL-6 mRNA levels and glycogen content were elevated in the hippocampus and no other areas of the brain, that the hippocampus was the source of IL-6 production in the brain both during and following exercise (Rasmussen et al., 2011). These results are important for the involvement of IL-6 in hippocampal function because they help establish plausibility that the cytokine is both secreted and signals in that region. The growth of new neurons through neurogenesis requires several molecular signaling events, primarily mediated through the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway (Leibinger et al., 2013). Current evidence suggests that IL-6 plays a role in activating the JAK/STAT pathway in hippocampal neurons, although further study is needed to confirm the exact role of IL-6 within this molecular signaling pathway.

One recently proposed hypothesis is that the long-term anti-inflammatory effects of exercise may be mediated via effects of exercise leading to a reduction in visceral fat mass (Rasmussen et al., 2011; Sarvas, Niccoli, Walser, Khaper, & Lees, 2014). It is

known that higher levels of visceral fat mass are associated with both brain inflammation and higher levels of pro-inflammatory cytokines, however exercise reduces both significantly. Given that several chronic disorders such as cardiovascular diseases, type 2 diabetes, dementia, and depression are associated with chronic low-grade systemic inflammation, the anti-inflammatory effects of IL-6 may represent a mechanism whereby exercise protects against these disorders (Pedersen & Steensberg, 2002; Petersen & Pedersen, 2005). This anti-inflammatory role of IL-6 has implications for neuronal cell survival and growth, and this effect warrants future research involving exercise and neurodegenerative disease. Several studies suggest evidence of IL-6 secretion and subsequent signaling through local neural immune cells in the CNS (Pedersen & Fischer, 2007; Reihmane & Dela, 2014).

Within the CNS, levels of IL-6 remain low under normal physiological conditions, however during brain injury, inflammation, hypoxia, and certain diseases, there is a marked increase in IL-6 levels with the predominant source of production coming from the astrocytes (Nybo et al., 2002). This same response is observed for IL-6 in response to exercise. An important aspect of astrocytes from studies with human cell cultures is their involvement in secreting factors such as neurotrophins and cytokines that act to signal neural cell proliferation (Nakanishi et al., 2007). This idea is consistent with the rationale that an increased presence of astrocyte-produced IL-6 in the brain is important for maintaining hippocampal neurogenesis, a process that could influence cognitive function (Pedersen & Febbraio, 2005). The question to answer is whether chronic exercise training can induce endogenous IL-6 production from astrocytes in the

hippocampus. A previous investigation by Nakanishi et al. (2007) found that the neuropoietic cytokines IL-6 and leukemia inhibitory factor (LIF) are secreted by astrocytes in the CNS. Both the astrocytes and microglia are known to secrete the IL-6 and LIF cytokines in the CNS, but it is not yet known whether both are secreted in the CNS in response to exercise.

Recent studies have illuminated the presence of another cytokine in the CNS, which is secreted by astrocytes and associated with oligodendrogenesis (Cohen & Fields, 2008; Pan et al., 2008). Leukemia inhibitory factor (LIF) is a pleiotropic cytokine involved in similar cell signaling processes as IL-6 (Pan et al., 2008). LIF plays a role in the exercise-induced inflammatory cascade, after it is actively produced by skeletal muscle during prolonged bouts of exercise (Pedersen & Febbraio, 2005). The signaling functions of LIF are diverse and it is produced by multiple organ tissues, such as neural tissue (Pan et al., 2008). It is currently speculated that LIF can be released into the circulation to travel and enter the CNS via the BBB, although this has not been entirely confirmed *in vivo* (Leibinger et al., 2013; Pan et al., 2008). This research is helpful toward further understanding the role of LIF, since it can be speculated that the cytokine may take part in neurogenic processes in the CNS. To test the idea that presence of LIF is upregulated in brain tissue following chronic exercise, where it can exert local effects on neural stem cells of the hippocampus, it must be first confirmed that significant concentrations of this protein are in hippocampal tissue. The idea that aerobic exercise training can increase the presence of this essential signaling molecule in the CNS has yet to be tested.

Interest in cytokines as a mediator of the effects of exercise on cognitive function is a relatively recent phenomenon. The original aim of this study was to investigate the plasticity of the hippocampus, a major player in learning and memory, with chronic exercise training in rats. The measurement of two cytokines, IL-6 and LIF, was intended to provide pilot data regarding candidate mediators of these exercise-specific effects.

Hypotheses

This study investigated rodents that followed a chronic exercise protocol and a group of sedentary controls. It was hypothesized that significant expression of the IL-6 protein would be detected in the hippocampus and positively correlated with cognitive performance in a hippocampal-dependent learning task. It was also predicted that LIF protein expression levels would be detected in the hippocampus tissue and that a significant positive correlation would be found between expression of LIF protein and cognitive performance in the exercise group.

The rationale for the association between IL-6 and LIF involves their shared binding receptor and molecular signaling activity. IL-6 and LIF are both known to be expressed endogenously in the brain (Nybo et al., 2002; Pan et al., 2008). Whether the expression of LIF protein can be upregulated in hippocampus tissue through chronic exercise training has yet to be explored explicitly. However, this was determined to be out of the scope of the current abilities/experience level of the investigator, so the measure of the protein levels through ELISA was not performed. Instead, it was agreed upon that a necessary step that could advance the project was determination of the protein concentration levels of the hippocampal tissue samples through BCA. This lead to a

series of BCAs that aimed to generate a standard curve to fit a range of different unknown protein sample concentration levels.

Objectives

Thus, the purpose of this study was to investigate the combined effects of exercise and nutrition on hippocampal-dependent learning and memory in rats. Methods of this study included use of a 5-6 chronic exercise protocol as well as two different protein meal compositions (low and high). The primary means for assessing effects of training and protein content of single meal on hippocampal-dependent memory was the Barnes maze task. Distance traveled around the Barnes maze and path latency were measured and analyzed to test for effects of the training protocol and diet composition.

CHAPTER II

REVIEW OF THE LITERATURE

As humans grow older, the structural integrity of their brain gradually declines (Kramer et al., 2006; Park, Polk, Mikels, Taylor, & Marshuetz, 2001). This age-related effect can leave increasing susceptibility to biological pathologies that aggravate cognitive dysfunction, such as Alzheimer's disease (Boyle et al., 2015). Research has been focused on finding effective and low-cost therapies that are related to reversal of the effects experienced during cognitive decline (Colcombe et al., 2006; Erickson, 2013).

Exercise and Cognition

A major example of one such therapy is aerobic exercise, an intervention in current literature supported to be both neuroprotective and helpful in maintaining integrity of white matter connections that can be negatively influenced during age-related cognitive decline (Erickson & Kramer, 2009; Voss, Heo, et al., 2013). The majority of what is known about aerobic exercise and cognitive function in humans has been elucidated by research studies that look at the internal architecture of the brain, where the primary structure consists of white matter tracts and connections (Kramer & Erickson, 2007; Voss, Heo, et al., 2013). These white matter connections constitute the neural circuitry, which allows neuron to neuron communication and feedback between the different brain structures (Chaddock-Heyman et al., 2014; Kramer & Erickson, 2007; Voss, Heo, et al., 2013).

There is a positive correlational relationship between regular aerobic exercise and white matter volume within specific regions of the brain that are known to function in cognitive processes (Erickson, 2013). A greater amount of white matter within a brain structure is associated with enhanced connectivity within its neural circuitry, which allows for faster processing speed and more efficient function (Erickson et al., 2011). This relationship between chronic aerobic exercise and cognitive function has been examined in humans through experimental measurement of the white matter volume in the hippocampus using neuroimaging techniques such as functional magnetic resonance imaging (fMRI) (Erickson & Kramer, 2009; Erickson et al., 2011). A surging area of interest regarding exercise and cognitive function is study of the hippocampus, a brain structure that is largely involved with cognitive functioning (Erickson et al., 2011). The hippocampus is an important part of the temporal lobe that plays a role in learning and memory. A region of the hippocampus known as the hippocampal formation is situated near the medial temporal lobe and is rich in hippocampal neurons (DeCarolis & Eisch, 2010; van Praag, Shubert, Zhao, & Gage, 2005). The cell death or apoptosis of hippocampal neurons is implicated in major symptoms of dementia and neurodegenerative diseases which include Alzheimer's disease (DeCarolis & Eisch, 2010; van Praag et al., 2005). Age-related cognitive decline has been associated with decreasing white matter volume in the hippocampus as well as declining growth of new neurons within its stem cell pool (Erickson et al., 2012; Szabo et al., 2011; van Praag et al., 2005). This loss of hippocampal white matter reduces overall structural integrity, which can lead to major pathological symptoms such as short-term memory loss and

difficulties with executive functions (Erickson et al., 2012; Voss, Heo, et al., 2013). The growth of new neurons is known as neurogenesis and is a crucial mechanism by which recovery of neural function is possible via enhancement of neuroplasticity (DeCarolis & Eisch, 2010; van Praag et al., 2005). Because the hippocampal nuclei deteriorate with age, it is important to identify ways to reverse this pattern of neural degeneration. The current literature points in the direction of chronic exercise training as a means to enhance both cognitive functioning and increase volume of the nuclei within the hippocampus, which is indicative of neurogenesis (Erickson, 2013; Erickson et al., 2011).

The study of chronic exercise and cognitive function in humans tends to focus on both longitudinal studies and randomized control trials, which examine the relationship with measures of hippocampal structure and aerobic fitness level in participants (Erickson & Kramer, 2009; Erickson et al., 2011). A common method used to look at cognitive function and examine its relationship with aerobic exercise training in humans is through the use of MRI, which is a non-invasive technique that images internal brain structure (Erickson et al., 2011). Erickson et al. (2011) designed a single-blind randomized control trial that investigated whether aerobic exercise training affected hippocampal volume and spatial memory, a type of memory associated with a properly functioning hippocampus (Erickson & Kramer, 2009; van Praag et al., 2005). The study compared a group of older adults across a moderate intensity, 3-day a week aerobic exercise intervention and a regime of stretching and toning exercises that served as a control. Results indicated a significant change in total hippocampal volume through MRI analysis following completion of a 1-year aerobic exercise intervention. Another notable

result was a significant increase in plasma blood levels of brain derived neurotrophic factor (BDNF) found in the exercise group; a biomarker for hippocampal neurogenesis, and improved spatial memory from the baseline levels prior to the intervention (Erickson et al., 2011). Studies such as this randomized control trial and others before it helped identify the use of moderate intensity aerobic exercise as a dependable protocol to investigate significant changes in structure and function of the hippocampus in humans following an exercise intervention (Colcombe et al., 2006; Erickson & Kramer, 2009; Hillman et al., 2008).

The investigation of aerobic exercise and its effects on cognitive performance have been examined in humans via several cognitive domains; including executive function and memory (Smith et al., 2010). Voss et al. (2013) conducted a study that looked at the relationship between a 1-year aerobic exercise intervention and cognitive function in older adults using a multitude of neurocognitive tests. They utilized diffusion tensor imaging (DTI) to obtain tractographic maps of participants' brain structure as a means to measure cerebral white matter integrity. The point of this work was to elucidate the effects of aerobic exercise on specific regions of the brain associated with cognitive function. Tests used included the forward and backward digit span task, spatial working memory task, task-switching, and the Wisconsin Card Sorting Task (Voss, Heo, et al., 2013). Findings included a significant association between improved aerobic fitness and change in white matter integrity especially in the prefrontal and temporal brain regions (Voss, Heo, et al., 2013). Given this evidence supporting that aerobic exercise results in improvements in white matter integrity, it is important to understand the specific

mechanisms that exercise is impacting at a neurobiological level. Although there is evidence to support mechanisms of moderate aerobic exercise in humans, there are more feasible approaches to identifying factors through the use of animal models. Following up the neuroimaging studies in humans with cellular, molecular and biochemically-based techniques using an animal model is common in translational neuroscience research. This allows for a closer look at the physiology of the brain and its neural circuitry, providing information on a microscale level that is not currently observable in human studies (Voss, Vivar, et al., 2013).

Neurobiological Mechanisms

Studies that examine specific biological mechanisms of aerobic exercise and cognitive function have advanced primarily through study of animal models, more specifically in rodents (Voss, Vivar, et al., 2013). A review by Voss et al. (2013) brought together the current state of the literature on human and animal models of exercise and cognition research. The primary topic of the review involves exercise-induced brain plasticity, an expression that is central to this area of research. Brain plasticity itself is an all-inclusive term, however exercise-induced brain plasticity is more specific as it only refers to changes related to physical exercise. Knowledge of what specific factors of exercise-induced brain plasticity are measurable in both animals and humans can help address important questions in the field. One of the questions being asked involves the comparison of environmental enrichment (EE) with aerobic exercise and how these treatments may affect neurogenic factors underlying spatial learning in rodents (Voss, Vivar, et al., 2013).

There is support from research using voluntary wheel running in comparison with EE that reveal it is the physical exercise alone that is significantly modulating neurogenic factors, which include synaptic plasticity, neurotrophin levels, spine density, and neurogenesis (van Praag, 2008; Voss, Vivar, et al., 2013). Research with the hippocampus accentuates the finding that exercise elicits both structural and functional changes that are advantageous to the formation of new memory and navigational skills (Erickson et al., 2011; Voss, Vivar, et al., 2013). It is imperative to answer the question regarding how exercise impacts neurogenesis such that spatial learning and memory are improved by exercise. Understanding the interplay between the biological and psychological effects of exercise on the hippocampus is paramount to advance our understanding of precisely how exercise benefits cognition.

Use of both voluntary and forced exercise paradigms in animal research has been shown to augment spatial memory in behavioral tests such as the Barnes maze and Morris water maze (van Praag, 2009). The knowledge of the cellular mechanisms that underlie changes to learning and memory has advanced through analysis of rat brain tissue in the hippocampus (van Praag, 2008). A study by van Praag and colleagues (1999) was among the first to examine the modifications to neuron structure in the hippocampus through studying exercised and sedentary mice brain tissue through both electrophysiological and immunostaining methods. This study and a follow-up study (van Praag et al., 2005) using aged mice found that there are quantifiable changes in long-term potentiation (LTP) with exercise training that could result in enhancement of spatial learning and memory. A major concurrent benefit of inducing LTP in the hippocampus through exercise is thought

to be neurogenesis. The idea that neurogenesis is involved in learning and memory was investigated by Kempermann et al. (1997) through a study by putting mice in an EE and quantifying changes in synaptic density in the CA1 and CA3 subfields of the hippocampus as well as the dentate gyrus in the hippocampus through immunohistochemical staining. Inducing neurogenesis through LTP involves multiple changes including growth factor gene expression, migration of new granule cells, and subsequent maturation of new neurons (Cotman, Berchtold, & Christie, 2007a; Wu et al., 2008). Research with exercise in both rats and mice suggests that the training induces these processes and makes LTP more likely to occur in the hippocampus (O'Callaghan et al., 2007; van Praag et al., 2005; Wu et al., 2008). The future direction of exercise and neurogenesis research is beginning to focus on identifying how exercise could be pro-neurogenic toward neuroinflammatory and neurodegenerative conditions such as Alzheimer's disease (Ryan & Nolan, 2015)

Neuroinflammation can be observed as a pathological mechanism during normal aging and in neurodegenerative conditions, it can negatively affect neurogenesis and cognitive function through damage to brain tissue (Ryan & Nolan, 2015). The pathological mechanism through which neuroinflammation propagates cellular damage is thought to involve cell-signaling factors known as cytokines. These cytokines are produced by cells of the innate immune system, which bind cell surface receptors and initiate a signaling cascade. A term used to describe a cytokine involved in propagating the inflammatory response is a pro-inflammatory cytokine. Pro-inflammatory cytokines include interleukin-6 (IL-6), interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha

(TNF- α), nitric oxide synthase 2 (NOS-2), cyclooxygenase 2 (COX2) and chemokine ligand 2 (CCL2) (Ryan & Nolan, 2015). These same cytokines can also be termed anti-inflammatory; they are antagonistic to pro-inflammatory processes as they reduce the inflammatory response cascades in cells (Petersen & Pedersen, 2005). Cytokines are pleiotropic proteins, which means they can serve multiple functions even those that are antagonistic of each other (Ryan & Nolan, 2015). The secretory cell that produces a cytokine is typically associated with a specific type of receptor to which it subsequently binds (Pedersen & Fischer, 2007; Petersen & Pedersen, 2006). Cytokines are produced primarily in the immune system, however recent studies in rodents support the secretion of cytokines through metabolic, neural, and endocrine cells (Pedersen & Fischer, 2007). The contraction of skeletal muscle during exercise elicits many physiological changes, including the release of various cytokines into the circulation (Petersen & Pedersen, 2005).

Exercise and Cytokines

Pederson et al. (2002) speculated that the release of the cytokine IL-6 during exercise was observed to target the brain as a hormone. It is now supported that IL-6 expression is upregulated in neural tissue following chronic exercise, primarily released by neurons in the hippocampus (Kalashnyk et al., 2014; Nybo et al., 2002). The skeletal muscle contracts during exercise and acts as a peptide-releasing endocrine organ (Pedersen & Febbraio, 2005; Pedersen & Fischer, 2007). Research by Pedersen and colleagues examined the possibility that IL-6 is secreted both by contracting skeletal

muscle and by brain tissue during exercise. IL-6 could be exerting its downstream effects via signaling pathways in the brain following exercise (Pedersen & Febbraio, 2005).

The investigation of the effects of cytokines on neural cells is rather new (Rasmussen et al., 2011). A cytokine is referred to as neuropoietic if its produced in a neural cell and binds to membrane-bound receptors of neurons (Pedersen & Febbraio, 2005; Rasmussen et al., 2011). The notion that cytokines have an effect in the CNS came through work with astrocyte cell cultures and rodent models which investigate brain inflammation (Leibinger et al., 2013; Nakanishi et al., 2007). IL-6 is one of these neuropoietic cytokines that responds to various forms of brain inflammation and mediates recruitment of cells/proteins involved in the immune response to migrate to the point of injury for repair of damaged tissue (Leibinger et al., 2013; Pan et al., 2008). IL-6 can signal as a pro-inflammatory factor during inflammation or bodily injury. The cytokine will be released by a microglia cell in response to activation of other signaling pathways, typically TNF or NFkB (Wu et al., 2008). During exercise, it has been shown that IL-6 is produced by muscle fibers via a TNF-independent pathway, which makes this signaling event different than the innate inflammatory response (Pedersen & Febbraio, 2005; Petersen & Pedersen, 2006). This is important to note because if IL-6 plays a role in signaling neurons in the hippocampus to undergo neurogenesis, the result of the signaling cascade could be different than the inflammatory response associated with apoptosis.

An anti-inflammatory response will activate a molecular signaling pathway downstream with antagonistic effects, like the JAK-STAT pathway (Leibinger et al., 2013). The anti-inflammatory response may impact the brain, consequently reducing the

response to factors that promote brain inflammation (Petersen & Pedersen, 2006). This is the plausibility for a mechanism that can protect the brain against degeneration (Rasmussen et al., 2011). This raises questions regarding possible endogenous release of IL-6 protein directly in the brain in response to exercise.

Recent studies investigating whether IL-6 is released in the brain during exercise found that not only is it released during exercise, it is released during the recovery period. These results are important for the involvement of IL-6 in hippocampal function because it helps establish plausibility that the cytokine is both secreted and signals in that region. The growth of new neurons through neurogenesis requires several molecular signaling events, primarily mediated through the JAK/STAT pathway (Leibinger et al., 2013). Current evidence suggests that IL-6 plays a role in activating the JAK/STAT pathway in hippocampal neurons, although further study is needed to confirm that mediation by IL-6 of this pathway is significant enough to influence neuron cell proliferation *in vivo*.

One recently proposed hypothesis is that the long-term anti-inflammatory effects of exercise may be mediated via effects of exercise leading to a reduction in brain inflammation (Rasmussen et al., 2011; Sarvas et al., 2014). It is known that higher levels of visceral fat mass are associated with both brain inflammation and higher levels of pro-inflammatory cytokines, however exercise reduces both significantly (Verstynen et al., 2012). The anti-inflammatory effects of IL-6 could represent a mechanism through which exercise protects against brain inflammation associated with high levels of pro-inflammatory factors (Pedersen & Steensberg, 2002; Petersen & Pedersen, 2005). This anti-inflammatory role of IL-6 has implications for neuronal cell survival and growth.

The effect can have implications for future research involving exercise and questions about whether it offers protection against neurodegeneration in the hippocampus.

Evidence from studies with microglia and astrocyte cell cultures suggest that IL-6 is produced by these cell types in the CNS and primarily in the hippocampus (Pedersen & Fischer, 2007; Reihmane & Dela, 2014).

Levels of IL-6 remain low under normal physiological conditions in the CNS, however during injury or inflammation, there is a marked increase in endogenous IL-6 levels (Nybo et al., 2002). This same response is observed for IL-6 in response to exercise (Reihmane & Dela, 2014). This idea is consistent with the rationale that an increased presence of astrocyte-produced IL-6 in the brain is important for maintaining hippocampal neurogenesis, a process that could influence cognitive function (Pedersen & Febbraio, 2005; Seguin, Brennan, Mangano, & Hayley, 2009). The literature supports the notion that chronic exercise training can induce endogenous IL-6 production from astrocytes in the hippocampus (Kalashnyk et al., 2014). The hypothesis that exercise might induce production of IL-6 in hippocampal tissue and initiate proliferation of new neurons, suggests that there are several mediating factors in response to exercise training and brain function.

Recent studies have observed presence of another cytokine factor in the CNS. (Cohen & Fields, 2008; Pan et al., 2008). The cytokine, known as leukemia inhibitory factor (LIF), is also a pleiotropic cytokine. Like IL-6, LIF is thought to be secreted by skeletal muscle both during and after prolonged exercise (Pedersen & Febbraio, 2005; Speisman, Kumar, Rani, Foster, & Ormerod, 2013). The binding and signaling functions

of LIF are similar to IL-6; it binds to the IL-6R and is involved in the JAK-STAT signaling pathway (Pan et al., 2008). It is currently speculated that LIF can be released into the circulation to travel and enter the CNS via the BBB, although this has not been entirely confirmed in vivo (Leibinger et al., 2013; Pan et al., 2008). LIF has been confirmed to be a factor released by skeletal muscle during exercise, much like IL-6. However, there is no current data that confirms an upregulation of LIF in brain tissue following exercise training. Because LIF is closely associated with IL-6 and its function in hippocampal-dependent learning, it can be postulated that LIF is significantly increased in the hippocampus during and after exercise (Cotman, Berchtold, & Christie, 2007b). The next step in research with LIF involves figuring out if it is present in the brain following exercise, namely the hippocampus, in order to figure out if it's indicative of changes to cognition associated with exercise.

It is known that nutrition plays a role in learning and memory, affecting hippocampal-dependent learning. There is speculation that protein content in the diet influences spatial memory and changes the plasticity of neurons associated with learning processes (Meeusen, 2014). Better understanding this potential relationship between protein in the diet and the amount necessary for effects on hippocampal-dependent memory will require further investigation (Monti, Baym, & Cohen, 2014). It is also important to note that there are synergistic interactions between exercise training and nutrition, meaning there are potential synergistic benefits from combining exercise training and different protein diet compositions when testing for positive effects on hippocampal-dependent learning and memory (Meeusen, 2014).

CHAPTER III

OUTLINE OF PROCEDURES

Experimental Methods

Young (5-6-week old) Male Sprague-Dawley rats (n=48) were housed individually under a 12 hr. dark/light schedule with *ad libitum* access to rat chow and water for the final 5 hours of the dark period. All animals were treated according to the guidelines of the University of North Carolina at Greensboro and federal policies. The animal housing facility is located at Eberhart Building 601, where the animals are individually housed. Data for this experiment were collected as part of a larger study that looked at protein content influences on cognition and chronic exercise influences on heart muscle. The dietary study tested different levels of protein in a single meal given to the animals prior to behavioral testing. The protein composition of a single meal was manipulated prior to behavioral testing and randomized for each animal to be either 15% casein (low protein) or 50% casein (high protein).

The exercise protocol used for this study is IACUC protocol 14-014, a protocol which is utilized by Dr. Joseph Starnes. There is a 6-7 week treatment period, which includes a 1-week familiarization period to acclimate the animals with running on the treadmill.

The total exercise treatment for this protocol is 1-week of familiarization plus the 5-6 weeks of training for a total period of 6-7 weeks. Each animal was placed on the treadmill for 5 minutes daily at a pace of 10 m/min during the familiarization period. After the familiarization period and group assignment of animals (trained and untrained), the 5-6-week treatment period began. Daily during the last hour of the light cycle, each animal of the trained group was placed into an individual lane on the treadmill, while untrained animals were placed individually on non-running lanes of the treadmill. Animals in the trained group underwent five consecutive days of exercise followed by two consecutive days of rest. Duration and intensity of the exercise were gradually increased over the 5-week period until each exercise session was 60 minutes in duration and performed at 30 meters/minute with a 6° grade. Animals were returned to their home cages following each exercise session. The animals in the untrained group remained in non-running lanes of the treadmill for the same duration as the trained group. The entire cohort, untrained and trained were randomly assigned to behavioral testing during either week 5 or week 6 of the exercise protocol. The behavioral testing was performed approximately two hours after the daily exercise session. Animals were tested after eating a single breakfast meal composed of a randomly assigned protein composition.

The Barnes maze was utilized as a spatial learning measure. The Barnes maze apparatus is a circular arena (~120 cm diameter) brightly lit from above, with 20 holes around the perimeter. One of the holes has an escape chamber where an animal can escape the brightly lit open arena. The position of the escape chamber does not change. The animals were placed individually in the center of the apparatus in a covered

container. The container was removed and each trial consisted of animals being allowed up to 2 minutes to find the escape chamber using only visual cues around the room. If the animal found the escape chamber before 2 minutes had elapsed, the video recording of the trial was stopped at that point and the time was recorded. Following conclusion of each individual trial on the maze, the animal was removed and returned to its home cage. The maze arena was cleaned with ethanol solution and the top, thin movable layer of the maze rotated clockwise to minimize possibility of scent trails to the escape chamber for the next animal. The latency and path length to escape was recorded by video recording software for each trial. There were 4 trials/day on 4 consecutive days followed by a probe trial (no escape chamber is present and time spent in the correct quadrant determined) on day 5 of testing.

There were a total of 4 cohorts of animals, consisting of 12 rodents per cohort. The animals of each cohort were randomly assigned to exercise (trained) or sedentary (untrained) conditions, as well as to receive a low protein meal (15% casein) or high protein meal (50% casein). The habituation period for the first week of the exercise protocol was conducted as stated earlier. Rodents of each cohort were randomly assigned to complete behavioral testing in the Barnes maze during week 5 or week 6. After the first behavioral testing week, sacrificing of the six animals tested during that week were scheduled and tissue samples were collected and stored. This was repeated for the second group of animals, allowing collection of tissue samples to follow conclusion of behavioral testing.

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and moved from their home cage to an operating room. If the animal was not showing sufficient signs of anesthesia, a follow-up injection of sodium pentobarbital was given to the animal. This procedure was performed on one animal at a time, for a total of two animals per sacrificing session on a given day. Each animal surgery was performed 3-4 hours after the animals' daily exercise training session, in order to allow for a return of physiological parameters to pre-exercise levels. The surgery began with an incision along the ventral abdominal cavity, where cuts were made toward the inner lining of the chest. This revealed the animals' heart, which was extracted while actively beating. Following heart removal, the animal was moved to the Harvard guillotine apparatus and decapitated. The skull of the animal was removed to reveal the brain, which were extracted following cutting of the optic nerves holding it to the skull. The entire animal brain was moved to a brain matrix where several dorsal slices were taken. A dorsal slice was taken 2 mm from the center of the brain sitting in the brain matrix to get a section of the hippocampus for tissue collection (see Figure 1). Samples was stored in a -80 C freezer immediately in Eppendorf tubes.

The bicinchoninic acid (BCA) biochemical assay was used to determine the total protein concentrations of 30-42 mg hippocampal tissue samples for use with enzyme-linked immunosorbent assay (ELISA). The purpose of the BCA is to quantify protein of an unknown sample using a standard curve generated through use of a known protein standard. For the assays in this study, the protein standard was bovine serum albumin (BSA) and the protocol was previously adapted for use with tissue homogenates. The tris

buffer solution was made using a recipe of 10 mM Tris (pH 7.4), 50 mM NaCl, and 2.5 mM MgCl₂ (Dominguez, Rivat, Pommier, Mauborgne, & Pohl, 2008). The recipe calls for protease inhibitor cocktails, but it was decided in this study to leave them out, because the inhibitors contain protein that could influence the results of the assay. These assays were conducted using hippocampal tissue collected from animals not associated with this study, in order to have expendable tissue samples to practice multiple runs of the assays. The final BCA used brain tissue samples that were from rat brain but not specifically hippocampus. For all BCAs, the standard curve required 7 microfuge tubes to make a serial dilution of the following concentrations; 25 ($\mu\text{g}/\mu\text{l}$), 12.5 ($\mu\text{g}/\mu\text{l}$), 6.25 ($\mu\text{g}/\mu\text{l}$), 3.125 ($\mu\text{g}/\mu\text{l}$), 1.56 ($\mu\text{g}/\mu\text{l}$), 0.78 ($\mu\text{g}/\mu\text{l}$), and a 0 (blank) tube. The BCA protocol calls for 14 microfuge tubes, in order to make duplicates of the standard curve. The standard curve was prepared by adding 25 (μl) of buffer solution to all 14 microfuge tubes. Next, 25 μl of the first tube was vortexed and transferred to the next tube and this mixing/transferring step was repeated until the sixth tube. Finally, 25 μl of volume from the 0.78 μg tube was pipetted out. While the standard curve was prepared, experimental protein samples were thawed on ice for 30 minutes. After the samples were thawed, 3 μl of the sample was added to 22 μl of buffer to make a total volume of 25 μl . BCA working solution was prepared by adding a proportional amount of BCA reagent A and B that corresponded with the total number of tubes to be used in the assay. 500 μl of BCA working solution was then added to each tube and each sample was vortexed thoroughly for several seconds. The samples were incubated in a 37 C° water bath for 30 minutes. Each tube was removed and wiped dry from the water bath after 30 minutes. Then each

sample was vortexed again briefly and 200 µl of each tube was pipetted into two separate wells of a microtiter plate. Lastly, the plate was immediately read by an Epoch plate reader at 562 nm using Gen5 software. The Epoch reader generates a printout for the BCA assay that contains two crucial parts; the standard curve with a linear fit and the protein concentration values. Because this assay calls for 3 µL of the sample protein to be diluted with 22 µL of buffer solution, it's important to remember to divide the calculated protein concentrations on the reader printout by 3 to get the actual µg/µL value. It's important to note that knowing the total amount of wet weight of the tissue is needed to get the total protein content of the sample. This entails taking the sample protein concentration value and multiplying it by the amount of buffer it's initially diluted into (200 with this assay) and dividing that result by the wet tissue weight (mg). This was the first opportunity for the investigator to learn to use the BCA assay to quantify protein concentration of the hippocampal tissue samples. The process of learning and executing this assay takes deliberate practice and in order to reach a satisfactory level of proficiency with the technique, multiple attempts were made. After several iterations of running the entire assay procedure, each step became more learned and easier for the investigator to perform efficiently. The results section of this paper lays out this learning process and explains specific steps taken to achieve proficiency with this assay protocol and its desired outcome.

The primary ELISA protocol that was selected during the planning stages for this study were assay kits for the cytokine IL-6 and LIF. The available assay kits are different for these cytokines, so two different protocols were considered. First, for IL-6, a protocol

that had been adapted by researchers and cited as reliable for use came from R&D systems (Dominguez et al., 2008). This protocol was simple in design even for an inexperienced individual and the company has excellent technical support and customer service. The ELISA assay considered for LIF came from Kamiya Biomedical, but was later determined to be too challenging and involved especially for an individual with little prior experience with the procedure. After determining the LIF assay was too difficult, it was not considered any further and alternate assays were reviewed. Because no alternative could be found, the plan to assay LIF was dropped. This led to an investigation into other factors, such as IL-10 and IL-1beta. However, ultimately the training required for this investigator to become competent with conducting ELISAs was deemed to be too great relative to the investigator's current level of expertise. Hence, the proposal was modified so that ELISAs were dropped and instead the investigator was asked to develop competence in conducting the BCAs. These results for the BCAs are reported herein.

Had the ELISAs been conducted, this would have required the following step. Brain tissue samples would be thawed on dry ice washed with Phosphate-Buffered solution (PBS) buffer, and then homogenized in preparation for ELISA assays. The homogenized tissue samples would be stored on ice prior to start of the assay protocol. IL-6 and LIF protein concentrations would be quantified using an ELISA immunoassay kit (Seattle, WA). Homogenized tissue samples of both trained and untrained groups would be pipetted into 96-well plates pre-coated with an IL-6 specific polyclonal antibody. This pipetting of samples onto each 96-well plate from both groups would be

randomized, in order to control for difference between separate assay kits. The samples would be run in duplicate on the plates, which would allow samples of both groups to be randomized across two 96-well plates. After reagent preparation and pipetting of tissue samples onto plates, there would be an incubation period of 1 hour at 37 C°. An automated washing period with a diluted wash solution would follow the incubation period. This procedure would be repeated five times with subsequent drying of the plate after each wash. Finally, addition of 50 µL of Substrate A and 50 µL of Substrate B to each well would be required prior to using ELISA plate reader for analysis of protein concentrations. The brain tissue samples would be handled and assayed in accord with procedures published by R&D systems.

All trials from the Barnes maze were recorded as videos using Topscan Suite software (CleverSys Inc.) and analyzed for total distance traveled, and path latency data. The software requires two things to be setup prior to analysis; a background image of the Barnes maze testing arena and arena profile. Each individual trial video was opened in the Topscan Suite software and automatically analyzed for variables listed above. The numbers were further organized in an Excel spreadsheet by experimental condition and prepared for statistical analysis.

A three-way mixed analysis of variance (ANOVA) was used to assess the results of the Barnes maze. The goal of this statistical analysis was to test for main effects for exercise (untrained, trained), protein content (low, high), and day (1, 2, 3, 4) and all possible interactions between exercise, protein content, and day.

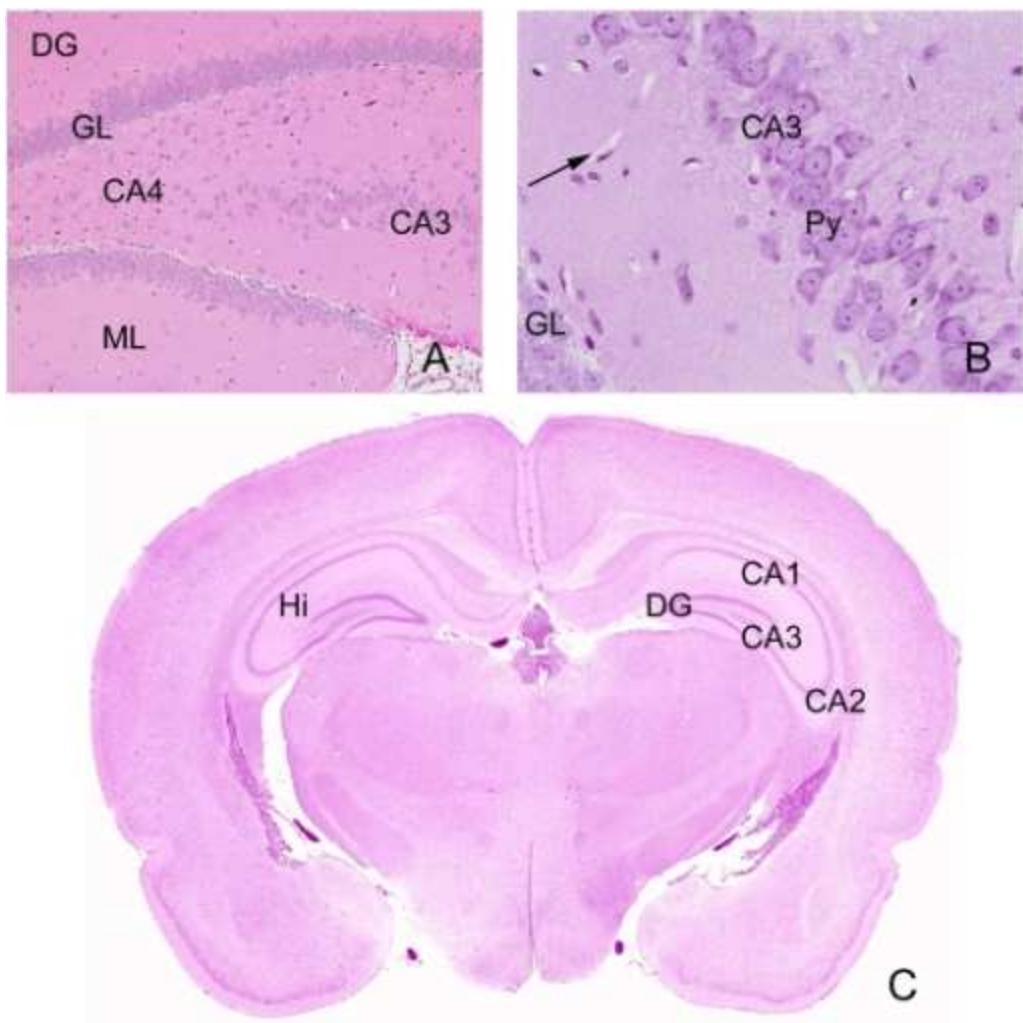


Figure 1. Dorsal Slice of the Rat Hippocampus Taken Approximately 2 mm from the Center of the Brain.

(https://openi.nlm.nih.gov/detailedresult.php?img=PMC2525487_md-6-180-f001&req=4).

CHAPTER IV

BCA CHAPTER

The BCA was used to determine the total protein concentrations of 30-42 mg hippocampal tissue samples for use with ELISAs. Before conducting the BCA, a standard curve was generated using both water and tris buffer.

This was done in order to determine if it would be more accurate to generate and compare protein concentrations on a standard curve using the same buffer that would be used to dilute the unknown samples (the tris buffer) as opposed to using a water buffer.

The first standard curve generated involved use of both water and tris buffer solutions to compare values between the two curves. Figure 2 depicts the two standard curves.

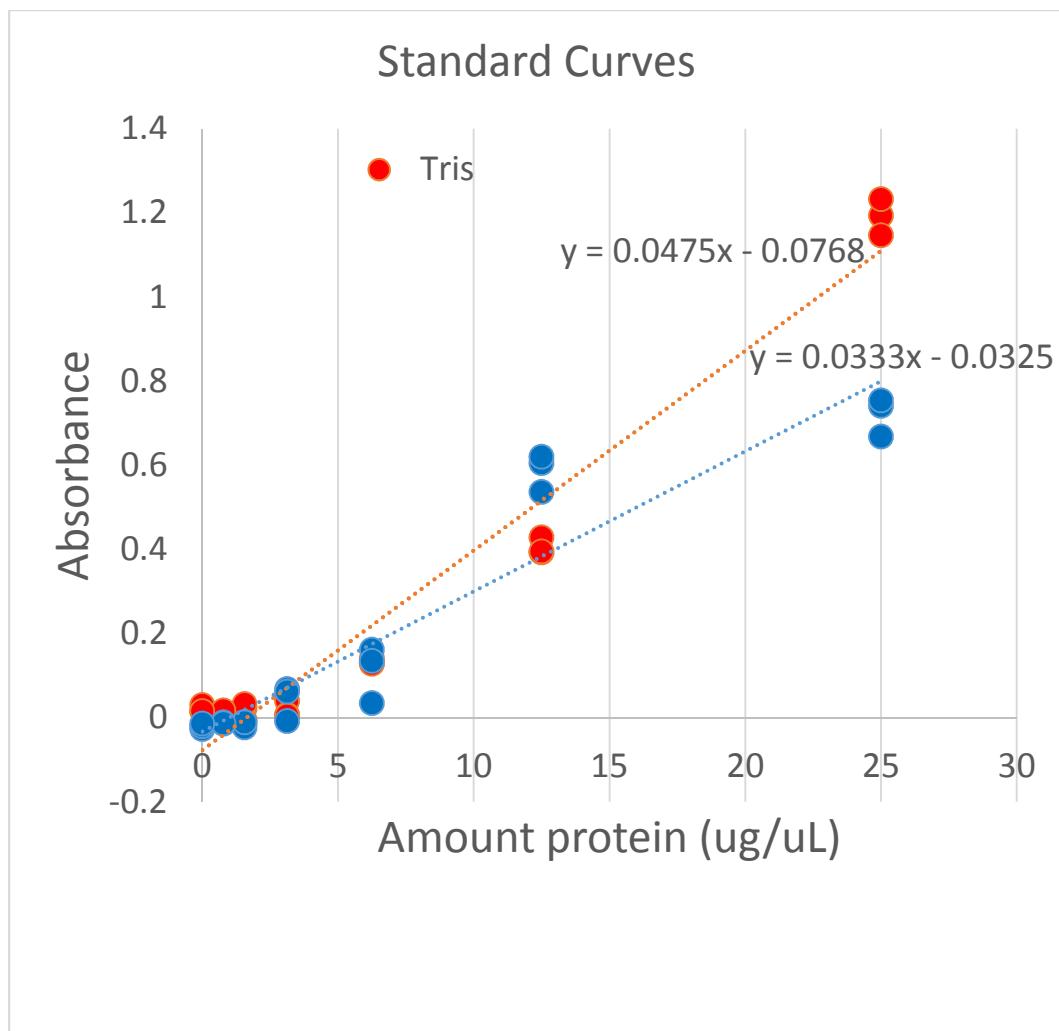


Figure 2. Standard Curve of Deionized Water and Tris Buffer in Triplicate.

Table 1. Average Absorbance Values for Both the Standard Curve and the Samples for BCA 1.

| BCA 1 Average Standard Curve and Sample Absorbance Values | | | | | | | | | | |
|---|--------|-------------|-----------------------------|----------|----------|----------|----------------------------------|----------|----------|----------|
| µg/mL | Water | Tris Buffer | Samples prepared with water | | | | Sample prepared with tris buffer | | | |
| 25.00 | 0.721 | 1.191 | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
| 12.50 | 0.587 | 0.406 | 1.202 | 0.608 | 1.194 | 0.388 | 0.814 | 0.567 | 0.747 | 0.489 |
| 6.250 | 0.111 | 0.133 | 1.377 | 0.630 | 1.290 | 0.431 | 0.820 | 0.600 | 0.733 | 0.368 |
| 3.125 | 0.041 | 0.017 | 1.511 | 0.640 | 1.624 | 0.569 | | | | |
| 1.560 | -0.014 | 0.016 | 1.538 | 0.634 | 1.604 | 0.604 | | | | |
| 0.780 | -0.013 | 0.015 | | | | | | | | |
| 0.000 | -0.020 | 0.020 | | | | | | | | |
| CV(%) | | | 0.461 | 3.970 | 1.190 | 15.70 | 0.461 | 3.970 | 1.190 | 15.70 |

From this figure, it can be observed that the two standard curves are different. This difference could be due to two reasons, which are not equally weighted as far as their overall impact. The first thing that could underlie the difference might be attributed to the technique and experience of the novice investigator; as human error could have changed how both curves turned out. A second possibility that could explain the differences in the two curves involves the protocol itself.

The use of deionized water as compared to the tris buffer solution when preparing the curves may also explain the difference. As can be seen by looking at Figure 2 more samples (as represented by the dots) fit closely along the tris curve than fall along the water curve.

However, there are a number of samples that are off the scale of both curves, making it difficult to calculate their respective concentrations. After considering these observations, it was determined that the tris buffer would be more accurate for fitting the unknown protein samples to a standard curve. Table 1 depicts the average absorbance values for this first BCA, giving averages for replicates of the samples run in quadruplicate on the assay plate.

Looking at this table in the water and tris columns, gives the values for the standard curve as you descend the column. It can be observed from this table that the tris curve does not contain any negative values near the zero of the curve, something that can be considered better when comparing to the negative values of the water curve.

The samples for all BCA assays are first diluted in 200 µL of tris buffer before homogenization, introducing an extra necessary step toward calculating the respective

protein concentration of the sample. This information from the standard curve and their corresponding absorbance values, contributed to the decision that going forward, all subsequent standard curves would be prepared with the tris buffer. Since the results from the first BCA indicated that the better method to prepare the standard curve was to use the tris buffer, it was clear that this would be easier to avoid any differences between deionized water and buffer solution when homogenizing the samples. In the first assay, it was also evident that there were several samples off the curve at the higher end of the range which made it difficult to determine the sample concentrations.

Wet weight of the tissue samples used in these BCA assays ranged from 30-52 mg.. The rat brain has similar uniform concentration of total protein throughout, so the size of the tissue sample has little influence on the concentration of protein within the tissue itself. After the standard curves are generated, the curve can be applicable to identify protein concentrations from a range of wet tissue weights.

This made it important to further dilute the samples by homogenizing differently, in order to get them to fit the curve. This led to the decision to compare my ability to determine the protein concentrations of the hippocampal samples by homogenizing them through a variety of methods.

Each step of preparation for a homogenization typically changes the total protein recovered for the assay from the sample, because each step alters the process of extracting protein. This introduces a certain level of variance to the final sample concentration from the assay, since homogenization steps can vary depending on the sample. The three methods decided upon to be tested included tissue samples that were

microsonicated only, use of a handheld tissue grinder only, as well as handheld tissue grinder followed by microsonication. The next thing to do was to prepare tissue samples with all three of these methods and conduct another BCA using the tris curve.

The hippocampal tissue samples were grouped for homogenization by the techniques stated above and each sample group was homogenized individually utilizing the assigned methods. Although one sample was planned and conducted with each of the three preparations, only two samples (one for handheld/microsonicator and one for microsonicator) were used to generate the standard curve and for the BCA results. Sample 1 was prepared using the handheld tissue grinder and the microsonicator together, while sample 3 was prepared using only the microsonicator. The sample with the other preparations did not produce results for all dilutions, so they were excluded from the final BCA results table and generated standard curve. This second BCA was conducted to evaluate how the protein concentrations of the hippocampal tissue samples would look when the supernatant of the homogenized samples was diluted with different volumes of tris buffer. A major reason behind the various dilutions was to ensure the samples would be diluted enough to fit various parts of the curve.

The samples in this assay were diluted in the following volumes of buffer; 90 μ L, 190 μ L, 290 μ L, 390 μ L, and 490 μ L. The total volumes were calculated by adding 10 μ L of supernatant to the volume of tris buffer resulting in total volumes of 100 μ L, 200 μ L, 300 μ L, 400 μ L, and 500 μ L. The standard curve in this second BCA was generated in duplicate using only the tris buffer used to prepare the samples. The following figure depicts the standard curve values calculated using the BCA protein standard.

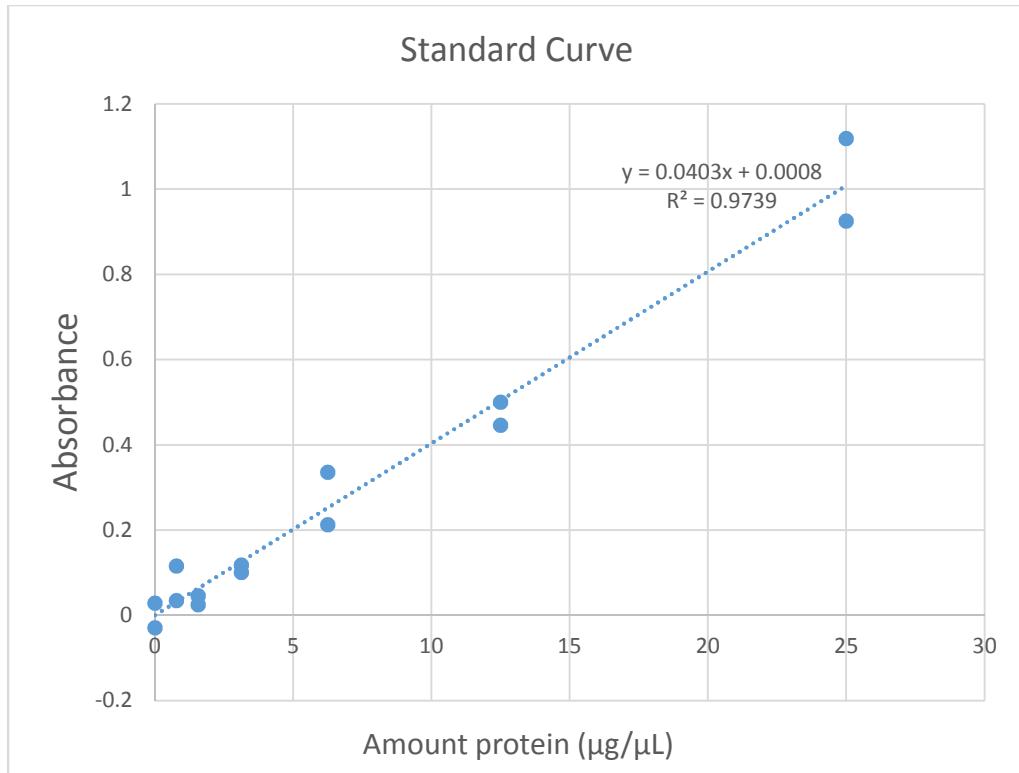


Figure 3. Standard Curve in Duplicate with Tris Buffer for the Second BCA.

Here you can see that the majority of the samples are close too or passed through by the line of best fit, with some off the line of best fit. This was an improvement over the curves of the first assay, as the best fit line for this curve passes through or is close to most samples (represented by the dots). Because the unknown protein samples used in this assay were diluted by various volumes of buffer and from the same homogenate, it was necessary to correct all five volumes for their respective dilution to see if the protein concentrations of the sample homogenate used to make these dilutions at the beginning of the assay were the same for all dilutions.

The reason concentrations for all protein samples should be equal after correcting for the dilution is because they are from the supernatant of the same homogenate. The

results from this second assay had a couple key issues that needed to be addressed prior to conducting any further assays. First, after correcting the sample concentrations for the dilution volumes to compare with the concentration of the homogenate, it was evident that the diluted samples did not match the sample supernatant from which they were diluted. This was observable when looking at the data for sample 1 and 3 in Table 2 in the corrected for dilution column, the numbers for the five different dilutions listed in this column are not the same, when they should be. This was an issue because those concentrations should match after correcting for the dilution, as noted above these samples are diluted from the same supernatant. Much like the differences in the two curves generated in the first assay, samples that differ in concentration after being corrected for their respective dilution might be the result of novice pipetting technique and related human error.

Table 2. Calculations for the Second BCA, Including the Sample Values Corrected for Dilutions, their Total Protein Concentration, and Protein Percent Recoveries.

| BCA 2 Values for Corrected Dilution, Total Protein, and Percent Recovery | | | | | | |
|--|--|----------|-------------------------|----------|----------------------|----------|
| Volume | Corrected for Dilution (absorbance) | | Total Protein µg/sample | | Percent Recovery (%) | |
| Dilution (µL) | Sample 1 | Sample 3 | Sample 1 | Sample 3 | Sample 1 | Sample 3 |
| 1:10 | 11.6 | 17.5 | 2324.33 | 3501.67 | 70.430 | 83.370 |
| 1:20 | 12.1 | 22.3 | 2413.33 | 4462.00 | 73.130 | 106.24 |
| 1:30 | 19.2 | 24.2 | 3848.33 | 4847.37 | 116.61 | 115.41 |
| 1:40 | 24.1 | 15.7 | 4825.70 | 3133.77 | 146.23 | 74.610 |
| 1:50 | 10.5 | 19.9 | 2091.67 | 3973.33 | 63.380 | 94.600 |

In the tables that represent the total protein concentrations for each assay, the value was obtained by multiplying the corrected for dilution value by 200 to account for

the first 200 μL dilution (see Tables 2, 5, 6). In order to understand how the different preparation methods turned out for the second BCA, looking at the correction for dilution column for sample 3 in Table 2, it can be observed that the range of the five values are closer together than sample 1. This suggests that the total protein concentration values in the next column for sample 3 are more reliable than sample 1, because they were calculated using the corrected dilution values. Sample 3 was a sample that was sonicated only, suggesting that this method might be better to use for preparing subsequent samples. After determining that the results with the sonicated only samples indicated less variance with protein concentrations compared to the other two methods, this method was used when homogenizing samples for subsequent assays. It can also be observed in the correction for dilution column under Sample 3 that the values were not the same, meaning it was possible that something went wrong with preparation due to human error.

Although the sonicated samples had the least difference in range when looking at the values for the corrected dilutions, there was still some variability between the values obtained. This foreshadowed the need for another assay that tested the same sample dilutions, but using only the sonication method to prepare the samples. The standard curve in Figure 3 for the second assay had more samples passing through and/close to the line of best fit than the first assay (which is depicted in Figure 2), which is an indication that it's a potentially better curve for determination of the unknown samples than the curve from the first assay. However, although the curve for the second assay had more samples passing through or near the line of best fit, this did not mean all of the samples tested in this assay were meeting this criterion. This was a likely indication that there

were errors with pipetting of the correct buffer volumes for the dilutions both into the sample test tubes and wells of the assay plate. This led to the determination that the volumes were not pipetted accurately and a third BCA was planned and executed.

The results in Figure 4 depict a third run of the BCA assay with the same hippocampal protein samples. 10 µL of supernatant was taken from samples prepared with sonication and diluted with tris buffer in the same exact manner as in the previous assay.

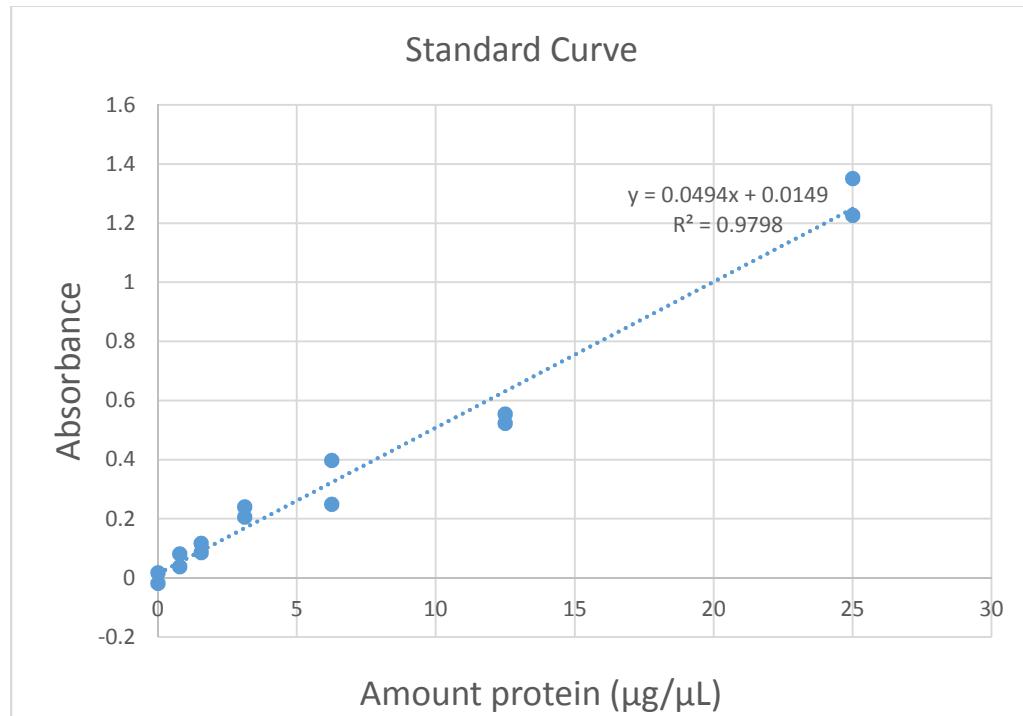


Figure 4. Standard Curve in Duplicate with Tris Buffer for the Third BCA.

Table 3. Average Absorbance Values for the Standard Curve and Samples of the Second BCA Assay.

| BCA 2 Average Absorbance Values | | | | | |
|--|--------------------|--------------|-----------------------------------|----------------------|----------|
| Protein Standard ($\mu\text{g/mL}$) | Absorbance (Curve) | | Dilution Volume (μL) | Absorbance (Samples) | |
| | Tris Curve 1 | Tris Curve 2 | | Sample 1 | Sample 3 |
| 25.00 | 0.925 | 1.119 | 100 | 0.157 | 0.200 |
| 12.50 | 0.446 | 0.500 | 200 | 0.064 | 0.134 |
| 6.250 | 0.213 | 0.336 | 300 | 0.066 | 0.091 |
| 3.125 | 0.101 | 0.118 | 400 | 0.089 | 0.056 |
| 1.560 | 0.025 | 0.046 | 500 | 0.029 | 0.052 |
| 0.780 | 0.035 | 0.116 | | | |
| 0.000 | -0.029 | 0.029 | | | |
| CV (%) | | | | 53.00 | 61.20 |

The standard curve from the third BCA had little error compared to the previous two BCAs, which can be observed by the best fit line, which shows that the sample dots are either close to or passed through by the best fit line. A good standard curve results from correct serial dilution. Table 4 depicts the average absorbance values from the third assay for the curve and the results from the serial dilution of the protein standard. The noticeable trend with the curves present in Figures 2-4 is that with each subsequent assay, the absorbance values for the samples; first BCA in table 1, second BCA in table 3, and third BCA in table 4, decrease significantly down the tris curve column as the values for the protein standard dilution decrease, due to more dilute protein.

The absorbance values for the curves represent the concentration of the known protein standard and are used to generate the standard curve. If there is a certain level of human error, such as with pipetting technique, dependent on with which dilution in the serial dilution the error occurred, could determine how many dilutions contain error in the curve. For example, if there is error with diluting the first protein value, 25 $\mu\text{g}/\mu\text{L}$, then

all subsequent dilutions (12.5, 6.25, 3.125, 1.56 and 0.78) could represent error through their detected absorbance values after the assay. It is imperative to minimize error during the serial dilution to obtain values representative of the known protein standard's concentration, in order to generate the most linear curve.

The general procedure for this assay was similar to the second assay, because the same sample dilutions were repeated except this time the sample homogenate was only prepared by sonication. Another difference with this assay involved taking 50 µL of homogenate straight from both samples prior to centrifugation. That way both sample dilutions would be run with tissue homogenate from sonication and the supernatant of those samples, in order to compare how this might differ in total protein concentration.

When looking at the average absorbance values for the sonicated samples of BCA 3 in table 4 and comparing them with the absorbance values for the standard curve, the sonication method produced a majority of samples with values that fit the standard curve. This was a good thing, but when correcting the sample values for their respective dilutions, the values were still not the same. Because the sample values were not the same after correcting for the dilutions, it was likely that there were still issues with the investigator's pipetting technique.

Table 4. Average Absorbance Values for the Standard Curve and Samples of the Third BCA Assay.

| Protein Standard ($\mu\text{g/mL}$) | BCA 3 Average Absorbance Values | | | | |
|--|---------------------------------|--------------|----------|----------|--------|
| | Absorbance | | | | |
| | Tris Curve 1 | Tris Curve 2 | Sample 1 | Sample 2 | CV (%) |
| 25.00 | 1.351 | 1.226 | 0.165 | 0.169 | 1.830 |
| 12.50 | 0.523 | 0.554 | 0.204 | 0.197 | 2.620 |
| 6.250 | 0.249 | 0.397 | 0.036 | 0.042 | 24.80 |
| 3.125 | 0.240 | 0.206 | 0.034 | 0.044 | 39.70 |
| 1.560 | 0.086 | 0.117 | 0.017 | 0.023 | 0.000 |
| 0.780 | 0.038 | 0.081 | | | |
| 0.000 | 0.018 | -0.018 | | | |

As was the case with the second assay, problems with pipetting technique can introduce variance to the sample values. This means that although the standard curve fit most of the samples, the variance with the corrected dilutions could indicate that not all of the volumes were pipetted correctly. If volumes are not pipetted correctly, it becomes difficult to get reliable values for interpreting the actual protein sample concentration. Addressing this issue with pipetting technique was one of the most important things for the next assay. A second issue involved the calculated percent recovery values, which were low overall as compared to the other assays. This result indicated issues that could stem from pipetting of the sample volumes or a missed step with calculating the dilution protein concentrations themselves.

It was necessary to examine the protein sample values and percent recoveries, in order to determine if the differences in the corrected dilutions could be attributed to problems with the procedure. After considering the results with the protein sample values and percent recoveries, it was determined that the pipetting was to blame for these results. These values can be observed in Table 5. The percent recoveries range from 8-167%, a very impractical range due to the high end being too high. It is expected that values will range between 50-60% for samples from supernatant and nearly 100% for samples from homogenate, which would constitute more reliable recovery rates.

For this assay, two new protein samples were taken from rat brain and sonicated as done with previous samples. The hippocampal tissue was not used as with the previous three assays due to a shortage of practice tissue, so alternative brain tissue was collected.

Table 5. Calculations for the Third BCA, Including the Sample Values Corrected for Dilutions, their Total Protein Concentration, and Protein Percent Recoveries.

| BCA 3 Values for Corrected Dilution, Total Protein, and Percent Recovery | | | | | | |
|--|--|----------|-------------------------|----------|----------------------|----------|
| Volume | Corrected for Dilution (absorbance) | | Total Protein µg/sample | | Percent Recovery (%) | |
| Dilution (µL) | Sample 1 | Sample 2 | Sample 1 | Sample 2 | Sample 1 | Sample 2 |
| 1:10 | 9.90 | 10.1 | 1973.3 | 2024.7 | 66.00 | 67.00 |
| 1:20 | 25.0 | 24.1 | 5000.0 | 4818.7 | 167.0 | 161.0 |
| 1:30 | 3.20 | 4.60 | 642.00 | 916.00 | 21.00 | 31.00 |
| 1:40 | 3.80 | 6.70 | 752.00 | 1338.7 | 25.00 | 45.00 |
| 1:50 | | 1.20 | | 240.00 | | 8.000 |

Since the objective of this assay is to determine unknown protein concentrations of various weights of rat brain tissue, the use of any brain tissue in general can suffice to

run the assay. This is because hippocampal tissue is not anymore concentrated in protein than other brain areas, making it an arbitrary part of the procedure. Figure 5 illustrates the protein curve obtained from the fourth BCA. Procedures for this fourth assay were the same as the third assay, except the centrifugation was reduced from 10000 g-force to 6000 g-force. Reducing the g-force applied to the samples will increase the likelihood that larger proteins will remain in the supernatant as opposed to getting pushed down to the pellet at the bottom on the tube. This was determined prior to this assay because there was a concern that the samples were being spun too much, which would result in losing some total protein.

When looking at this figure, the best fit line of the standard curve passes through the majority of the samples run in the assay. Also, samples that were not exactly passed through by the best fit line were very close to the line, indicating that they were in range of the curve. This was a desirable result, which meant that all samples fell within range of the curve. It is now possible to determine the unknown protein concentrations based on the highest and lowest ends of this curve, something that was missing from the previous curves with samples not in range of the curves values. In order to determine the accuracy of recovering the protein from its homogenization, the percent recovery values were calculated and can be seen in Table 6.

The values for diluted tissue homogenates (samples identified with an 'h') are expected to be higher than the diluted tissue supernatant (samples identified with an 's'). As can be seen from Table 6, this was the case, as the average percent recovery for tissue homogenate was 85% and was 45% for the supernatant samples. These values were not

perfect though, as the expected rate for tissue homogenate recovery is close to 100%, and with 85% in this assay, that suggested there were some errors in the results. The expected recovery range for supernatant samples is between 50-60%, so the 45% was also slightly under this range.

Like the previous assays, the corrections for the various dilutions were made, and are represented in Table 6. It is expected that each corrected for dilution value in each individual column for each sample would be the same, but in this case they still were not equal. However, it can be noticed that the percent recoveries for the supernatant samples are closer in range (31-58 and 20-65) versus (21-167 and 31-161) than the previous assays. This helped support that the samples in the fourth assay could more accurately reflect the calculated total protein concentration values than samples in the previous three assays since there is less difference with percent of protein recovered during procedures with the samples in this assay.

The improvement in the corrections for dilutions, standard curve, and percent recoveries were all indicative of improvement in pipetting technique throughout the assay by the novice investigator. Each standard curve improved across the multiple attempts, meaning that techniques improved with more practice.

The results from this last assay yielded a preparation method for the hippocampal tissue samples to be used with ELISA, as well as a standard curve to fit the unknown protein samples and calculate their respective concentrations. This will be applicable to a range of tissue weights, since the majority of the hippocampal samples collected for ELISA will vary in wet tissue weight. The reason its applicable to a range of tissue

weights stems from the use of a variety of dilutions, meaning a wide range of different tissue concentrations (high and low) can be calculated using this curve.

Table 6. Calculations for the Fourth BCA, Including the Sample Values Corrected for Dilutions, their Total Protein Concentration, and Protein Percent Recoveries.

| Volume Dilution (μ L) | BCA 4 Values for Corrected Dilution, Total Protein, and Percent Recovery | | | | | | | | | | | |
|----------------------------------|--|----------------|----------------|----------------|------------------------------|----------------|----------------|----------------|----------------------|----------------|-----------------|------|
| | Corrected for Dilution (absorbance) | | | | Total Protein μ g/sample | | | | Percent Recovery (%) | | | |
| Sample 1(h) | Sample 2(h) | Sample 3(s) | Sample 4(s) | Sample 1(h) | Sample 2(h) | Sample 3(s) | Sample 4(s) | Sample 1(h) | Sample 2(h) | Sample 3(s) | Sample 4 (s) | |
| 1:10 | 17.71 | 14.43 | 12.26 | 3542.3 | 2886.0 | 2452.3 | 93.0 | | | 50.0 | 65.0 | |
| 1:20 | 15.61 | 20.84 | 12.55 | 9.510 | 3122.7 | 4168.7 | 2509.3 | 1902.7 | 82.0 | 72.0 | 43.0 | 50.0 |
| 1:30 | 15.31 | 17.98 | 8.970 | 12.56 | 3061.0 | 3595.0 | 1794.0 | 2511.0 | 81.0 | 62.0 | 31.0 | 66.0 |
| 1:40 | | 14.27 | 9.030 | 4.670 | | 2853.3 | 1805.3 | 934.70 | | 49.0 | 31.0 | 25.0 |
| 1:50 | | 17.39 | 16.83 | 3.820 | | 3478.3 | 3366.7 | 763.30 | | 60.0 | 58.0 | 20.0 |

Note. (h) refers to homogenate sample, and (s) refers to supernatant of sample.

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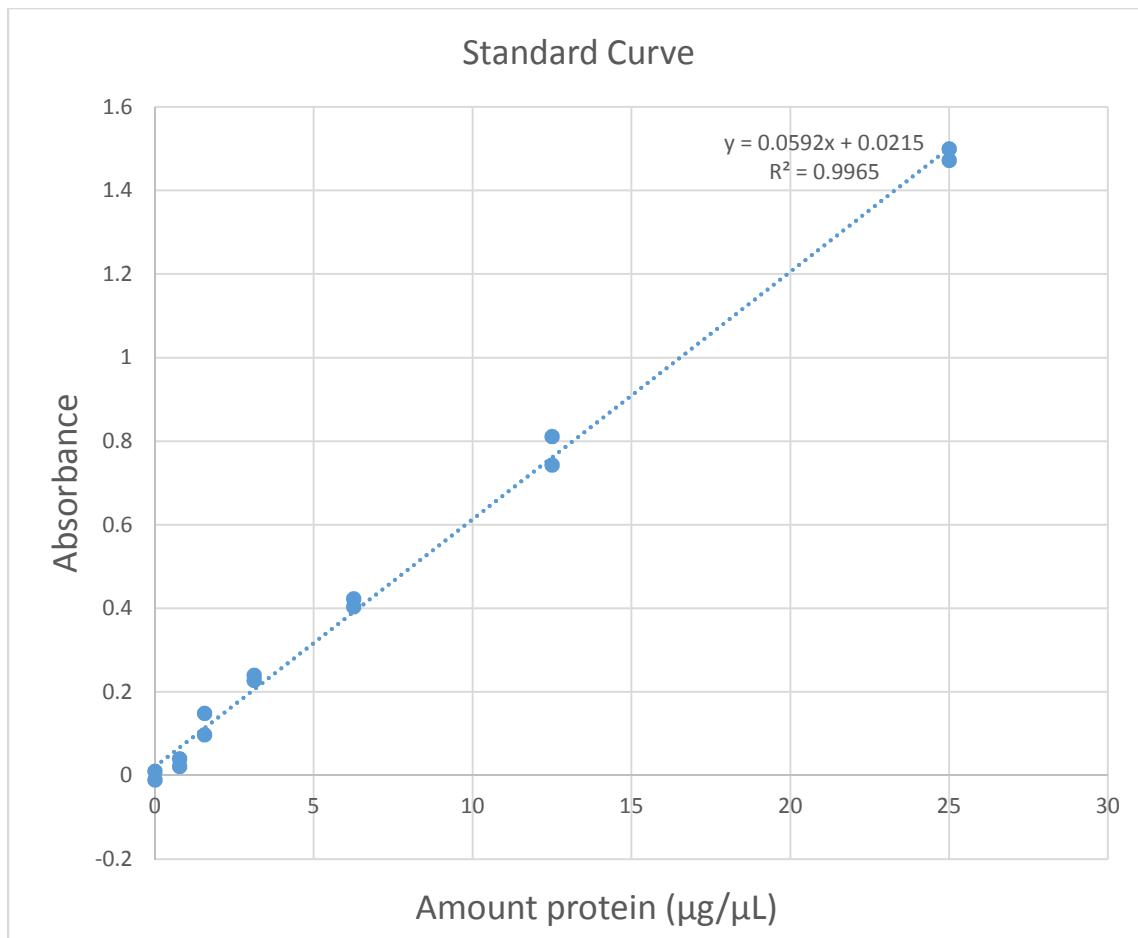


Figure 5. Standard Curve in Duplicate with Tris Buffer for the Fourth BCA.

CHAPTER V

RESEARCH ARTICLE

Chronic aerobic exercise is known to positively influence cognitive performance with benefits particularly evident for measures of hippocampal-dependent memory. There is evidence of enhancement to cognitive performance through dietary modification. Although the effects of chronic exercise on learning and memory are documented, it is unclear what impact protein content of a single meal may have on cognitive function and in combination with regular exercise. The purpose of this study was to evaluate the combined effects of chronic exercise and nutrition on hippocampal-dependent learning and memory in rodents. Six-week old male adult Sprague-Dawley rats ($n=29$) were randomly assigned to a 5 or 6-week forced exercise protocol (Ex) or to a sedentary control condition (Sed). During the last week of exercise, hippocampal-dependent memory was assessed 60 min after a low (15% casein) or high (50% casein) protein meal in the Barnes maze on 4 consecutive days and with a probe trial on the fifth day of testing. Results from this study indicated a main effect for days ($p<0.05$), but no effect of training. There was an observed trend that low protein trained animals performed worse than the other groups. Future studies could investigate different intensities of exercise and volumes of training, in addition to considering the combined effects of exercise and diet on hippocampal-dependent memory.

Introduction

There is a gradual, progressive decline in cognitive function with advancing age (Colcombe et al., 2006). This decline may be partially explained by decreases in the volume of the hippocampus with advancing age, leading to declines in memory function (Erickson et al., 2009; Erickson et al., 2011). This is evidenced by worsening performance in hippocampal-dependent tasks such as those that require efficient spatial memory and function (Erickson et al., 2011; Kramer et al., 2006).

Modifiable lifestyle habits like physical exercise have been investigated in pursuit of developing interventions that help prevent age-related cognitive decline and neurodegenerative disorders such as Alzheimer's (Erickson et al., 2011; Kramer et al., 2006). Aerobic exercise has some documented support to specifically improve cognitive function by increasing volume of the hippocampus in aging adults and improving spatial memory performance with individuals who exercise versus those who remain sedentary (Erickson et al., 2011; Szabo et al., 2011). Hence, it's imperative to further understand the effects of exercise on hippocampal-dependent memory, knowing that changes in hippocampal structure are responsive to exercise and are correlated with improved function (Erickson et al., 2011; Gomes da Silva et al., 2012).

The use of spatial memory paradigms to measure hippocampal-dependent function is commonplace with both human and animal research testing the efficacy of interventions like exercise (Erickson et al., 2011; Gomes da Silva et al., 2012). Animal research investigating hippocampal-dependent memory uses a variety of behavioral paradigms, most commonly the Morris Water Maze (MWM), and the Barnes Maze (BM)

(Harrison, Hosseini, & McDonald, 2009). Both of these hippocampal-dependent tasks have been tested in studies that examine the effects of exercise on cognitive performance (Chorna et al., 2013; Kaidah, Soejono, & Partadiredja, 2016). Results from these studies find that there are significant improvements to spatial learning and memory following chronic exercise training in rodents (Kaidah et al., 2016).

Dietary modification is another lifestyle habit that is garnering attention with regards to improving cognitive function (Tucker, 2016). Age-related cognitive decline has been correlated with nutritional deficiencies, with which a marked deficiency of various dietary factors like antioxidants can increase risk for age-related decline (Nooyens et al., 2015). With relation to learning and memory function, dietary factors that are supported by research to improve cognitive function and reduce risk of age-related decline include omega-3-fatty acids, vitamin D, carotenoids, as well as phytonutrients typically found in a variety of vegetables (Latimer et al., 2014; Tucker, 2016; van de Rest, van der Zwaluw, & de Groot, 2013). It is known that higher protein diets can reduce risk for mild cognitive impairment (MCI) in the elderly, due to their role in maintaining neuronal membrane integrity (Roberts et al., 2012). On the contrary, diets low in necessary proteins can increase the risk for MCI and subsequently dementia, as lack of this macronutrient affects neuronal structure and consequently neuronal function (Roberts et al., 2012; Tucker, 2016). Low intake of protein has been documented to be associated with impaired synthesis of various neurotransmitters in the brain, due to fewer available biological precursors to initiate synthesis processes (Roberts et al., 2012). Impairments in cognitive function with relation to low protein intake include declines in

memory function (Tucker, 2016). It is necessary to understand how dietary modifications with protein might influence cognitive function when the level of protein is adjusted.

Learning and memory performance are often tested in response to dietary modifications by using similar behavioral paradigms as have been used with other lifestyle habits in both humans and animals. Recent studies in humans have looked at the role of antioxidants and omega-3 fatty acids in improving cognitive flexibility to fight risk of age-related decline. These studies primarily used neuropsychological tests of memory to measure how cognitive performance changes longitudinally after regular consumption of these nutrients (Nooyens et al., 2015; Oulhaj, Jerneren, Refsum, Smith, & de Jager, 2016). Although results with these supplements are promising, more work needs to be done testing out the efficacy of these dietary modifications. One way to test a dietary modification is with an animal model, as this allows for more control over the administration of diet.

The past research with animal models of dietary modification has investigated administration of a variety of dietary factors (Latimer et al., 2014; Oulhaj et al., 2016; Rendeiro et al., 2012). Several individual dietary modifications have been linked to cognitive function, including foods rich in flavonoids, diets rich in omega-3-fatty acids, and large doses of Vitamin D (Latimer et al., 2014; Oulhaj et al., 2016; Rendeiro et al., 2012).

Traditionally, exercise and diet are studied independently in regards to their effects on cognitive function, but it's becoming clear that synergistic interactions between these two lifestyle habits could result in significantly enhanced benefits to brain health

that help stave off age-related cognitive decline (Meeusen, 2014). Exercise has been evidenced to interact with dietary interventions, enhancing their positive effects on brain function as well as ameliorate age-related cognitive losses (Meeusen, 2014). Nutrition provides essential building blocks for maintaining efficient neuronal structure and function, and adequate nutrition is linked with improved exercise performance (Meeusen, 2014; Oulhaj et al., 2016).

These studies with exercise and nutrition aim to find combined effects that are more significant for hippocampal-dependent memory than either factor alone. A recent study by Klein et al. (2016) tested the effects of long-term exercise on reversing cognitive impairment in mice fed a high fat diet. They found significant positive changes in cognitive function of mice fed the high fat diet who were chronically exercised, meaning that the long-term exercise altered the effects of the high fat diet on cognitive function (Klein et al., 2016). Results from this study suggested that there can be benefits to cognitive performance outcomes when using one of the interventions (exercise) to blunt the effects of another (diet modification). Another study by Veasey et al. (2015) combined aerobic exercise with vitamin supplementation to test effects on cognitive performance and mood state measured pre- and post-exercise, substrate metabolism during exercise, and affect measured during exercise and post-exercise. It was found that although exercising alone when in a fasted state leads to increased mental fatigue post-exercise, this can be both be significantly reduced and cognitive function actually improved post-exercise when individuals are administered a multivitamin supplement (Veasey et al., 2015).

There is still much to be unraveled regarding the synergistic effects of exercise and nutrition, especially with protein content. Little is currently known about how much protein should be consumed for optimal benefits toward memory performance. Even less information is available about the combined effects with chronic exercise training, which could reveal a relationship between the two. The objective of the following study was to test a combination of chronic exercise training and different protein meals (high and low) in rodents, in order to determine any relationship between the two on hippocampal-dependent learning and memory.

Methods

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Animal Care and Use Committee of the University of North Carolina at Greensboro. Five-six-week old male Sprague-Dawley rats ($n=48$) were housed individually under a 12 hr. dark/light schedule with *ad libitum* access to rat chow and water. The animals were randomly assigned to an exercise condition (trained or untrained) and a diet condition (15% casein or 50% casein).

The exercise treatment for this protocol was 1-week of familiarization plus 5 or 6 weeks of training for a total period of 6-7 weeks. During the familiarization period, each animal in the trained group was placed on the treadmill for 5 minutes daily at a pace of 10 m/min. Exercise training took place during onset of the dark cycle, and each animal of the trained group was placed into individual lanes on the treadmill, while untrained animals were placed individually on non-running lanes of the treadmill. Animals had five

consecutive days of exercise or time in the non-running lanes followed by two consecutive days of rest. Duration and intensity of the exercise was gradually increased over a 5-week period until each exercise session plateaued at 60 minutes in duration and was performed at 30 meters/minute up a 6° grade. Animals were returned to their home cages following each session. The animals in the untrained group remained in non-running lanes of the treadmill for the same duration as the trained group. Behavioral testing for any given cohort was conducted over a two-week period. Hence, the cohort, consisting of untrained and trained rats, was randomly assigned to behavioral testing during either week 5 or week 6 of the exercise protocol. The behavioral testing was performed approximately two hours after the daily exercise session.

The Barnes maze was utilized as the spatial learning measure for this study. The Barnes maze apparatus is a circular arena (~120 cm diameter) brightly lit from above, with 20 holes around the perimeter. One of the holes has an escape chamber where an animal can escape the brightly-lit open arena. The position of the escape chamber does not change. The animals were placed individually in the center of the apparatus in a covered container. After the animal was positioned at the center, the container was lifted and the bright room light was immediately switched on as an aversive stimulus during the maze testing period. Each trial consisted of animals being allowed up to 120 seconds to find the escape chamber using only visual cues represented by four pictorial images, which were placed around the white curtain surrounding the interior of the maze. If the animal found the escape chamber before 120 seconds had elapsed, the video recording of the trial was stopped at that point and the time was recorded. However, if the animal

failed to find the escape chamber after 120 seconds, it was slowly guided to the correct target hole and allowed to enter for 10 seconds. Following conclusion of each individual trial on the maze (whether guided or entering chamber on its own), the animal was removed and returned to its home cage. The maze arena was thoroughly cleaned with ethanol solution and the top, thin movable layer of the maze rotated clockwise to minimize possibility of scent trails being left behind on the maze that could influence the trial of the subsequent animal. The latency and path length to escape was recorded by video recording software for each trial. There were 2 trials per day for 4 consecutive days followed by a probe trial only (no escape chamber is present and time spent in the correct quadrant determined) on day 5 of testing.

Animals' normally received rat chow (Harlan Teklad inc.) containing 18-22% soluble protein and ad libitum access to water, this diet was given on a daily basis during a 5-hr period at the end of the dark cycle. The protein content of the animal's daily chow was modified with different levels of casein before each session of behavioral testing (see Table 9). Animals fasted during their light cycle performed the exercise protocol during the last hour of the light cycle. During behavioral testing, the first meal was provided 1 hour into the dark cycle. The meal provided 20% of their average daily caloric intake. There was then a 4.5-hour period where the animals had no food, during which the behavioral testing took place. The behavioral testing occurred 1 hour after the first meal was administered. This first meal that was administered to each animal had been previously randomized to consist of either 15% casein or 50% casein.

A three-way mixed analysis of variance (ANOVA) was used to assess the results of the Barnes maze. The goal of this statistical analysis was to test for main effects for exercise (untrained, trained), protein content (low, high), and day (1, 2, 3, 4), and for all possible interactions between exercise, protein diet, and day. All statistical analyses were conducted using SPSS version 23 software.

Results

During the acquisition trials of the four days of behavioral testing before the probe trial day, animals in all groups learned to find the escape chamber located under one of the 24 total holes on the Barnes maze platform. This was observable through the reduction in the path length and latency to find the escape chamber across days of testing.

For path latency, results from the Barnes maze yielded a significant main effect for day, $F(2.67, 82.79)=7.08$, $p=0.000$ (see Figure 5), but the main effects for exercise, $F(1,31)=1.24$ and diet, $F(1,31)=0.666$ were not significant, $p>0.05$. None of the interactions were statistically significant (see Table 5).

The distance traveled to the escape chamber was also calculated and expressed as an average of two trials on each individual day. Results indicated that the main effect for day was significant, $F(2.62, 78.50)=3.43$, $p=0.03$, but the main effects for exercise, $F(1, 30)=2.20$, and diet, $F(1, 30)=0.11$, were not significant, $p>0.05$ (see Figure 6). None of the interactions were statistically significant (see Table 6).

Table 7. Day x Exercise Interaction and Values from Statistical Analysis for Path Latency.

| Day x Exercise Interaction Values for Path Latency | | | | |
|--|--------------|----------------|--------|-------|
| Effect | df numerator | df denominator | F | P |
| Exercise x Diet | 1 | 31 | 0.0887 | 0.354 |
| Day x Diet | 2.67 | 82.79 | 0.399 | 0.731 |
| Day x Exercise x Diet | 2.67 | 82.79 | 0.074 | 0.964 |
| Day x Exercise | 2.67 | 82.79 | 0.423 | 0.714 |

Table 8. Day x Exercise Interaction and Values from Statistical Analysis for Distance.

| Day x Exercise Interaction Values for Distance | | | | |
|--|--------------|----------------|------|------|
| Effect | df numerator | df denominator | F | P |
| Exercise x Diet | 1 | 30 | 0.36 | 0.56 |
| Day x Diet | 2.62 | 78.50 | 0.48 | 0.67 |
| Day x Exercise x Diet | 2.62 | 78.50 | 0.24 | 0.84 |
| Day x Exercise | 2.62 | 78.50 | 0.67 | 0.55 |

Table 9. Composition of Protein Diets (High and Low) that were Fed as Meals to the Animals in the Study.

| Protein Diets (Low/High) | | |
|--------------------------|------------|------------|
| | Casein 15% | Casein 50% |
| | g | g |
| Casein | 175.4 | 584.8 |
| Corn Starch | 434.4 | 179.4 |
| Maltodextrin | 144.3 | 57.9 |
| Sucrose | 109.3 | 48.2 |
| Cellulose | 50.0 | 50.0 |
| Soybean Oil | 90.0 | 90.0 |
| Mineral Mix (Casein) | 35.0 | 35.0 |
| Vitamin Mix | 10.0 | 10.0 |
| Choline bitartrate | 2.5 | 2.5 |
| L-Cystine | 2.3 | 7.5 |
| | | |
| | gm% | gm% |
| Protein | 15 | 50 |
| Carbohydrate | 75 | 35 |
| Fat | 10 | 10 |
| | | |
| kcal/g | 3.9 | 3.8 |

Note. (gm) refers to the unit gram.

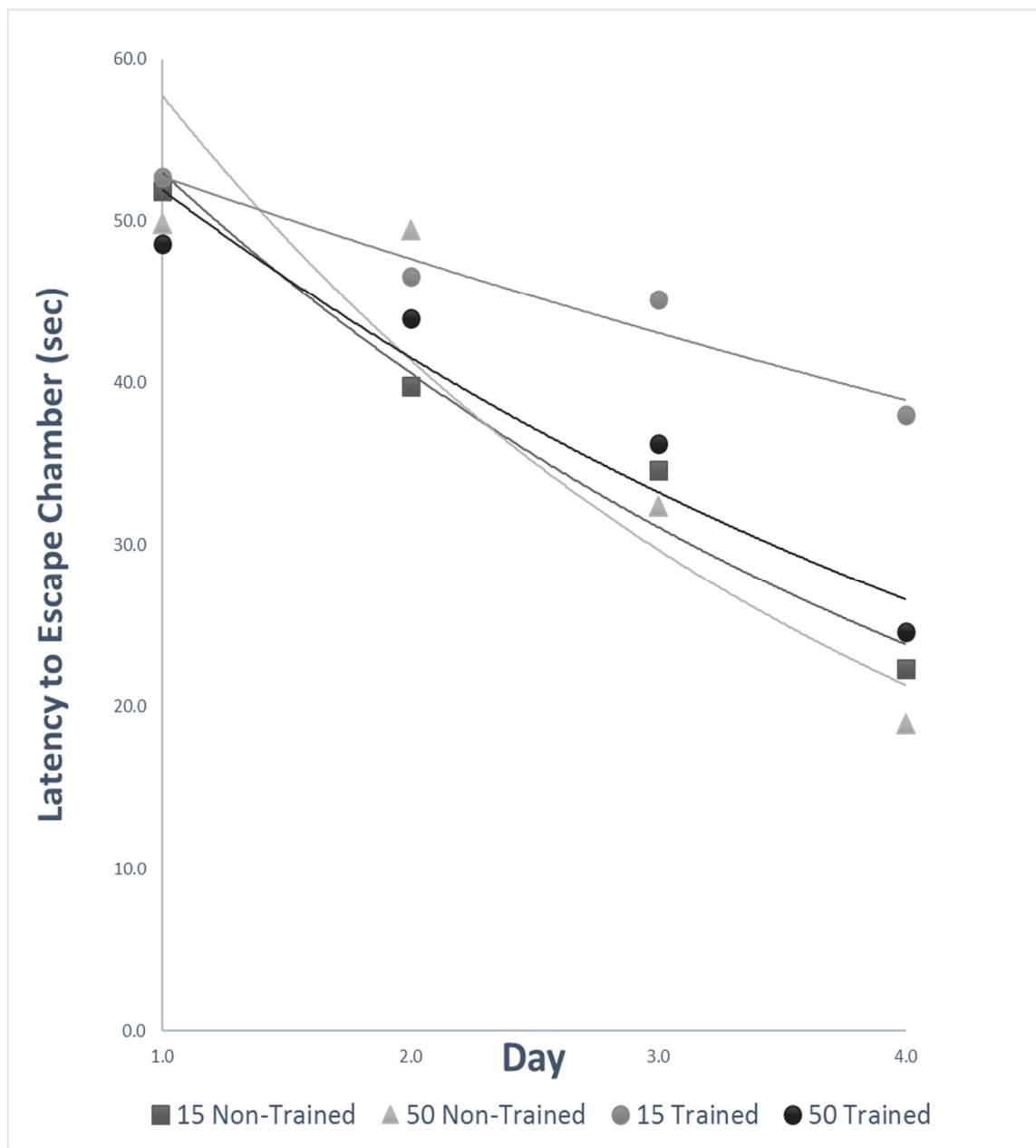


Figure 6. The Average Latencies for Days 1 through 4 of the Barnes Maze Task for All Groups of Animals.

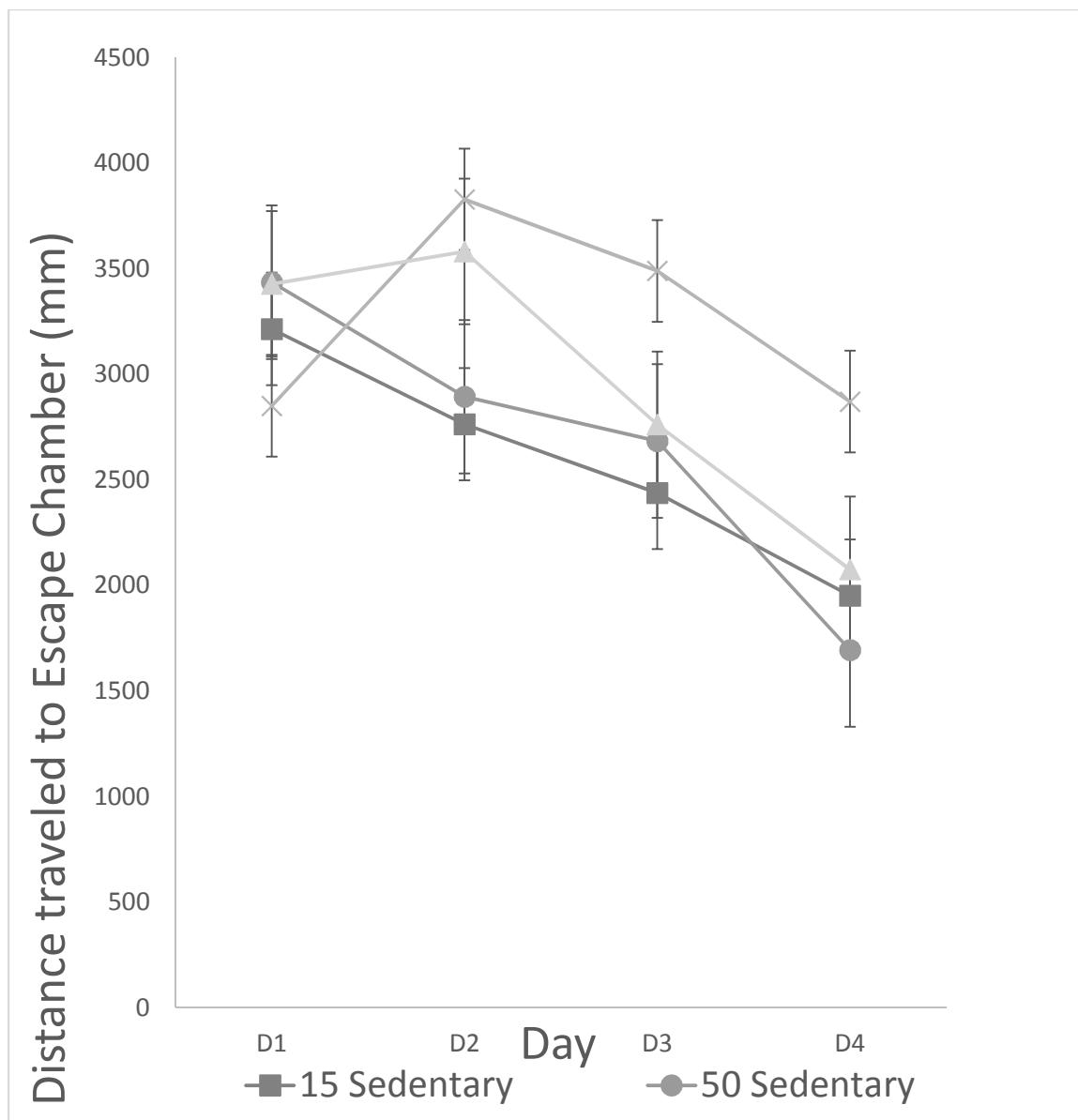


Figure 7. Average Distance Traveled to Escape Chamber for All Groups Between Day 1 and Day 4.

Discussion

The objective of this study was to test the effects of exercise training and modification of the protein content of a single meal on hippocampal-dependent memory using a rodent model. A 5-6-week chronic exercise protocol was utilized along with a single protein meal of either high or low protein prior to each day of spatial memory testing.

There was no significant effect of exercise training in this study, which is a result that does not follow suit with other literature, which has reported significant effects of exercise training on spatial memory performance (Aguiar Jr et al., 2011; Cetinkaya et al., 2013; Kaidah et al., 2016). These results suggest that spatial learning was not enhanced by the chronic exercise protocol utilized in this study. No effect of training might be indicative that something may be different with our exercise protocol in comparison with other studies that got significant effects. The animals used in this study were of a young age (6 weeks at start), which could have limited the likelihood of observing any effects of exercise on memory performance. There is evidence of more pronounced and significant effects of aerobic exercise training on spatial memory for older rodents (Driscoll et al., 2006; Gomes da Silva et al., 2013).

Another possible explanation for the different results that was considered related to the exercise intensity. However, there is evidence that the rodents in this study had a substantial gain in aerobic capacity in response to the training protocol. In this study, the rate of speed for the treadmill was set to plateau at 30 meters per minute by the end of week 3 and remained steady through week 6. Mitochondria content of the animals was

measured via cytochrome *c* oxidase activity in the gastrocnemius muscle using a Clark-type oxygen electrode and showed a 78.7% increase in activity compared to the relative sedentary values (Starnes, 2016). Again, these findings indicate that an effect of exercise was found with this training protocol, however it is not clear why this was not detected with the behavioral test. Another possibility is that this exercise protocol was too short in length in comparison with others that had more weeks of training (Aral Latife Arzu, 2014; Chennaoui et al., 2015; Kaidah et al., 2016). The protocol used in this study also peaked in volume of training at 60 minutes per session, which might not be long enough as evidenced by studies using longer periods (Chennaoui et al., 2015). Although gains in aerobic capacity were achieved in this study, to observe changes in the behavioral measure it may have been necessary to have more added volume of training(Chennaoui et al., 2015).

If volume of the exercise protocol was insufficient, it would be important to address this in further studies by testing different volumes, lengths, and intensities of training under similar conditions. Since no effect of training was detected with the Barnes maze, the results do not follow with what has been previously reported (Cechella, Leite, Gai, & Zeni, 2014; Cetinkaya et al., 2013; Kaidah et al., 2016). Obtaining more data with different exercise protocols could prove useful toward determining what might underlie the null effect of training. The primary means of testing spatial learning and memory in this study was the Barnes maze, a task that has been previously proclaimed to be less aversive than the Morris water maze (Harrison et al., 2009). This selection was made to have a test for hippocampal-dependent learning that wasn't as stressful and involves less

physical activity than the Morris water maze (Chorna et al., 2013). However, because the task was less aversive, it is possible that the animals tested using this spatial memory paradigm were not motivated enough to find the escape chamber. This might have been exacerbated by the fact that the animals in this study were handled extensively for 6-7 weeks. This could influence their exploratory behavior in the maze, because it is known that regular handling modifies the stress response. In a future study, it would be important to test for aversiveness in the Barnes maze because this might be critical for observing the benefits of exercise in the task. Then a further study would be needed to better determine the possible role of stress in the spatial learning and exercise relationship. As reported previously, there is little stress response in animals during the Barnes maze, especially in comparison with the Morris water maze indicating that level of stress was of little concern for animals in this study (Harrison et al., 2009; O'Callaghan et al., 2007).

Although every group improved their performance on the Barnes maze over the course of days 1 through 4, the low protein trained group performed the worst overall, as can be observed in Figure 5. This group also had the longest distance traveled on average in the maze, making for an interesting trend. After observing this trend, there was a possibility that this group was notably poor in comparison with the other conditions. One explanation for this result could be that chronic aerobic training may require more protein than accessible to provide the necessary biological precursors to support memory processes (Meeusen, 2014). Another explanation could be that during exercise, the muscle used up available amino acids, resulting in less essential amino acids left available for brain cells (Monti et al., 2014). It is also possible that the 1-hour time period

prior to the Barnes maze task was not enough time to digest the protein meal, in order to have essential amino acids readily available. Further research could test various timings of the meal prior to the Barnes maze to better determine if there is a significant difference with meal timing and digestion of protein. Dietary factors such as protein are involved in regulating several brain mechanisms including neurotransmitter synthesis, synaptic transmission, fluidity of neuronal membranes, and signal-transduction pathways (Meeusen, 2014). Because protein in the diet may be crucial to neuronal health and brain function, understanding its influence on brain processes with aerobic exercise could shed light on the mechanisms that maintain efficient cognitive functioning (Monti et al., 2014). This trend with low protein and aerobic exercise could be worth exploring in further studies, in order to more fully understand their association with memory function (Monti et al., 2014). Another possibility involves the timing of the protein meal with the bout of exercise, testing different timings for the protein meal relative to the timing of the exercise could shed more light on when the protein is most available to benefit memory.

Figure 5 illustrated the performance of all groups in the Barnes maze and an observable trend from this graph involved the high protein trained group improving in memory performance much faster than the high protein untrained group. This can be observed in the figure by looking at the line of best fit for the high protein trained animals, where they start out with high latency, but end up near the two untrained groups at day 4. It could be useful to know this because if those in the high protein trained group are improving faster, there might be an effect with the combination treatment that could be observed with more data. This would help support the original hypothesis of high

protein/trained animals performing better in the spatial memory task than other groups alone, because it can be speculated that something in the high protein/trained condition maybe influencing the group to improve in the task the fastest.

If further results show that this finding for the low protein trained animals is reliable, this may indicate that chronic exercise training necessitates more protein to provide essential biological precursors to support memory processes. Because it is not entirely clear whether the results from our study support any specific trend, future studies may need to collect more data by increasing the number of animals in both the exercise and diet groups. A reason for doing this involves the null results with training in the Barnes maze, possibly resulting from too small of a sample size. The use of a larger sample could potentially increase the power of statistical analyzes, allowing for a clearer interpretation of the results from this study, especially to determine if the null results in the Barnes maze for training were just due to simply not having enough animals. Also, since no effect of training was detected, it might be useful in further studies to test out different training volumes to better replicate training protocols used with related studies that observed significant training effects.

After considering the results from this study, it is suggested that future research examine the combined effects of chronic exercise training and the protein-content of meals eaten prior to engaging memory related tasks to optimize potential performance. It is also imperative to better understand how the relationship between chronic exercise and protein diet composition influence cognitive performance through future studies that test how different conditions of each factor interact with cognitive function.

CHAPTER VI

DISCUSSION

The process of writing this thesis manuscript included multiple meetings to discuss potential methods and the direction that the project took changed as more information was understood about the ability level of the investigator. Beginning with a proposal that included both behavioral and cellular analyzes, it was soon determined that the timeline for the training of the investigator was too extensive to allow for the accomplishment of all that was originally agreed upon. Reasons behind this included both the current ability and experience level of the investigator, individual progress with reaching milestones of proposed work, and unforeseen complications with proposed methods.

After investigating the ELISAs and which avenue to take regarding their execution, the decision was made for the investigator to forego completing them. This led to the conductance of the BCA assays, a technique that was new to the investigator and one that could help with understanding an approach for preparing the hippocampal tissue samples for the ELISA analysis. This new direction involved completing several BCA assays with different tissue preparation methods and sample dilutions to find a standard curve that would fit the unknown hippocampal tissue chunks. These BCA results first identified that generating the curve with the tris buffer is most efficient as this is the buffer used to dilute the unknown samples.

The next idea emerging from the BCA assays was homogenization of the samples with sonication, a necessary step prior to centrifugation and collection of the supernatant. This helps release more protein for collection, increasing the available protein to be assayed. Dilution of the sample homogenate and its supernatant revealed a more accurate picture of the protein concentrations. After comparing dilutions of 1:10, 1:20, 1:30, 1:40, and 1:50 that fell on a standard curve of 0-25 µL/µg protein, a method for determining tissue sample sizes between 20-60 mg was devised. Results from the fourth and final BCA had the most close-fit curve for the tissue samples, ranging in values from 50-93% recovery. The higher percent recoveries were present for the sample homogenate, while the lower values (between 30-65%) were for the supernatant. Both of these results were nearly what was expected, higher percent recoveries for the homogenate arise from less dilute samples. Thus, the recommended method of homogenization for sample preparation would have the following steps; First, dilute the samples in 200 µL of tris buffer. Then, sonicate the samples until a uniform liquid is obtained. Third, centrifuge the samples for 10 minutes at 6000 g-force to obtain the supernatant. Pipette the supernatant and store it in -80 C° or use immediately to run a protein assay.

The Barnes maze results from this study illustrated an interesting trend with the low protein trained animals, in which they did not perform as well as any other group. Their performance was evident in Figure 6 because it can be observed that they are the worst group overall, and although not a statistically significant difference, it was worth considering. The trained group improved faster than any other group, another trend in the

data that could be observed from Figure 6. This suggested that the trained animals were getting better at finding the escape chamber than other groups over time.

Another factor that could have influenced the null result from the Barnes maze was the possibility that it was not aversive enough for the animals. In the literature, it is supported that the Morris water maze is more stressful than the Barnes maze because of a significant spike in corticosterone levels in animals following the last day of training versus animals on the last day of Barnes maze training (Harrison et al., 2009).

In the future, if the results with the Barnes maze followed the same trend noted above with more animals, that may help indicate that chronic exercise training requires more protein intake to yield biological precursors to support memory function. Future studies would need to collect more data with the Barnes maze by increasing the number of animals overall, because results from the current study are based on a small sample size. No effect of training was found in the study, so it might be useful in further studies to investigate different exercise intensities and training volumes to better replicate training protocols used with the studies that observed significant training effects. It also could be interesting to more deeply test how the relationship between chronic exercise and protein diet composition influence cognitive performance through further studies that test different conditions of each factor and their interaction with cognitive function.

The results from the last BCA assay provided a potential preparation method for the hippocampal tissue samples to be used with ELISA. A standard curve was also produced to fit the unknown protein samples and calculate their respective concentrations across a range of tissue weights. A wide range of low and high concentrations emerged

from various dilutions of unknown protein samples and were fit to the standard curve, meaning the curve has workable endpoints.

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