4-2024

Beneficial Effects of Exercise on Glial Cell Line-Derived Neurotrophic Factor Content and Endplate Morphology in Skeletal Muscle of Aging Male and Female Rats

Juliana M. VanGyseghem
Western Michigan University

BENEFICIAL EFFECTS OF EXERCISE ON GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR CONTENT AND ENDPLATE MORPHOLOGY IN SKELETAL MUSCLE OF AGING MALE AND FEMALE RATS

Juliana M. VanGyseghem, Ph.D.

Western Michigan University, 2024

Premenopausal women display lower incidence and severity of neurological disease compared to men of the same age, yet these populations are often treated similarly. A decline in neuromuscular function is associated with aging, which may be partially explained by a decline in neurotrophic factor expression with age. A possible way to maintain neuroprotection would be to regulate the production and release of a target-derived neurotrophic factor, such as glial cell line-derived neurotrophic factor (GDNF). GDNF has been shown to be the most potent survival factor for motor neurons and GDNF content of skeletal muscle has been shown to increase with exercise.

This study examined GDNF levels and endplate morphology in skeletal muscle of males and females at different ages and with exercise. We hypothesize that the neuromuscular junction (NMJ) area and endplate dispersion will increase with sedentary aging in both sexes. We also hypothesize that prior to reproductive senescence, GDNF content will be higher in female rats than age-matched males. Furthermore, we hypothesize that exercise will increase estrogen levels and GDNF expression in skeletal muscle.

Hindlimb muscles soleus (SOL) and plantaris (PLA), and serum estrogen were taken from sedentary and exercised male and female Sprague-Dawley rats from 4 to 78 weeks of age. Sedentary groups consisted of 4, 6, 8, 12, 52, and 78-week-old females, and 4, 6, 8, and 12-week-old males. Exercise groups consisted of male and female 4-week-old animals that voluntarily exercised in a running wheel for 2 weeks, 8-week-old animals exercised for 4 weeks, and for females a 52-week-old group exercised for 26 weeks. Right SOL and PLA were used for

immunohistochemical analysis. Acetylcholine receptors at the NMJ were stained with α-bungarotoxin. Left SOL and PLA were used for analysis of GDNF protein content by using enzyme-linked immunosorbent assay (ELISA). Trunk blood was taken, and the serum was used for quantification of estrogen levels using ELISA.

Our findings indicate that as individuals age, there is a noticeable change in the area and dispersion of endplates. Through exercise, we saw endplate area increase, coupled with a reduction in dispersion. However, in male rats, exercise did not significantly change endplate dispersion. Our study stands to be a pioneering investigation into the connection between endplate structure, aging, and exercise in female rats. Our results additionally reveal that levels of GDNF protein were higher in younger females compared to age-matched males. Physical activity increased GDNF levels in both sexes, consistently maintaining higher levels in females up until 12 weeks of age, when exercised males displayed the highest GDNF content overall. Furthermore, exercise induced an increase in estradiol levels. These collective findings contribute to the expanding narrative highlighting the positive effects of exercise across the lifespan, benefiting the individual both neurologically and physically.

BENEFICIAL EFFECTS OF EXERCISE ON GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR CONTENT AND ENDPLATE MORPHOLOGY IN SKELETAL MUSCLE OF AGING MALE AND FEMALE RATS

by

Juliana M. VanGyseghem

A dissertation submitted to the Graduate College
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Biological Sciences
Western Michigan University
April 2024

Doctoral Committee:

John M. Spitsbergen, Ph.D., Chair
Pamela Hoppe, Ph.D.
Monica McCullough, Ph.D.
Christopher Pearl, Ph.D.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr John Spitsbergen. From the very beginning, I knew what to expect from him as a mentor, beginning with his ‘open door’ policy. While juggling many things and having an overflowing plate of duties and responsibilities, he always made himself available for his grad students.

I would also like to thank my committee members, Dr. Hoppe, Dr. McCullough, and Dr. Pearl. Dr. Hoppe, thank you for always pushing me to know more and learn. Dr. Pearl, thank you for your invaluable help as my estrogen guy and for helping me make sense of some of the data. Dr. McCullough, I cannot thank you enough for the all-around support you provided. From mentorship as an educator, as a mom and woman in science and the constant encouragement you gave spurred me on to where we are now. Thank you all for your vital role in this process!

To Dr Jellies. Thank you for your wisdom and insight. You have helped me become a more critical thinker and have enhanced my articulation skills. I will miss our chats!

To my lab family. Alberto, you taught me everything I know. The amount of time you gave me, while finishing your own PhD, was sacrificial and generous. You supported and encouraged me, in the highs and the lows. To all the women scientists who showed me it was possible; Sydney, Susanne, Bonnie, Tara, Sam, and Alyssa. And a huge shout-out to the PhD-Moms- Alanna, Sarah and Amy G. You all kept me afloat whether it was walks, tots, dance parties, hugs, or the look of sympathetic understanding… it takes a village and I have the best one.

To my church family. Holly, Hannah, Kayla, Dawnie, Kim, Amber, Allena, Ashley, and Alexis. You all came into my life at the right exact moment. Thankful for a God who sees me, loves me, and has provided me with such wonderful people.

To my oldest friend, Organic Amy. You have stuck with me for all the transitions and transformations that life has thrown our way. Thank you for always checking in, spurring me on and being a listening ear.

엄마의 이타심과 그 모든 희생 때문에 제가 여기까지 왔어요. 고맙고 사랑해요.

To Mom and Dad. For always asking me how research was going, even if it didn't make any sense to you. And for raising me in such a way to know I can do hard things. Holly… for having the vision to know when to start and for being my biggest advocate and cheerleader. You have been that for me since day 1, and you are a huge reason why I am the way I am (in a good way).

My own family. To my kids who saw every side of what grad school looks like. For their understanding and all the ways they stepped up to help. I hope this will inspire them to reach for their dreams and work hard to achieve them. And, to the biggest hero of them all, my wonderful husband, Dr. VanGyseghem. For the many nights that I would come home and say "Grad school is hard" and you knew exactly what that meant. For endlessly troubleshooting equipment in the lab so that I could continue my research. For believing in me. Always. Especially when I didn't believe in myself. And for being everything I needed in every moment. Thank you.

Juliana M. VanGyseghem

TABLE OF CONTENTS

Table of Contents — Continued

CHAPTER

Table of Contents — Continued

CHAPTER

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The focus of the research in our laboratory is to understand the regulation of neurotrophic factor expression in target tissue of the peripheral nervous system, and the role they play in helping maintain a healthy nervous system as we age.

Aging Population and Healthcare Costs

Aging is defined as the process of becoming older. It's something we all do, whether we like it or not. Ironically when we're younger we can't age quickly enough, but as we get older, we wish time would slow down. There comes a point in aging when it is no longer beneficial to us, as our molecular and cellular processes begin to damage, leading to a decrease in physical and mental capacities. Not only that but getting older is very expensive. According to a report in 2019 the life expectancy for both males and females has continued to be on an upward trend. The World Health Organization estimates, by the year 2030, that about 17% of people will be over the age of 60 years old, raising the elderly population level to close to 1.4 billion people. To extrapolate even further, by the year 2050 the overall population size will increase to 2.1 billion and the aging population that is 80 years old or higher will be close to 246 million. So, what does that mean? Recent studies from the Centers for Medicare and Medicaid have shown that the US spends a little over 4 trillion dollars on healthcare and healthcare-related costs. Interestingly, about 81% of it is spent on personal healthcare. This has been steadily increasing due to our aging population. Not only that, but it raises the question of the quality of life. Just because we are living longer, does that mean our quality of life is good?

Sarcopenia, senile muscle atrophy, is seen in the elderly and can cause a difference in the quality of life (T. Gustafsson & Ulfhake, 2021; Marcell, 2003). Sarcopenia is defined as the loss

of muscle mass, and strength due to aging. In 2010 it was estimated that almost half of the elderly population, about 18 million people in the USA were affected by sarcopenia (Janssen et al., 2004).

One might ask, why does sarcopenia occur? There are some camps of thought that the reason why sarcopenia occurs is because of the loss of communication between the neuron and the muscle. Atrophy occurs when protein synthesis is less than the rate of protein degradation, leading to a decrease in total muscle protein, muscle mass, and then impaired functional ability (Sartori et al., 2021). Atrophy has been seen after reduced neural stimulation, hindlimb unloading, immobilization, and the loss of nerve connection(Cisterna et al., 2014; Gao et al., 2018; Tomanek & Lung, 1974).

Just as getting older is a natural occurrence, there is something that happens within your body called neural plasticity. Neural plasticity is where neurons make contact, retract, make contact, and retract with the target tissue. While there is nothing inherently wrong with this process, during the aging process the time that the neuron stays retracted increases (Hunter et al., 2016). The prolonged loss of motor neurons (MNs) connecting to target tissue has been shown to limit the target tissue's ability to sprout and regenerate, leading to decreased muscle mass and strength, known as sarcopenia (Hunter et al., 2016).

Neuromuscular Junction

The neuromuscular junction (NMJ) is the area that links the MN and skeletal muscle fibers. It is a specialized area of the motor unit, where the MN innervates the skeletal muscle. The NMJ is where the nerve communicates with the muscle. Communication at the NMJ involves a synapse from an axon that is extending from a MN in the spinal cord. The end of the axon reaches the skeletal muscle fibers and makes contact through an area called the motor

endplate. There is a small space between the axon terminal and motor endplate called the synaptic cleft. In the synaptic cleft is the site where communication between the presynaptic neuron and postsynaptic junction of the skeletal muscle occurs.

Communication between the neuron and skeletal muscle begins with the depolarization of the axon terminal. After depolarization, voltage-gated calcium channels open allowing calcium to enter the terminal. There are synaptic vesicles that contain the neurotransmitter, acetylcholine (ACh), that get triggered to move towards the edge of the terminal upon the entry of calcium. Once the vesicles have reached the edge of the terminal, they bind with the membrane. Here the synaptic vesicle fuses with the membrane and releases ACh by exocytosis. The ACh will travel across to the synaptic cleft where it will bind with ACh-receptors. This leads to muscle fiber excitation which will result in muscle contraction and then ultimately movement.

NMJ Morphological and Functional Changes

The NMJ functions as a crucial link between nerves and skeletal muscles, facilitating the effective conversion of chemical signals into electrical signals in skeletal muscle. This process is vital for normal muscle contraction and then maintenance of muscle mass. Research suggests that disruptions in the structure and function of the NMJ play a significant role in the decline of muscle mass and muscle power associated with sarcopenia (Casati et al., 2019; Faulkner et al., 2007).

For optimal signal transmission, changes occur in the pre-synaptic membranes and post-synaptic membranes have a branching pattern known as the ‘pretzel-like’ configuration (Rudolf et al., 2014). Studies by Rowan et al. (2011) indicated that the weakening of nerve connections in aging muscles, known as denervation, is a primary contributor to muscle fiber atrophy. As a result of degenerating nerve terminals and muscle fiber loss that come with age, post-synaptic

ACh-receptors undergo structural reorganization, referred to as ‘fragmentation’ (Rudolf et al., 2014). Furthermore, Deschenes et al. (2011) observed changes in the distribution of ACh-receptors preceding the age-related muscle alterations seen in sarcopenia, including an increase in ACh-receptor area and perimeter.

NMJ and Aging

NMJs are a good indicator of motor health on a systemic level. With healthy NMJs ACh-receptors are densely clustered in continuous, winding structures that are almost ‘pretzel-like’ (Rudolf et al., 2014). However, in aged or dystrophic muscle the NMJ becomes more fragmented, with less continuous connections.

One potential factor contributing to fragmentation might be motor neuron death. Declining motor neuron health results in the disconnection of muscle fiber innervation, followed by reinnervation from adjacent neurons, forming new neuronal sprouts that target the same post-synaptic structure (Rudolf et al., 2014). The NMJ is the site in which effective transmission from chemical signals in nerve terminals to electrical signals in skeletal muscle is essential for normal muscle contraction and muscle maintenance. Current understanding in the field suggests that motor neuron death is the leading cause of denervation in aging sarcopenia because of the fast decline in the number of alpha motor neuron cell bodies in the spinal cords of humans older than 60 years old (Berger & Doherty, 2010; Kawamura et al., 1977; Tomlinson & Irving, 1977). Having NMJ structure and function impairment is suggested to play an important role in sarcopenia-related decline of muscle mass and muscle power (Casati et al., 2019; Faulkner et al., 2007).

With senescence, increased levels of dispersion is a sign of weakened motor unit recruitment. With fewer MNs and MUs, to be able to recruit the same fiber the remaining nerve

terminal is branching to reinnervate denervated muscle. The loss of innervating motor neurons and possibly decrease levels of trophic signaling between neuron and muscle, may explain an increase in sarcopenia among the elderly (Lexell, 1993).

Neurotrophic Factors

Neurotrophic factors (NFs) are a family of biomolecules that support the growth, neuronal survival, regulate cell proliferation and differentiation of both developing and mature neurons, modulate axonal and dendritic outgrowth and regulate synaptic plasticity (Henderson et al., 1994; Morcuende et al., 2013; Yan et al., 1995; Zhu et al., 2008). There are different families of NFs defined by their unique cell signaling mechanisms. One of those families are neurotrophins which include brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). The family of ciliary neurotrophic factor family includes ciliary neurotrophic factor (CNTF) leukemia inhibitor factor (LIF), interleukin-6 (IL-6), prolactin, growth hormone, leptin, interferons and oncostatin M. Another family is the glial cell line-derived neurotrophic factor family ligands (GFLs) which includes artemin, neurturin, persephin and glial cell line-derived neurotrophic factor, GDNF, the neurotrophic factor that I will be focusing on in my studies (Cobianchi et al., 2017; Oppenheim et al., 1995).

GDNF

GDNF was first identified in 1993 in cultured B49 rat glial cells that were found to enhance the survival and differentiation of dopaminergic neurons by promoting dopamine uptake in embryonic midbrain dopaminergic neurons (L. F. Lin et al., 1993; L. H. Lin et al., 1994). It was characterized as a distant member of the transforming growth factor-β superfamily (L. F. Lin et al., 1993). GDNF is a dimeric protein linked by disulfide bonds, featuring seven conserved

cysteine residues. Its molecular weight is 30 kDa, which undergoes glycosylation as well (L. F. Lin et al., 1993). There are two forms of GDNF, in which the mature form is cleaved for secretion and expressed in two splice variants. The larger of the two is $GDNF_{633}$ and a truncated form that is missing 78-base pair sequence, $GDNF_{555}$. These are derived from a single RNA (Springer et al., 1995). These two different isoforms are expressed differentially in rat skeletal muscle. In healthy skeletal muscle the predominant isoform is $GDNF_{555}$ while $GDNF_{633}$ becomes the predominant isoform following denervation (Springer et al., 1995).

While GDNF has been shown to have major effects on enteric, sympathetic and dopaminergic neurons, it has also been identified as a potent neurotrophic factor for regulating MN survival in the peripheral nervous system (Henderson et al., 1994). GDNF prevents MN apoptosis during development *in vivo* (Oppenheim et al., 1995), decreases the loss of MNs in animal models of motor neuropathy and degeneration, rescues MNs from axotomy-induced cell death, and protects MNs from chronic degeneration (M. S. Airaksinen & Saarma, 2002; L. Li et al., 1995; Ruven et al., 2018; Sariola & Saarma, 2003; Trupp et al., 1995).

After the discovery of GDNF, many researchers began identifying which cell types in the mammalian body contained and produced GDNF. GDNF protein is found throughout both the central and peripheral nervous systems. It has been found within the normal growth and morphogenesis of the ureteric bud in developing kidneys and in Sertoli cells in the testis (Costantini, 2010; Meng et al., 2000). Synthesis and secretion of GDNF has been found in many cell types such as astrocytes, oligodendrocytes, Schwann cells, MNs, and skeletal muscle (Henderson et al., 1994; Yamamoto et al., 1996; Yan et al., 1995).

GDNF Synthesis

GDNF, initially synthesized as a 211-amino acid precursor within mammalian cells, is a secreted protein. Its pre-sequence guides it to the endoplasmic reticulum to facilitate secretion. Following secretion from the endoplasmic reticulum, the protein undergoes a process involving sulfide bond-mediated folding and dimerization. Subsequently, N-linked glycosylation modifies the protein, which then undergoes proteolytic cleavage to transform into its final mature form, consisting of 134 amino acids (L. F. Lin et al., 1993; L. H. Lin et al., 1994; Piccinini et al., 2013).

GDNF Receptors and Signaling

Members of GFLs have low amino acid sequence homology, but function as homodimers of the activation of tyrosine kinase rearranged during transfection (RET) receptor. The RET receptor is expressed in the central and peripheral nervous system during development (Sanicola et al., 1997). GDNF, like the rest of the GFLs use RET as a signaling receptor but RET can only be activated in the presence of co-receptor glycosylphosphatidylinositol-linked (GPI) GDNF receptor α (GFRα) (Sariola & Saarma, 2003). GRFα receptors are bound to the plasma membrane via GPI anchors (Poteryaev et al., 1999) which contain three globular cysteine-rich domains, with the second domain being most commonly bound by GLFs, which is crucial for RET binding (M. Airaksinen et al., 1999; Peterziel et al., 2001).

GDNF has two regions that make contact with the GFRα receptor. One of the regions is where the N-linked glycosylation takes place (Eigenbrot & Gerber, 1997; Parkash et al., 2008; Silvian et al., 2006). The other region is the C-terminal of the mature GDNF which is very important for its binding property to GFRα1 and activation of RET (Eketjall et al., 1999; Parkash et al., 2008). It is in the C-terminal of mature GDNF that we find cysteines Cys131 and Cys133

which participate in the formation of a ring structure by linking with Cys68 and Cys72 (Oh-hashi et al., 2009).

Glial cell line-derived factor family ligands (GFLs) express high affinity specificity for one of the four main GFRα receptors (GFRα1-4). GDNF binds to GFRα1, NRTN binds to GFRα2, ARNT binds to GFRα3, and PSPN binds to GFRα4. The binding of the GFLs recruits RET to the lipid rafts, which promotes phosphorylation and dimerization of the tyrosine residues and triggers its association with Src family kinases (SFK) required for downstream signaling (Tansey et al., 2000). SFKs only interact with RET when the receptor is located within the membrane microdomain (Tansey et al., 2000). The RET-SFK relationship in downstream signaling caused by GDNF has been studied and found that SFK activity, in particular p60Src, was needed for GDNF-mediated Akt and MAPK phosphorylation (Encinas et al., 2001). The activation of RET triggers the mitogen activated protein kinase (MAPK), phosphoinositositide-3-kinase (PI-3K), Erk and Akt pathways which are attributed to act in promotion of cell survival (M. S. Airaksinen & Saarma, 2002; M. Kim & Kim, 2018; Sariola & Saarma, 2003). Src was found to be critical for GFL-mediated neuronal survival, when a PI-3K inhibitor was used and it prevented GFL-mediated and Src-mediated neuronal survival (Encinas et al., 2001).

While GFRα is more widely expressed when compared to RET in the nervous system there is another way that GFRα receptors can signal (Trupp et al., 1997; Yu et al., 1998). GFRα receptors could signal independent from RET via a novel transmembrane protein (Poteryaev et al., 1999; Trupp et al., 1999). Studies performed by Paratcha et al., (2003) that involved RET-independent signaling by GDNF, in both glial and neuronal cells, showed a similarity between intracellular pathways that were activated by the neural cell adhesion molecule (NCAM), $p140^{NCAM}$ NCAM isoform. Neural cell adhesion molecule (NCAM) is an adhesion molecule

found in the nervous system and skeletal muscle, and it is involved in cell migration, synaptic plasticity, and neurite outgrowth during development (Crossin & Krushel, 2000; Ronn et al., 2000; Schachner, 1997). NCAM isoform interacts directly with GDNF family ligands along with GFRα receptors, and mediates GDNF signaling in the absence of RET. GDNF, acting via NCAM pathway, has been shown to promote Schwann cell migration and axonal growth in cortical and hippocampal neurons (Paratcha et al., 2003). GFLs exhibit a lower binding affinity for the NCAM isoform and do not initiate intracellular signaling upon direct binding. Nevertheless, in the absence of GDNF, the interaction between GFRα1 receptors and NCAM leads to the suppression of NCAM-mediated cell adhesion, allowing for potential variations in physiological outcomes.

GDNF mRNA and protein are found to be localized to the postsynaptic element of the NMJ in skeletal muscle. However, RET and GFRα1 mRNA are found in the MN and their proteins are concentrated in nerve endings at the presynaptic terminal of the NMJ (Baudet et al., 2008; Suzuki et al., 1998). Because of the location and quantity of RET and GFRα1 receptor at the NMJ and how closely GDNF is expressed there it suggests that there is an important trophic role in nourishing and maintaining the neuromuscular synapse.

GDNF and Skeletal Muscle

GDNF can be expressed by numerous target tissues, including skeletal muscle. In 1995, researchers successfully employed PCR from adult skeletal muscle identifying two distinct isoforms of GDNF (Springer et al.) One variant ($GDNF_{633}$) exhibited elevated expression in denervated rat skeletal muscle after 1-2 weeks of axotomy (Springer et al., 1995).

In 2004 Zhao et al. investigated GDNF mRNA expression across four distinct types of skeletal muscle, including healthy skeletal muscle, denervated muscle, denervated muscle that

received sensory input, and denervated muscle in which innervation was immediately repaired. The findings indicated that denervated muscle displayed the highest GDNF expression, followed by muscle receiving sensory input, and finally, muscle undergoing immediate repair. The category of healthy skeletal muscle exhibited the lowest GDNF mRNA levels, emphasizing GDNF's heightened expression during reinnervation at the NMJ (Zhao et al., 2004). The results from experiments performed by Springer and Zhao provide further evidence that diverse GDNF isoform expression is influenced by muscle innervation. This highlights GDNF's role as a neurotrophic factor originating from target tissues such as skeletal muscle. Moreover, trophic factor is dynamic following different injuries.

Another study that looked at the effects of denervated human skeletal muscle and GDNF upregulation was done in 1998 by Lie & Weis. They were looking at GDNF expression transcripts in healthy skeletal muscle, denervated skeletal muscle, and muscle from patients with Duchenne muscular dystrophy (DMD). DMD is an inherited genetic condition resulting in changes to the dystrophin protein. These changes ultimately lead to the deterioration of muscles and a state of physical frailty. What Lie and Weiss saw was that GDNF expression was significantly higher in muscle that was denervated when compared to normal or DMD affected muscle. A possible explanation for why this might have occurred is that there is still some amount of innervation with DMD patients so there is less need to try and attract the nerve back to the muscle, when compared to a completely denervated muscle. While other researchers had shown the effects of GDNF expression in denervated muscle, Lie and Weiss showed that GDNF mRNA expression in humans was similar to patterns that were observed in rats (Lie & Weis, 1998; Springer et al., 1995; Zhao et al., 2004).

So, what happens to muscle fibers when there is an excess of GDNF? In 1998 (Nguyen et al., 1998)used a mouse model that overexpressed GDNF in muscle fiber leading to a hyperinnervation at motor endplates. This was unique because when overexpressing other neurotrophic factors, such as NT-3 and NT-4 it did not result in the same hyperinnervation of muscle fibers (Nguyen et al., 1998). Keller Peck et al. in 2001 found similar results following injections of GDNF, there were multiple axons per one NMJ, as compared to the control. They concluded that it was the overexpression of GDNF that caused hyperinnervation because when they stopped the injections postnatally after day 10 the number of axons to individual NMJ decreased.

Retrograde Transport of GDNF

GDNF in skeletal muscle has shown retrograde transport, even in aging animals (Henderson et al., 1994; Nguyen et al., 1998). This follows the neurotrophic factor theory where target tissues are providing protection via neurotrophic support (Oppenheim, 1991; Purves et al., 1998). Upon internalization, GDNF and its receptors becomes confined within signaling endosomes which are transported towards the cell body via motor protein (C. Wu et al., 2009; Zahavi et al., 2015, 2017). Research has demonstrated that GDNF is subjected to both retrograde and anterograde transport. The implications of anterograde transport on the physiological well-being of MNs remain less explored, necessitating further investigations into its effects (Haase et al., 2002; Leitner et al., 1999; Rind & von Bartheld, 2002; Russell et al., 2000; Zahavi et al., 2015). However, what we know about GDNF and retrograde transport has been shown in the following experiments. In 1999 Leitner et al. began studying retrograde transport *in vivo* of some of the members of the GDNF family. This was conducted in the spinal cord of Sprague-Dawley using radiolabeled GDNF and neurturin injected into the sciatic region to determine retrograde

transport in MNs in the lumbar region by use of autoradiography. When looking at the spinal cord of the injected rats the results showed that more radiolabeled GDNF than neurturin was located to ventral MNs. This indicated that there are physiological differences in the actions of GFLs and that there is retrograde activity from GDNF (Leitner et al., 1999).

Zahavi used a microfluidic platform and saw the different effects of GDNF dependent on where it was applied, either on cell bodies or muscle cells (2015). This *in vitro* microfluidic platform is a compartmental system that allows for neuronal cell body separation from their axons and synapses are quickly becoming a useful tool for researchers (Taylor et al., 2005). It is beneficial because it allows researchers to study local versus distal signals, and monitor retrograde and anterograde transport (Taylor et al., 2005). When introduced into the soma compartment, GDNF triggers the activation of survival pathway signaling through AKT. Conversely, when administered to the muscle compartment, GDNF fosters axonal growth at the axon tips and the innervation of muscle cells, as indicated by Zahavi et al. in 2015. Additionally, GDNF exhibited the capability to be visually traced in a retrograde manner from muscle cells back to neurons.

GDNF and Motor Neuron Protection

GDNF is generated and released by skeletal muscle and undergoes a process of binding, internalization, and retrograde transportation in both motor and sensory neurons (Yan et al., 1995). The receptor for GDNF, is present in all spinal cord motor neurons from an early developmental stage. This presence significantly impacts the survival, axonal guidance, and growth of motor neurons as they age (Trupp et al., 1999; Yan et al., 1995). RET receptors are primarily found in presynaptic nerve terminals. Absence of these receptors results in underdeveloped presynaptic connections during developmental stages and the subsequent loss of

endplates after birth (Baudet et al., 2008). Experimental removal of RET in cranial motor neurons underscores its importance in both developmental and postnatal motor neuron survival. This removal leads to an ongoing reduction in motor neuron count after birth (Moore et al., 1996).

In comparison to other neurotrophic factors (Henderson et al., 1994; Oppenheim et al., 2000), GDNF has demonstrated remarkable effectiveness in safeguarding motor neurons against programmed cell death. Treatment with GDNF subsequent to motor neuron axotomy not only averted cell loss and atrophy compared to untreated cells but also induced significant hypertrophy in the injured motor neurons (Oppenheim et al., 1995). Among all the neurotrophic factors, GDNF was the only one capable of preserving all of the motor neurons after axotomy. This preservation comes with maintenance of normal soma size and the prevention of neuronal atrophy (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995).

In 2008, Baudet et al. demonstrated the significance of GDNF-mediated RET signaling in both synapse maturation and motor neuron survival. This was observed through experiments involving RET-deficient mice, where nearly half of their neuromuscular synapses were found to be missing. Furthermore, the absence of RET had a detrimental impact on motor neuron terminal sprouting. Numerous additional studies have shown the importance of GDNF/GFRα1/RET signaling in the development and maintenance of the NMJ. This has been demonstrated by introducing GDNF through injections or overexpression, leading to an augmentation in endplate size and an increase in terminal axonal sprouting, which resulted in hyperinnervation of endplates (Keller Peck et al., 2001; Nguyen et al., 1998; Zwick et al., 2001).

Following synaptic activity, there is an observed elevation in neurotrophic factors that potentially contribute to an enhancement in transmission (Schinder & Poo, 2000). The secretion

of these neurotrophic factors have been shown to elicit an increase in the release of neurotransmitters from presynaptic nerve terminals (Wang & Poo, 1997). Similar outcomes have been demonstrated in *in vitro* experiments, where neurotrophic factors increase in response to chronic depolarization in cultured environments (Vianney et al., 2014), as well as in *in vivo* experiments, where neurotrophic factors rise after periods of exercise (Gomez Pinilla et al., 2002; McCullough et al., 2011; Wehrwein et al., 2002).

Exercise

The National Institute of Health-National Institute of Aging (National Institute of Health, n.d.) reports that exercise can improve mental health by reducing feeling of depression or stress, increase energy level and improve sleep. Exercise has also been shown to improve brain health, decrease risk of disease, strengthen bone and muscle and improve the ability to do everyday activities. Cardiovascular disease, Type 2 Diabetes, and some cancers have been shown to improve with exercise, as reported by the CDC (Centers for Disease Control and Prevention, 2022). It is important to exercise during aging as it preserves muscle functionality (Bann et al., 2015; Drey et al., 2016; Trappe et al., 1996). With aging, exercise can be limited, but it still is an effective preventative or intervention plan as reviewed by (Bao et al., 2020). While the physical benefits of exercise are known, exercise has been shown to help maintain neurological health as well. Exercise has been shown to slow down the degradation effects at the NMJ, where the MNs communicate with muscle fibers. A possible explanation could be that there is an increase in neurotrophic factor expression in skeletal muscle of exercised individuals when compared to

sedentary (Gyorkos & Spitsbergen, 2014; Love et al., 2003; McCullough et al., 2011; Mrowczynski, 2019; Stanga et al., 2020).

Exercise and NMJ

Different types of exercise, such as endurance exercise (running) (Andonian & Fahim, 1987; Deschenes et al., 1993) and resistance exercise (weight lifting) (Deschenes et al., 2000) have resulted in significant expansion of pre-and post-synaptic components of the NMJ. Exercise has also been found to modulate electrophysiological parameters, such as increased quantal content (Dorlochter et al., 1991; Fahim, 1997), which indicate that with altered activity structural and functional adaptions are linked together. It has also been shown that different types of activity had differing effects on pre- and post-synaptic structures of NMJ of motor neurons.

A study performed by Deschenes et al. in 2006 showed that with exercise pre-synaptic features were altered, while post-synaptic structure of the NMJ on skeletal muscle did not. In the pre-synaptic apparatus it showed increased length of nerve terminal branching and complexity of those branching patterns. This supports the work that was done in that lab previously in 1993 (Deschenes et al.) and in 2003 (Deschenes & Wilson). They showed that increased staining of ACh containing pre-synaptic vesicles corresponded with increased nerve terminal branching. This suggests that in order to store more neurotransmitter there must be increased branching to deliver neural impulses to those additional vesicles (Deschenes et al., 2006). This seems like a likely response as exercise requires an enhanced store of ACh, and between the pre-synaptic branch length and ACh containing vesicles, it would make sense that exercised rats would show increased branch length and complexity.

Exercise and Motor Unit Protection

Exercise could potentially be neuroprotective in the central and peripheral nervous system. Exercise has been shown to mitigate negative effects of neurogenerative disorders including dementia (Buchman et al., 2012; M. Chang et al., 2010; Y. K. Chang et al., 2012; Larson et al., 2006; Lewis et al., 2020; Rolland et al., 2007), Alzheimer's disease (Cui et al., 2018; De la Rosa et al., 2020; Nation et al., 2011) and Parkinson's disease (Goodwin et al., 2008; Palasz et al., 2019; Real et al., 2017; Sung et al., 2012). This is an exciting possibility as exercise could provide a non-invasive and easily accessible treatment to protect against the onset of various neuromuscular disabilities and disease.

Naturally, the NMJ shows plasticity, and it has been shown that exercise can help maintain synapse structure and function as well as improve recovery from injury and reduce degenerative changes. Exercise has been shown to increase pre- and post-synaptic element size at the NMJ (Deschenes et al., 1993), increase size and degree of branching of presynaptic nerve terminals (Andonian & Fahim, 1987), and increase the quanta release of the neurotransmitter, acetylcholine (Dorlochter et al., 1991) of aged NMJ.

The type of exercise has been shown to make a difference in the neuromuscular function. High-intensity exercise, the type that recruits large motor cell bodies, such as resistance training, seems to have the biggest effect on neuromuscular function in elderly individuals (Einsiedel & Luff, 1994; Gardiner et al., 1984). Resistance training has been shown to increase maximum motor neuron firing frequency, rapid muscle force production, fine motor control, central muscle activation, muscle cross-section area, and volume in all fiber types and induces the same relative gains in anatomical muscle size in both young and old (Aagaard et al., 2010). As this is important to how it affects denervation, resistance training and having consistent physical

activity have been shown to keep the size of fast twitch myofibers when compared to age-matched sedentary controls.

Fast twitch and slow twitch myofibers have different denervating processes, especially when it comes to aging. Slow motor units seem to be retained longer in life when compared to fast motor units (Dalton et al., 2008). A potential explanation for the difference between muscle fiber types could be how much neurotrophic factor is available to help maintain and protect motor units.

GDNF and Exercise

Exercise and GDNF have shown, on their own, to induce similar changes in the neuromuscular system. These positive changes include enhancing the maintenance of synapses, inducing axonal sprouting, and increasing endplate complexity and size (Andonian & Fahim, 1987; Deschenes et al., 1993; Keller Peck et al., 2001; Zwick et al., 2001). Exercise has been demonstrated to increase levels of GDNF expression within skeletal muscle and the spinal cord (McCullough et al., 2011, 2013; Wehrwein et al., 2002), and that GDNF expression depends on the type and level of intensity of activity and may depend on if myofiber types are being recruited (McCullough et al., 2011; Wehrwein et al., 2002). This was shown by McCullough et al. in 2011 when levels of GDNF expression increased in SOL (a predominately slow twitch muscle) after low intensity exercise but decreased in the EDL (a predominately fast twitch muscle). The run training used in these experiments was categorized as slow speed (10 m/min), which may not have been a high enough intensity to recruit the fast twitch myofibers, which ultimately resulted in a decrease in GDNF protein content. The other type of exercise training that was selected was swimming, which has been shown to recruit fast twitch dorsiflexion more than run training due to the higher cycling rate and having to overcome resistance from the water

(Gruner & Altman, 1980; Roy et al., 1991). To have recruitment of fast twitch myofibers from running that are similar to that of swimming the speed of the treadmill would be ~67m/min (Roy et al., 1991).

Skeletal Muscle Classification

In 1966 Bergstrom & Hultman developed a surgical technique that made it possible to get human skeletal muscle biopsy samples which were used for histological and biochemical analyses. However, physical differences, such as color and length of contraction, were observed as early as 1873 by Louis Antoine Ranvier in skeletal muscle of rabbits. In the early 1920's muscle fiber was starting to get categorized as red or white, which was then related to how much myoglobin was found within each fiber (Needham, 1926). It was from these early observations that gave rise to the classifications of slow-twitch and fast-twitch muscle by way of histochemical staining (Engel, 1962). Slow-twitch muscle fibers stained dark, or red, while fast-twitch muscle fibers stained light or pale.

Fast-twitch

Fast-twitch can be broken into two separate categories. Fast twitch IIA (fast-oxidative glycolytic) and fast twitch IIX (Fast glycolytic). These categories are based from differential myosin heavy chain gene expression (Talbot & Maves, 2016) and sarcomere contractile machinery (Tajsharghi, 2008). Fast twitch muscles are used for more powerful and dynamic movements, such as lifting heavy weights or quick sprinting movements. For sprinters 80% of muscle fiber was fast twitch muscle fiber, and power lifters had 60% more fast twitch fiber makeup when compared to endurance athletes (Costill et al., 1976; Widrick et al., 2002).

Many neuromuscular disorders affect primarily target Type II muscle fibers. For instance, in Duchenne muscular dystrophy, Type II fibers are the initial ones to deteriorate (Marini et al.,

1991; Pedemonte et al., 1999; Webster et al., 1988). In myotonic dystrophy type 2, there is atrophy observed in Type II fibers (Pisani et al., 2008; Vihola et al., 2003) Additionally, aging and sarcopenia result in Type 2 fiber loss and atrophy, including a reduction in the diameter of the remaining Type 2 fibers (Lexell, 1995; Nilwik et al., 2013). Experiments conducted in rat models have demonstrated that denervation can induce specific atrophy in particular fiber types, depending on the muscle group under examination (Ciciliot et al., 2013). The nature of the injury, the muscle group affected, and the time elapsed since the injury or period of rest may all exert an influence on how specific muscle fiber types are impacted (Biering-Sorensen et al., 2009).

Slow-twitch

The counterpart to fast-twitch, are slow-twitch muscle fibers. Whereas fast-twitch is used for short, powerful bursts of energy, slow-twitch is used more in endurance activities. Long and middle distance runners have been shown to have 60-70% slow twitch composition, with has high as 90-95% in sports that require the highest aerobic and endurance capacities (Aagaard & Andersen, 1998; Bergh et al., 1978; Fry et al., 2003). In slow twitch muscles there's a higher mitochondria volume densities and capillary-fiber contact length (Sullivan & Pittman, 1987).

Just as certain diseases primarily impact fast-twitch muscle fibers, there are others that similarly affect slow-twitch fibers. Examples of such diseases include obesity and type 2 diabetes, which are associated with reduced proportions of Type 1 muscle fibers (Hickey et al., 1995; Oberbach et al., 2006; Tanner et al., 2002). Additionally, spinal cord and muscle inactivity have been demonstrated to cause atrophy in Type 1 muscle fibers (Gallagher et al., 2005; Grimby et al., 1976). This indicates that various muscle diseases don't stem solely from general muscle degeneration but often result from specific defects within the affected tissues.

The specificity of how certain diseases impact an individual appears to be linked to the proportion or quantity of different muscle fiber types. While it remains unclear why specific fibers are more susceptible to those diseases, it's worth noting that males and females have different ratios of fast-twitch to slow-twitch muscle fibers. This difference could potentially contribute to variations in disease susceptibility among individuals.

Motor Neuron

Somatic motor neurons have their cell bodies in the central nervous system and project their axons to skeletal muscle. There are three different types of somatic motor neurons; alpha, beta, and gamma. Alpha motor neurons innervate extrafusal motor fibers and their cell bodies are located in the ventral horn of the spinal cord, and will be the focus of this dissertation. The motor neuron and all of the muscle fibers that it innervates are called the motor unit. Motor unit categories are split into slow, fast fatiguing and fast fatigue-resistant.

The different types of motor units innervate different types of muscle fibers. Slow motor units stimulate small muscle fibers, which are slow-twitch muscles. This type of muscle is used for endurance, as it contracts slowly, using small amounts of energy (Stifani, 2014), which is why it is so resistant to fatigue. This muscle type is oxidative which means it uses oxygen and characteristically look red. The fast fatigue-resistance motor units are considered to be intermediate (Burke et al., 1973), and stimulate moderate-sized muscle fibers which don't contract as quickly as fast-fatiguing motor units. Contraction of fatigue resistant muscle fibers can be sustained for much longer than slow motor units, and they can generate more force. In fatigue resistant motor units energy is gained from both oxidative and glycolytic pathways. The last type of motor unit is fast fatiguing, which consists of the largest fiber size which is used for quick powerful actions (Burke et al., 1973; Eccles et al., 1960; Lee & Heckman, 1998; Stifani,

2014). This type of motor unit does not require oxygen, but generates energy strictly through glycolytic pathways. The muscle fibers have a white appearance because they have low levels of hemoglobin that is required for oxidative energy.

Multiple distinct signaling pathways contribute to the regulation of skeletal muscle fiber type. Among these regulatory pathways are the Ras/mitogen-activated protein kinase (MAPK) pathway (Murgia et al., 2000), calcineurin pathway (Chin et al., 1998; Naya et al., 2000), calcium-calmodulin-dependent protein kinase IV pathway (H. Wu et al., 2002), and the peroxisome proliferator γ coactivator 1 (PGC-1) pathway (J. Lin et al., 2002). The Ras/MAPK pathway serves as a vital connection between motor neurons, signaling systems, and processes related to excitation and transcription regulation. This connection facilitates the nerve-dependent activation of the slow program during muscle regeneration (Murgia et al., 2000).

Difference Between Male and Female Skeletal Muscle Composition

Differences between male and female skeletal muscle include energy metabolism, fiber type composition, and contractile speed (Esbjornsson et al., 1993; Green et al., 1984; Komi & Karlsson, 1978; Kuiper et al., 1996; Liljedahl et al., 1996). Females have a higher percentage of slow-twitch muscle fibers when compared to males. Another difference includes males having an overall larger muscle size than females. Male skeletal muscle typically have higher maximum power output and female muscles are generally more resistant to fatigue and recover faster (Fulco et al., 1999; Glenmark et al., 2004; Hakkinen, 1993; Linnamo et al., 1998).

There is also a difference in mitochondrial structure and function between males and females. There are several instances that point to differences in multiple organs and cell types, but in particular, hearts from healthy female mice have a higher proportion of large mitochondria when compared to males (Dworatzek et al., 2014). Females displayed decreased rate of reactive

oxygen species in cardiac and skeletal muscle under stressed conditions compared to males (Colom et al., 2007; Kander et al., 2017; Lagranha et al., 2010). Female rats shown higher mitochondrial DNA and protein contents in skeletal muscle (Colom et al., 2007).

While all the mechanisms behind the sex-related difference in skeletal muscle are unknown, many of the differences are likely due to the differences in sex hormone levels.

Estrogen Synthesis and Receptors

Estrogen is a steroid hormone important to the development and regulation of female reproductive changes and secondary sex characteristics (Huether & McCance, 2019). There are four known types of estrogen, estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4). Estrone (E1) is present during menopause as the predominant circulating estrogen. Estradiol (E2), is the predominant estrogen during reproductive years in terms of both absolute serum levels as well as estrogenic activity. During pregnancy, estriol (E3) is the most prevalent estrogen type, however, estetrol (E4) is also present and is only produced during this period. It may be noted that estradiol is ten times more potent than estrone and one hundred times more potent than estriol (Labhart, 2012). While forms of estrogen are present in both males and females, there is approximately four times the amount of estrogen in females as males. These hormone levels are regulated by the negative feedback effect of estrogen on the hypothalamus and pituitary gland (Delgado & Lopez-Ojeda, 2022). In females, estrogen is primarily produced by the ovaries (Kendall & Eston, 2002) and by the placenta during pregnancy (Marieb, 2013).

Estrogen synthesis begins with cholesterol in specialized cells in the ovary called theca interna cells. Production of estrogens start with the synthesis of pregnenolone from cholesterol, which is then converted to progesterone by 3-beta-hydroxysteroid dehydrogenase. The progesterone is converted to androgens, which will then be converted to estrogen by aromatase.

Once estrogen has been synthesized in the ovary, it enters systemic circulation as either a free hormone or bound to sex hormone-binding globulin (SHBG). Steroid hormones readily diffuse across the cell membrane and free estrogen, or estrogen bound to sex hormone-binding globulin (SHBG), is characterized by unregulated diffusion. The actions of estrogen are mediated by the estrogen receptor (ER), a dimeric nuclear protein that binds to DNA and controls gene expression. Within the cell cytoplasm, estrogen binds to and activates ERs which modulate the expression of many genes (Whitehead & Nussey, 2001). Further, estrogen initiates a physiological response from the cell by binding to an alpha-estrogen receptor (ESR1) or a beta-estrogen receptor (ESR2). This binding forms an activated estrogen-ER complex which enters the nucleus to bind nucleotide sequences known as estrogen response elements (ERE). It may be noted that the physiological response is dependent upon the presence and the type of ER available for binding in the cell.

As mentioned previously, ERs are synthesized in two protein forms, ESR1 and ESR2, which function as transcription factors when bound with their ligand (Mangelsdorf et al., 1995). They are found in many different cell types with ESR1 present in the uterus and pituitary gland primarily, prostate (stroma), ovary, testes, bone, breast (mammary gland), cervix, vagina, white adipose tissue, liver, hypothalamus, and muscle (Hamilton et al., 2017); ESR2 is expressed in the colon, prostate (epithelium), testes, salivary glands, bone marrow, central nervous system, immune system and vascular endothelium (Couse et al., 1997; J. A. Gustafsson, 2003; Weihua et al., 2003). Additionally, estrogen can bind to and activate rapid-signaling membrane estrogen receptors (mERs) leading to modulation of intracellular signaling cascades (Soltysik & Czekaj, 2013) such as GPER (GPR30) (Levin, 2015; Prossnitz et al., 2007). The predominant mechanism of estrogen action is through nuclear ER expression in estrogen target organs (Mangelsdorf et al.,

1995). It should be noted that both ESR1 and ESR2 are both expressed in human skeletal muscle at the mRNA level, but ESR2 is only found at the protein level (Wiik et al., 2003).

Estrogen vs. Estradiol

Estrogen has widespread systemic benefits which aid in the longer life span of females compared to males (Regan & Partridge, 2013). The hormone serves protective roles by preventing oxidative stress, which is thought to contribute to longevity in females (Vina et al., 2006). Additionally, the antioxidant and membrane-stabilizing properties of estrogen prevent muscle damage (MacNeil et al., 2011). Estrogen aids in repair and recovery by mitigating inflammatory responses, while estradiol affects satellite cell activation and proliferation to enhance growth and recovery potential of cells (MacNeil et al., 2011; Tiidus, 2001). Moreover, estradiol and age affect myosin function in women (Enns & Tiidus, 2010). The actions of both estrogen and insulin-like growth factor 1 (IGF-1) (Lemoine et al., 2002; Sitnick et al., 2006; Wiik et al., 2009) are hypothesized to play a role in muscle strength (Longcope, 1998).

Estrogen and Skeletal Muscle

Many studies have pointed to estradiol as an important contributor to muscle strength and function. For example, Moran et al. (2007) saw significant decrements in the force-generating capacity of hind-limb muscles in mice when estradiol was diminished. With estradiol replacement, full muscle strength was recovered, suggesting estradiol is an important hormone affecting muscle contractile function. These results were consistent when controlling for physical and muscular activities of the mice (Greising et al., 2011). Wiik et al. (2005) suggested that

ESR1 and ESR2 expression is altered by functional demands on muscle when a study of endurance training resulted in increased expression of both receptors in human skeletal muscle.

In addition to its role in muscle contraction, estradiol also affects antioxidant enzyme levels in skeletal muscle (Baltgalvis et al., 2010). Antioxidants enzyme levels and *esr1* gene expression respond to changes in estradiol levels both acutely and chronically, further supporting the influence of estrogen-mediated mechanisms in muscle contractility (Baltgalvis et al., 2010). Wise et al. (2001) showed the importance of estradiol synthesis to not only tissues of the central nervous system but also to the peripheral nervous system, specifically, the NMJ. A meta-analysis performed by Greising et al. (2009) observed greater muscle strength for postmenopausal women on estrogen hormone therapy compared to those without treatment, suggesting an important role of estrogen in muscle strength.

Estrogen and Aging Females

The endocrine system plays a major role in cellular interactions, metabolism, and growth, explaining why changes in hormone levels contribute to the aging process. As women go through menopause, they exhibit an accelerated decline in muscle mass and strength (Calmels et al., 1995; Carville et al., 2006; Cooper et al., 2008; Greeves et al., 1999; Kurina et al., 2004; Samson et al., 2000; Skelton et al., 1999). This may be related to the considerable decline in estrogen levels, with an average of 80% estrogen loss during the first year of menopause (Cauley et al., 1989; Phillips et al., 1993; Vina et al., 2006). This subsequently causes decreased muscle function (Lemoine et al., 2003; Sipila, 2003). Specifically, higher endogenous estrogen levels have been associated with increased muscle strength and lower rates of fall-related limb fractures in 75-year-old women (Sipila, 2003), with age-related losses in muscle strength being implicated with declines in estrogen levels (Lowe et al., 2010). However, reviews by Meeuwsen et al.

(2000) and Enns & Tiidus (2010) revealed conflicting results on the estrogen-specific effects on skeletal muscle. It was demonstrated that the benefits of estradiol on skeletal muscle structure and contractile function heavily depended on the species, study type, age, muscle size, and muscle fiber type.

Estrogen may stimulate muscle repair and regenerative processes, however, the mechanisms by which it influences processes involved in muscle damage, inflammation, and repair require further investigation (Clarkson & Hubal, 2001). One proposed mechanism is that estrogen does this by acting as an antioxidant that limits the oxidative damage of muscle (Vina et al., 2006). Furthermore, estrogen may act as a membrane stabilizer and govern the regulation of downstream genes and molecular targets (Enns & Tiidus, 2010). With age, androgen and estrogen levels decrease, causing an associated decline in muscle and bone mass and strength, further demonstrating the critical role of these hormones throughout the aging process.

Estrogen and Aging Males

Estrogen is produced in men by aromatization, a process by which the limbic system and brain tissues convert testosterone into estradiol (Naftolin et al., 1971) and Leydig cells of testis (Ryan et al., 1972). Nilsson et al., (2001) first described the expression of both ESR1 and ESR2 in the testes. It was later confirmed by Cooke et al. (2017) that ESR1 was imperative to the function of efferent ducts and epididymal functions. When ESR1 was disrupted, it resulted in abnormal sperm due to the loss of ion transport and water reabsorption, ultimately affecting male fertility. In addition, ESR1 had nonreproductive effects on targets such as brain, adipose, skeletal

muscle, bone, cardiovascular and immune tissues. ESR2, however, primarily influenced epithelial differentiation in the prostate and seminiferous epithelium.

It has been established that estrogens are important regulators of bone health in both men and women (Clarke & Khosla, 2010; Vandenput & Ohlsson, 2009). Testosterone levels decline with age in men, suggesting a decrease in systemic estrogen levels. However, sufficient levels of estrogen may be available locally to bone or skeletal muscle depending on the availability of testosterone. For instance, the risk of fracture in aging men is inversely correlated with the decreased serum levels of estrogens and androgens with age (Vandenput & Ohlsson, 2009). It should be noted that most studies have been conducted on animals rather than humans thus the role of estrogen in aging males has not been fully investigated.

Estrogen and Aging/Menopause

Estrogen may be an important factor contributing to the sex differences observed in brain aging and neurodegeneration (Zarate et al., 2017). Moreover, the neuroprotective actions of estrogens are apparent during aging and menopause. The loss of estrogen with menopause is associated with mitochondrial dysfunction, neuroinflammation, synaptic decline, cognitive impairment, and increased risk of age-related disorders (Zarate et al., 2017).

Disease susceptibility differs in women depending on whether they are pre- or post-menopause, possibly due to the loss of estrogen. For example, it is suggested that the higher prevalence and greater severity of Alzheimer's disease (AD) in women is due to the postmenopausal reduction in sex steroid hormone concentration (Brann et al., 2007; R. Li & Singh, 2014; Tang et al., 1996). The risk of developing Parkinson's disease (PD) is also influenced by estrogen, exhibiting a lower risk in premenopausal versus postmenopausal men and women. This may be explained in part by the estrogen-induced inhibition of microglial

activation and neuroinflammation system leading to reduced oxidative stress, neuroinflammation, and neurodegeneration of dopaminergic neurons in murine models of PD (Labandeira-Garcia et al., 2016; Rodriguez-Perez et al., 2010). Taken together, both preclinical and clinical data indicate that both aging and menopause lead to increased neuroinflammation, which may contribute to sex differences in age-related neurological diseases such as AD and PD (Zarate et al., 2017).

Numerous studies have further shown that pre-menopausal women are protected against stroke relative to men. However, stroke incidence increases in women following menopause (Di Carlo et al., 2003; Murphy et al., 2004; Niewada et al., 2005; Roquer et al., 2003). Not only does the incidence increase post-menopause, but worse outcomes are also observed compared to men, resulting in significantly higher disability and fatality rates (Di Carlo et al., 2003; Hochner-Celnikier et al., 2005; Niewada et al., 2005; Roquer et al., 2003). Women are clearly at a greater risk of disease and degeneration post-menopause, highlighting differences in aging between the sexes.

Sex Hormones and Neurosteroidogenesis During Aging

In addition to the previously discussed processes, neurosteroid synthesis is also affected by aging. Steroidogenesis begins with a rate limiting step in which cholesterol is transported from the outer to the inner mitochondrial membrane. During each step, various enzymes are differentially expressed in neurons and glia in a regional and pathophysiological manner. Under normal physiological conditions, neurons are the main sites for brain estrogen production from testosterone, relying on the high expression of the enzyme aromatase (Azac-Fonseca et al., 2016). Therefore, targeting key enzymes involved in brain estrogen production has been

proposed as a method to improve brain function during aging and to prevent onset of neurodegenerative disease (Veiga et al., 2004).

Mechanism for Estrogen Neuroprotection

In various neuropathological conditions such as Alzheimer's disease, Parkinson's disease, traumatic brain injury, stroke, and multiple sclerosis, estradiol has demonstrated the ability to enhance the expression of genes involved in synaptogenesis, axonal repair, and synaptic plasticity, namely Bcl2, TrkB, and cadherin-2 (J. Feng et al., 2013; Khan et al., 2015; Saraceno et al., 2018). Moreover, estradiol exhibits neuroprotective effects by combating oxidative stress, reducing the production of reactive oxygen species, and thereby safeguarding mitochondrial function (Rettberg et al., 2014; Simpkins et al., 2010). Furthermore, estradiol has been observed to potentially stimulate the release of neurotrophic factors, including glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1), and brain-derived neurotrophic factor (BDNF). These neurotrophic factors play crucial roles in neuronal protection and the restoration of damaged neuronal circuits under pathological conditions giving estradiol an important role in neuroprotection (Arevalo et al., 2015; Yuan et al., 2019). While we know estrogen receptors are present on skeletal muscle, it has not yet been elucidated how GDNF may be a downstream target of estrogen.

Knowledge of cellular mechanisms first included ligand receptor binding, activation, direct DNA binding and gene regulation, but has now expanded to non-DNA binding or tethering, cellular non-genomic signaling, and receptor mediated non-ligand hormone activities (Hewitt et al., 2016). Three major genomic ER-mediated transcriptional regulation mechanisms have been characterized primarily for ESR1. First, the classical mechanism involves the direct binding of DNA to regulatory elements, leading to an activated estrogen-estrogen receptor

complex that can recruit additional factors involved in transcriptional regulation. The second mechanism, tethering, occurs when there is indirect binding to transcription factors like AP1 or Sp1 binding sites already bound to DNA. Lastly, ligand-independent receptor activation is a mechanism proposed to involve altered phosphorylation of sites on the ER protein. This regulates gene expression in the absence of ligand through membrane growth factor receptor mediated intracellular signaling pathways allowing growth factors to phosphorylate ER (Hamilton et al., 2017). Estrogen also binds to and activates membrane ERα or GPR 30, introducing the rapid intracellular signaling pathway. Coupling of non-genomic and genomic signaling mechanisms may explain the complementation of different cellular signaling pathways, eliciting the broad spectrum of responses to estrogen. Studies that have administered estrogen to ovariectomized (OVX) mouse uterine tissues showed some gene regulation at intervening timepoints, however, most fall within early (within 2 hours of administration) or late (within 12-24 hours of administration) clusters (Hewitt et al., 2003).

The two mechanisms include rapid non-genomic effects and genomic effects. Rapid effects are believed to possess widespread significance in various diverse cells and processes that include vasorelaxation in endothelial cells (S. E. Kim & Rhee, 2015), neuroprotection in neuronal cells (Prokai & Simpkins, 2007), prolactin secretion from pituitary tumor cells (Watsona et al., 1999), cell cycle stimulation in breast cancer cells (Simpkins et al., 2008), cell proliferation and differentiation, bone conservation (Prossnitz & Barton, 2011), and sperm motility (Luconi et al., 1999). Furthermore, studies have identified proteins and receptors that mediate non-genomic effects of estradiol, indicating the presence of ERs on the plasma membrane of neuronal cells in rats (A. Kumar et al., 2018). According to Pedram et al. (2009), mice lacking both ESR1 and ESR2 displayed no binding of E2 to the cell membrane or

cytoplasm and a failure to activate PI3K signaling pathways. These results were further supported by in vitro studies where ESR1 and ESR2 were required for E2 binding in MCF7 cells, that is, ER-positive breast cancer cells that produce GPR30. Collectively, these findings support the importance of ERs in the mediation of estradiol mechanisms.

Through non-genomic actions, estradiol performs cell-type specific activation of various signaling pathways like PLC/protein kinase C, SRC/extracellular activated kinase (ERK), phosphatidyl-inositol 3 kinase (PI3K)/Akt, and p38/mitogen activated protein kinase (MAPK) (Acconcia & Marino, 2011). According to some studies, estrogen mediates membrane signaling through GPER, specifically, GPR30, which causes alteration in calcium influx and cAMP signaling (Haas et al., 2009). In a study using ER-negative, GPR30-positive breast cancer cells, MAPK/ERK phosphorylation and PI3K/Akt activation were elicited by estradiol, thereby suggesting that GPR30 mediates estrogen signaling (Y. Feng & Gregor, 1997). Treatments have been shown to be stopped in culture by the inhibition of either the MAPK/ERK or Akt signaling pathway (Clark et al., 2014; Kelly & Levin, 2001).

The second mechanism mediating estradiol actions was originally thought to cause genomic effects through ERs acting as ligand-driven transcription factors (O'Malley, 1967). It was later discovered that activation and dimerization of ERs is initiated by estradiol binding to chaperone-bound, inactive ERs (V. Kumar et al., 1986). Activated ERs then bind to specific EREs in the promoter regions of estradiol responsive genes where gene transcription is modulated by altering the rate of recruitment of general transcription factors and coregulators (Klein-Hitpass et al., 1989). Additionally, estradiol bound ERs can indirectly influence transcription, especially genes lacking EREs. It should be noted that this genomic mechanism is a slow process, usually taking 12-24 hours for physiological effects to be apparent.

CHAPTER II

Voluntary Exercise Increases GDNF Protein Content and Endplate Area in Hindlimb Muscle of Male and Female Rats

Juliana M. VanGyseghem and John M. Spitsbergen

Department of Biological Sciences, Western Michigan University, Kalamazoo, MI 49008

John.spitsbergen@wmich.edu

Office phone: (269) 387-5648

3443 Wood Hall, Mail Stop 5410

Department of Biological Sciences
Western Michigan University
1903 W Michigan Ave
Kalamazoo MI 49008-5410 USA

Conflict of interest statement: The authors declare no competing financial interests.

Cover letter:

Males and females differ hormonally and neurologically as they age yet are given the same treatment for many neurological diseases. The conceptual advancements from this study include comparisons of changes in GDNF content and endplate morphology in response to exercise, in male and female rats. The focus of this study was to compare young and old ages as well as primarily fast-twitch, plantaris, and primarily slow-twitch, soleus, hindlimb muscles.

Our significant findings are females had higher levels of GDNF content in both types of muscle compared to age-matched males. Exercise impacted GDNF content of skeletal muscle in both sexes. Exercise altered endplate morphology in hindlimb muscles in females but not in males.

This study highlights biological sex differences in neuroprotection observed with exercise, which may help in the development of novel therapeutic regimens for each sex.

I have read and have abided by the statement of ethical standards for manuscripts submitted to Neuroscience, as well as the other statement that all authors have approved the final article.

Abstract:

Increased expression of target-derived neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), may help protect against the age-related decline in neuromuscular function. The purpose of this study was to compare GDNF protein content in male and female rat skeletal muscle and investigate morphological changes of the neuromuscular junction (NMJ) with age and voluntary exercise. Male and female Sprague-Dawley rats were exercised in voluntary running wheels for 2 weeks, 4 weeks, or 6 months. Plantaris (PLA) and soleus (SOL) were removed and processed for GDNF protein content in skeletal muscle by an enzyme-linked immunosorbent assay. Tissues were bound with α-bungarotoxin to analyze the structure of the NMJ. GDNF protein levels were higher in hindlimb skeletal muscles from sedentary females as compared to muscle from age-matched males. In SOL muscle of 6-week-old sedentary females the GDNF protein concentration (pg/g tissue weight) was 45.75±8.16, which was significantly higher than age-matched males at 1.55±0.02. For both sexes, exercised animals had higher GDNF protein concentrations in PLA and SOL than their age-matched sedentary counterparts. Exercised 12-week-old males had the highest overall GDNF protein concentration of 98.20±1.36. Endplate area increased with sedentary aging for both sexes. There was no difference in endplate dispersion with sedentary aging in male rats. However, in female rats, endplate dispersion increased with sedentary aging. The results indicate that there are sex differences in levels of GDNF expression. Because GDNF concentration is higher in female rats, this may explain why females are more protected against diseases with a neurological component.

Keywords: neuromuscular junction, endplate morphology

Significance statement: Aging is associated with a decline in structure and function at the neuromuscular junction, which may be explained in part by a reduction in neurotrophic factor expression with age. It has been suggested that increased expression of target-derived neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), may help protect against the age-related decline in neuromuscular function. Although males and females are often treated similarly, it has been shown that premenopausal women display lower incidence and severity of neurological disease compared to men of the same age. Results of previous

studies have shown that exercise can increase GDNF levels in skeletal muscle from male rats; however, little is known concerning the impact of exercise on GDNF expression in skeletal muscle from female rats.

Introduction:

Sex differences in skeletal muscle structure and function: There are anatomical and physiological differences in skeletal muscle between males and females, including differences in the size of skeletal muscle fibers (Glenmark et al., 2004) with males showing a significantly larger cross-sectional area compared to females (Simoneau & Bouchard, 1989; Staron et al., 2000). Additionally, there are differences in the composition of fast-twitch (Type II) versus slow-twitch (Type I) muscles in males and females (Haizlip et al., 2015) where females have a higher percentage of slow-twitch muscles than males. Females also tend to recover more quickly than males from fatigue and have higher endurance (Laurent et al., 2010), and it is suggested that this shorter recovery time may be due to the higher number of Type I skeletal muscle fibers in females.

Sex differences in response to exercise: There are also differences in how males and females recover after exercise and muscle damage. The presence of creatine kinase (CK) in the bloodstream is an indicator of damaged muscle, and studies have shown that levels of this marker were significantly higher after exercise and skeletal muscle injury in male rats than in female rats (Amelink et al., 1990; Amelink & Bar, 1986; Bar et al., 1988). However, the levels of CK, as a response to injury, are the same for ovariectomized female rats as for male rats, suggesting that estrogen plays a role in the prevention of and recovery from muscle damage.

Exercise and Glial cell line-derived neurotrophic factor production: Neurotrophic factors are a family of biomolecules that support the growth, survival, and differentiation of both developing and mature neurons, and play a role in maintaining skeletal muscle health. GDNF plays a versatile role in many cell and tissue types, including skeletal muscle, Schwann cells, and motor neuron axons, cell bodies, and synapses (Henderson et al., 1994; Nguyen et al., 1998; Nosrat et al., 1996; Springer et al., 1994, 1995; Suter-Crazzolara & Unsicker, 1994; Suzuki et al., 1998; Trupp et al., 1995). GDNF has been shown to save somatic motor neurons from naturally occurring cell death (Oppenheim et al., 2000), and axotomy-induced cell death (Oppenheim et al., 1995), and protects motor neurons from chronic degeneration (Corse et al., 1999). Exercise has been linked to increased glial cell line-derived neurotrophic factor (GDNF) protein concentrations in skeletal muscle (Wehrwein et al., 2002).

Different modalities of exercise appear to have differential effects on GDNF expression in muscles of different phenotypes. For example, low-intensity exercise led to an increase in GDNF in the SOL, a predominately slow-twitch muscle, while decreasing GDNF content in EDL, a predominantly fast-twitch muscle (McCullough et al., 2011). By increasing the intensity of exercise, by using running wheels with added resistance, fast-twitch muscles showed clear evidence of recruitment (hypertrophy) and increased GDNF content (Gyorkos et al., 2014).

Even without considering the effects of exercise, simply the age of the individual affects levels of GDNF expression (Cintron-Colon & Spitsbergen, 2019). Studies have shown that both an increased loss of somatic motor neurons (Jacob, 1998; Johnson et al., 1999) and a decrease in neural plasticity (Johnson et al., 1999) with increased age. While the complete mechanism for the loss of skeletal muscle with age is unknown, it has been suggested that a decrease in neurotropic signaling could be a contributing factor (Bergman et al., 1999). Yet, with all these interesting findings of the beneficial effects of exercise on the neuromuscular apparatus, little is still known about the difference between males and females at the cellular level, and the neurotrophic mechanisms that are altered or regulated differently in each sex.

Hypotheses: The purpose of this study is to examine the effect of age and exercise on GDNF content and endplate morphology in skeletal muscles from male and female rats. Our hypothesis is that levels of GDNF protein expression in skeletal muscle will be higher in young female rats than in age-matched male rats. We also hypothesize that exercise will cause a greater increase in skeletal muscle GDNF protein content in females compared to males.

Experimental Procedures:

Methods and Materials

Subjects

All animal experiments were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council) and protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University. Male and female Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Male and female

rats were acquired at different times, so they were not housed together. Female rats were acquired at 4 weeks of age, 8 weeks of age, and 52 weeks of age. Male rats were acquired at 4 weeks of age and 8 weeks of age. All rats were considered pre-puberty at 6 weeks of age and younger and adolescent at 8-12 weeks of age (Sengupta, 2013). Females at 52 and 78 weeks of age were considered to be aged as they are going through reproductive senescence. Animals were acquired and acclimated to their environment before testing began. Rats in each age group were randomly separated into a sedentary group, which were maintained in cages without access to running wheels (n=6), and an exercise group which had access to running wheels (n=6). Animals were exposed to a 12-hour light/dark cycle and had access to food and water *ad libitum*.

Voluntary Exercise Protocol

Voluntary exercise protocols lasted for a two-week period, four-week period, or six-month period. All the animals completed the entire duration of the study. All rats were housed in clear polycarbonate living chambers (19" x 10.5" x 8"). The running wheels (Lafayette Instruments, Lafayette, IN) were attached to the living chambers and were freely accessible at all times throughout the study. Voluntary exercise was chosen as the training type because it has been shown that rats are internally motivated and do not need external stimuli to induce running (Legerlotz et al., 2008; Sherwin, 1998). Sensors were placed on the running wheel where a computer recorded the distance run and running speed using software from Lafayette Instruments.

Tissue Collection and Processing

Following completion of exercise training protocols, exercised and sedentary rats were weighed and euthanized (CO_2 asphyxiation, followed by thoracotomy) and the predominately slow-twitch SOL and predominantly fast-twitch PLA muscles were removed.

The PLA and SOL muscles of the right side were frozen at resting length, stored at -80 °C, and later processed for analysis for endplate morphology. The PLA and SOL muscles from the left side were processed for GDNF protein content analysis via ELISA. Each muscle was weighed, dipped in liquid nitrogen, smashed into a fine powder, and homogenized with sample processing buffer (0.55 mol/L NaCl, 0.02 mol/L NaH_2PO_4, 0.08 mol/L Na_2HPO_4, 2 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L benzethonium chloride, 2 mmol/L benzamidine, 20

KIU/mL aprotinin, 0.5% bovine serum albumin (BSA), and 0.05% Tween-20). Homogenates were centrifuged and supernatants were collected and stored at -80 °C until ready for analysis.

Enzyme-Linked Immunosorbent Assays (ELISAs)

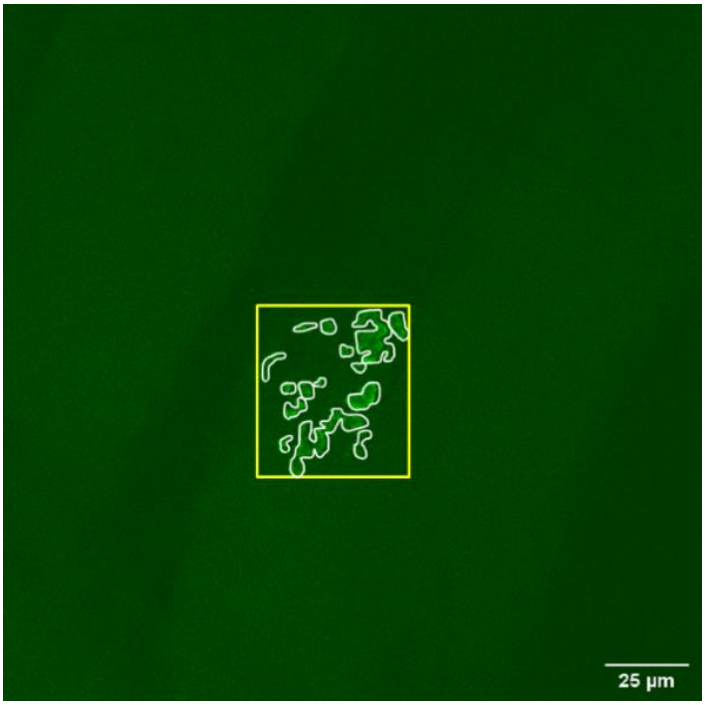
To determine the concentration of GDNF protein in SOL and PLA muscles ELISAs were run according to the manufacturer's (R&D System, Minneapolis, MN) specifications. Briefly, a 96-well plate was bound overnight with monoclonal anti-GDNF antibody (primary antibody) overnight. The plate was then washed and blocked with BSA. A series of standards were made in sample processing buffer and added to the top of the plate to create a standard curve. Study samples were then added to the remainder of the plate. After incubation, plates were washed and secondary antibody (biotinylated anti-GDNF) was added and incubated overnight. The plate was washed, and horseradish peroxidase conjugated to streptavidin (Pierce, Rockford, IL) was added. The plate was washed again before adding tetramethylbenzidine color reagent (Sigma). The reaction was stopped using 0.1 M HCl, and absorbance was measured at 450 nm.

Morphological Analysis of Neuromuscular Junction

The SOL and PLA muscles taken from the right side of the body were used for imaging. These were embedded in optimum cutting temperature compound (Sakura Finetek, Torrance, CA) and sectioned using a Leica Cryostat. All sections were thaw-mounted on HistoBond Microscope Slides (VWR; 195 International, Bridgeport, NJ, USA), vacuum sealed, and stored overnight at 4 °C.

Rat muscle was sectioned longitudinally (50 µm) and fixed in 4% paraformaldehyde at room temperature for 1 hour, washed in phosphate-buffered saline (PBS) (3 x 5 minutes), and incubated in buffer containing 10% donkey serum, 4% BSA, 0.5% Triton X-100 in PBS for 30 minutes, in a humidified chamber, at room temperature. α-bungarotoxin conjugated to AlexaFluor488 (Invitrogen B13422 diluted 1:200) was used to visualize endplate receptors. Slides were washed the next day in PBS and prepared in PBS:Glycerol (1:1) and a glass coverslip for microscopy. Slides were viewed by use of a Nikon Eclipse E750 confocal microscope.

Images from fifty random endplates were captured for SOL and PLA muscles using confocal microscopy. Each endplate was visually analyzed to make sure that it was within the longitudinal border of the myofiber before being scanned and stored. Each endplate was analyzed using Image J software as previously described (Deschenes et al., 2006). Saturation threshold, dispersion, and box area computation were performed as previously described (Gyorkos & Spitsbergen, 2014). Briefly, dispersion of endplates was measured by inverse bean-to-box ratio. The box was drawn so that the endplate was inscribed within the box. The bean was drawn so that it traced the perimeter of the endplate. The ratio between the two is a metric of how well the box represents the bean, or of how much of the box is taken up by the bean. Having a lower bean-to-box ratio indicates a higher degree of dispersion.



Statistical Analysis

All data were reported as the mean ± standard error of the mean (SEM). Data were analyzed using a two-way analysis of variance (ANOVA) and Tukey's post-hoc test to assess statistical significance between different groups. Differences were considered statistically significant at $p<0.05$. Where appropriate data was fit with a linear regression and correlation and variance were determined.

Results

Distance Run by Male and Female Rats: Animals were given voluntary access to running wheels where distance was recorded via software on a computer. During the course of the exercise program, there was no difference observed in the distance run by age-matched male rats compared to female rats. The 4–6-week-old female rats ran 38,200 m ±8,500 m/week, 8–12-week-old females ran 58,000 m±9,000 m/week and 52-78 week-old females ran 4,700 m±1,000 m/week. The distance run by 8–12-week-old females was greater than that run by 52-78 week-old females.

The 4-6 week-old male rats ran 22,000 m±7,000 m/week and the 8-12 week-old males ran 104,000 m±42,000 m/week. The distance run by the 8-12 week-old male rats was greater than that run by the 4-6 week-old males.

Animal Body Weight and Muscle Weight: The animal's body weight and relative muscle weight were measured in order to determine any changes with age and exercise. Both male and female sedentary rats grew at the expected rates for their age range between 4 and 12 weeks of age. Exercised young male rats (4-6 week-old) did not show any difference in body weight after two weeks of voluntary running, but adolescent male rats (8-12 week-old) did show a decrease in body weight when compared to sedentary male controls at the same age. In female rats, there was no effect of exercise on body weight.

Relative muscle weights (g) were examined to determine if exercise had differential effects on the primarily slow phenotype SOL compared to the primarily fast phenotype PLA muscles. In male rats, exercise had no effect on the relative weight of SOL muscle between 4 and 6 weeks of age. However, relative weight was higher in exercised versus sedentary for male rates

between 8 and 12 weeks of age (0.514±0.023 and 0.424±0.017, respectively). In male rats exercise had no effect on relative tissue weight in PLA. In female rats, relative weight of SOL muscle was higher in 4-6 week-old exercised versus sedentary female rats (0.670±0.011 and 0.519±0.042, respectively). Exercise had no effect on relative weight in 8-12 week-old female rats but increased weight in exercised 52-78 week-old female rats when compared to age-matched sedentary (0.587±0.04 and 0.403±0.03, respectively). In female rats, exercise had no effect on relative muscle weight in 4-6 week-old, and 8-12 week-old PLA. However, relative weight of PLA muscle was higher in exercised versus sedentary with exercise 52-78 females rats (1.142±0.05 and 0.934±0.057, respectively).

Effects of Sedentary Aging on GDNF Content of Skeletal Muscle: SOL (slow-twitch) and PLA (fast-twitch) were used for GDNF protein quantification in both male and female rats. In

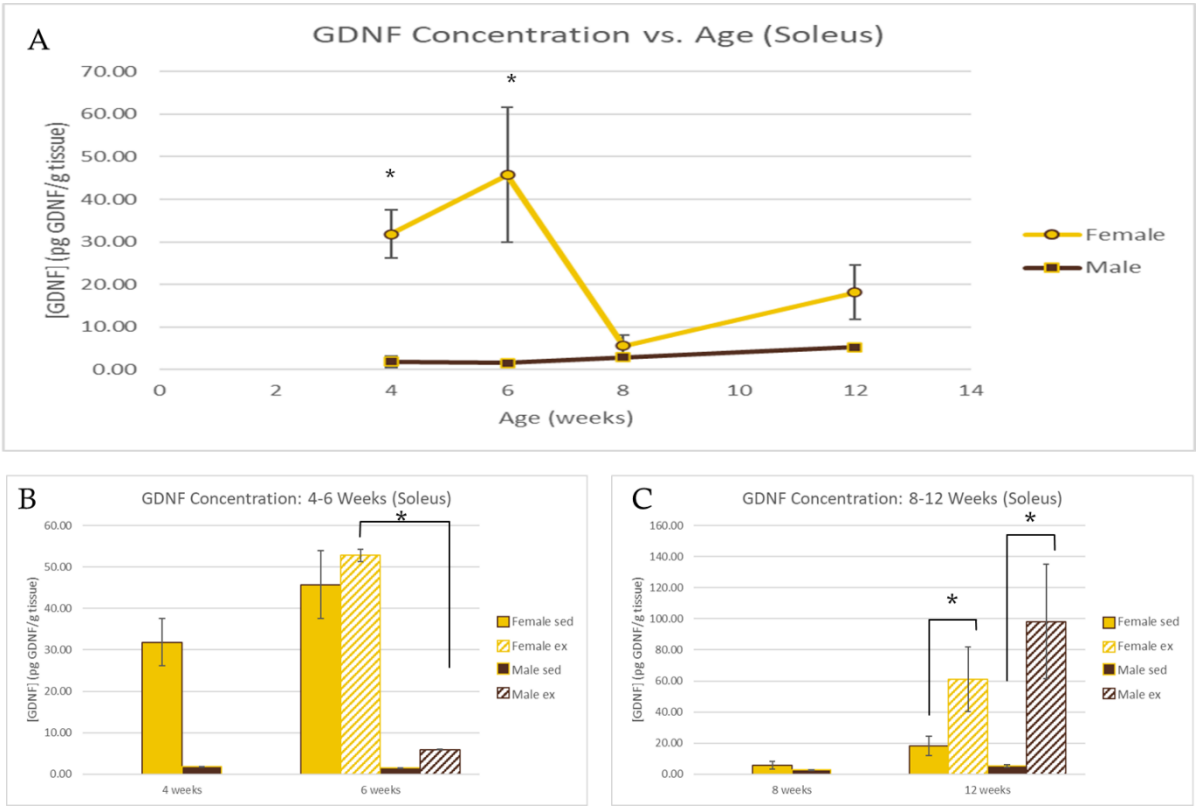


Figure 1: Effects of exercise on GDNF protein content in SOL muscle of male and female rats. GDNF protein concentration was determined by ELISA. Panel A: GDNF concentration (pg/g tissue weight) in SOL muscle with sedentary aging. GDNF protein concentration is higher in SOL muscle from 4- and 6-week-old sedentary female rats compared to that in age-matched males. Panel B: Effects of exercise on SOL GDNF concentration in 4–6-week-old animals. Exercised 6-week-old females had higher GDNF protein concentration compared to age-matched males. Panel C: Effects of exercise on SOL GDNF concentration in 8–12-week-old animals. GDNF protein concentration is greater in 12-week-old exercised males and females compared to age-matched sedentary controls. Asterisk (*) indicates a significant difference ($p<0.05$).

male rats GDNF protein content of SOL (Figure 1) and PLA (Figure 2) showed no difference with sedentary aging.

In females there was a decline in GDNF content of SOL at 8 and 12 weeks of age when compared to levels measured at 4 weeks of age (Figure 1). In female rats, the content of GDNF in PLA remained the same with sedentary aging (Figure 2). In older female rats (52-78 week-old) GDNF protein content was below levels of detection in sedentary and exercised females which is why data for those time points are not shown on the graphs

In 4-week-old sedentary females GDNF protein content was higher in the SOL muscle (31.84± 5.68 pg/g tissue weight) when compared to age-matched sedentary males (1.85 ± 1.29 pg/g tissue weight). Similarly, the GDNF protein content of 6-week-old sedentary female (45.75± 15.85 pg/g tissue weight) was also higher than sedentary age-matched males (1.55± 0.80 pg/g tissue weight). There was no difference in GDNF levels in 8–12-week-old female rats compared to age-matched males.

Although there were no differences in the levels of GDNF content in the PLA between different ages of the same-sex populations, there were differences when males and females were compared to each other. Overall, GDNF protein content was higher in female PLA than in male PLA across all ages. Specifically, there was higher levels of GDNF protein content in 4-week-old sedentary females than in sedentary males of the same age. At 12 weeks, there were higher levels of GDNF protein concentration in sedentary females than in sedentary males. (Figure 2)

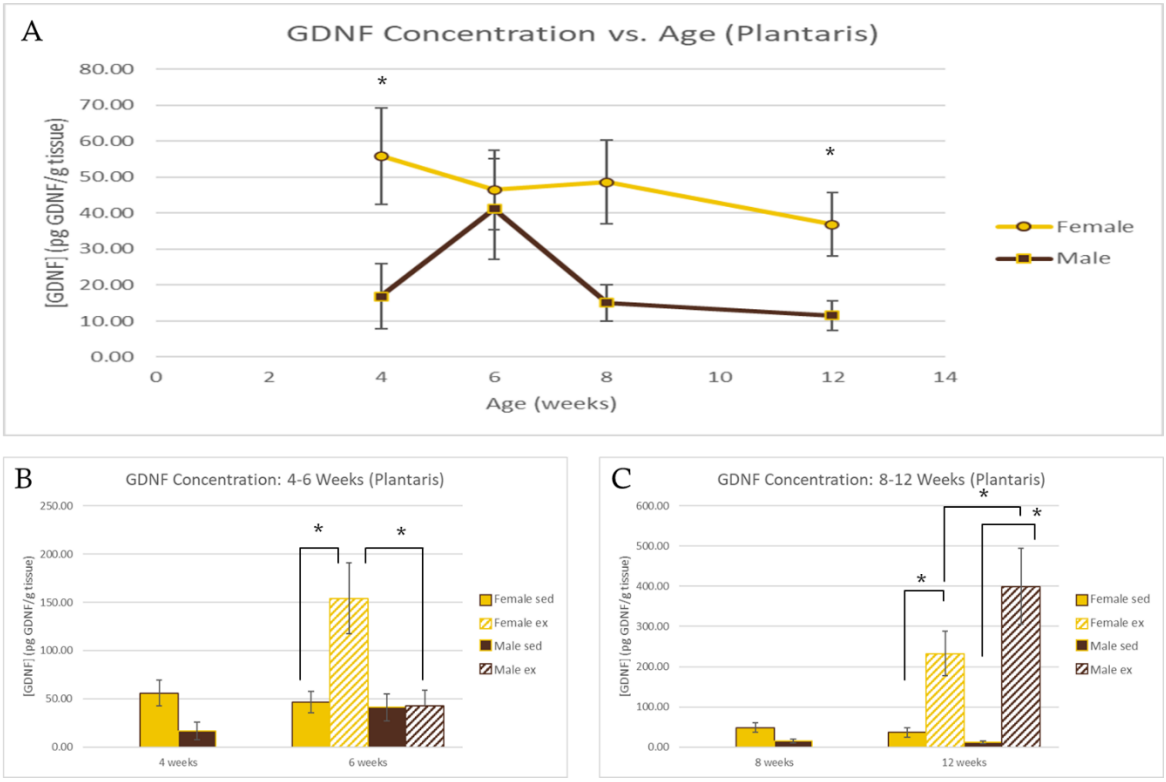


Figure 2: Effects of exercise on GDNF protein content in PLA of male and female rats. GDNF protein concentration was determined by ELISA. Panel A: GDNF concentration (pg/g tissue weight) in PLA muscle with sedentary aging. In female rats, GDNF protein concentration in 4 and 12 week-old sedentary is higher compared to age-matched males. Panel B: Effects of exercise on PLA GDNF concentration in 4-6 week-old animals. Exercised 6 week old female rats had higher concentrations of GDNF compared to age-matched sedentary female and exercised 6 week males. Panel C: Effects of exercise on PLA GDNF concentration in 8-12 week-old animals. Exercised 12 week-old female rats had higher GDNF concentration when compared to age-matched sedentary. Exercised male rats showed higher GDNF concentration when compared to age-matched males and age-matched exercised females. Asterisk (*) indicates a significant difference ($p<0.05$).

Effect of Exercise on GDNF Content of Skeletal Muscle

In male rats exercise increased GDNF protein content of the SOL and PLA muscle in 12-week-old rats when compared to sedentary. However, in female rats exercised increased GDNF protein content of the SOL and PLA muscle at both 6 and 12 weeks of age when compared to age-matched sedentary.

There was no difference in GDNF concentration in the SOL of 6-week-old exercised male and female rats when compared to age-matched sedentary controls of the same sex. However, four weeks of voluntary exercise over the 8–12-week-old age range led to an increase in GDNF protein concentration in the SOL of both male and female rats (Figure 1).

Within the 4-6-week-old age range, GDNF protein concentration in the SOL was about 300% higher in female rats than in their age-matched male counterparts for both sedentary and exercised groups.

There were increases in the level of GDNF protein content in the PLA of 12-week-old exercised males when compared to age-matched sedentary male rats. Likewise, there were increases in GDNF concentration in the PLA of the 6-week-old and 12-week-old exercised females when compared to age-matched sedentary female rats. Among exercised female rats, there was a trend toward higher levels of GDNF protein content in the PLA when compared to the GDNF content in the PLA of age-matched males (Figure 2).

Effects of Sedentary Aging on Endplate Dispersion

Endplate size and dispersion varied with age in the sedentary male rats. In 4-week-old sedentary males, the SOL contained smaller and less disperse endplates compared to those observed 12-week-old males (Figure 3). In males, endplates had the highest degree of dispersion in SOL muscle at 6-weeks of age. Endplate dispersion did not increase in the SOL of the older time points of sedentary males in comparison to the level of dispersion in the SOL of the 6-week-old sedentary male rats (Figure 5).

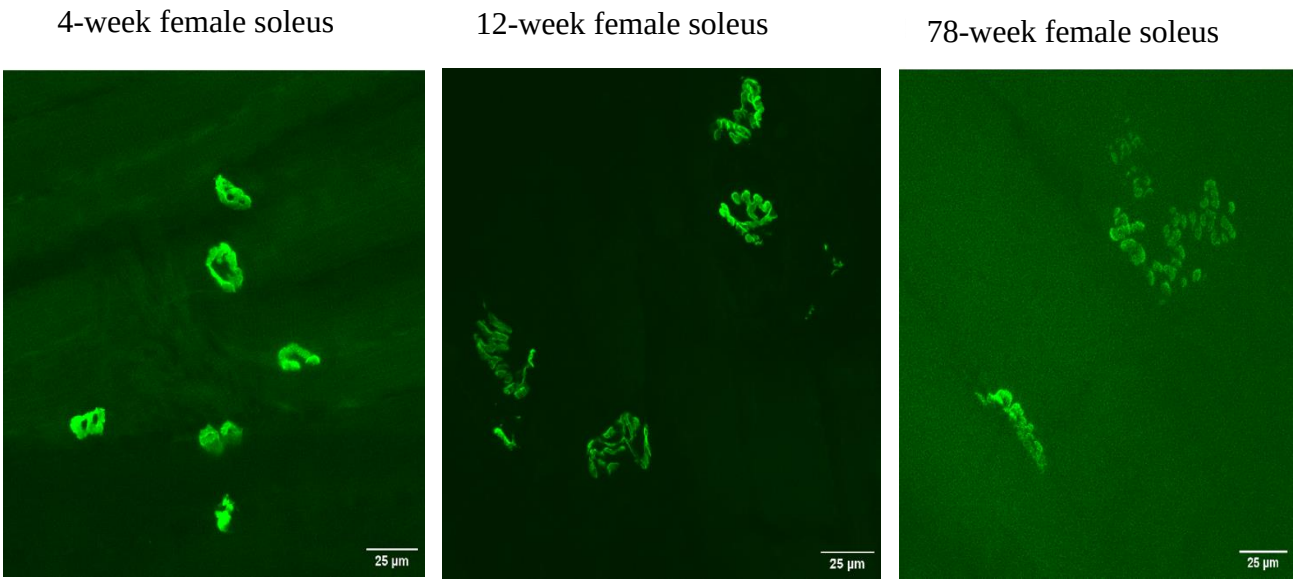


Figure 3: Endplate morphology in SOL from sedentary female rats. Acetylcholine receptors at the neuromuscular junction were visualized using α-bungarotoxin. Images were captured using Nikon Eclipse Confocal Microscope and were analyzed using the software ImageJ.

In males, there was no difference between endplate dispersion in the PLA of 4-week-old and 12-week-old sedentary rats (Figure 7). However, the endplates were more dispersed in the PLA of 6-week-old sedentary male rats when compared to the 4-week-old sedentary males (Figure 7).

In female rats, with sedentary aging there were similar trends in changes seen in endplate size and dispersion. The endplates were generally smaller with less dispersion in the SOL of 4-week-olds, as compared to the larger and more disperse endplates in the SOL of 12-week-old sedentary female rats. While the endplates were expectedly larger and it should be noted that there was a higher degree of dispersion in the SOL of sedentary 78-week-old females (Figure 5). The endplates were 30% more dispersed in the SOL of 78-week-old sedentary females (0.115±0.01) compared to the level of dispersion seen in the SOL of 4-week-old sedentary females (0.42±0.01)

In females, the level of endplate dispersion increased in the PLA of aging rats. There was no difference in the level of endplate dispersion in the PLA of 4-week-old sedentary females (0.48±0.01) compared to 6-week-old sedentary females (0.47±0.01). However, there was a difference in levels of endplate dispersion when comparing the 4-week-old sedentary females to all other older time points above 6 weeks of age. In sedentary females, endplate dispersion was the highest at 52 weeks of age (0.22±0.01). The level of endplate dispersion seemed to have become less fragmented in the PLA of the 78-week-old sedentary females (0.36±0.01) and was similar to the levels of endplate dispersion in the PLA of the 12-week-old sedentary females (0.35±0.01). However, the degree of dispersion was similar in the PLA between 4-week, 12-week and 78-week old sedentary females (Figure 7).

Effects of Sedentary Aging on Endplate Area

In males, endplate area in SOL (Figure 4) and PLA (Figure 6) muscle from 8-, 12-week-old male sedentary rats, was greater than that in 4-week-old rats. Interestingly, the endplate area was found to increase in the SOL of sedentary 4-week-old females to 12 weeks of age. However, endplate area decreased in the SOL of 78-week-old sedentary females (Figure 4). Endplate area increased in the PLA of 12-week-old (163.77±17.96) sedentary males compared to the endplate area in the PLA of 4-week-old sedentary males (96.34±5.23). The endplate area in the PLA of

older sedentary females was larger at all time points in comparison to the 4-week-old sedentary females (Figure 6).

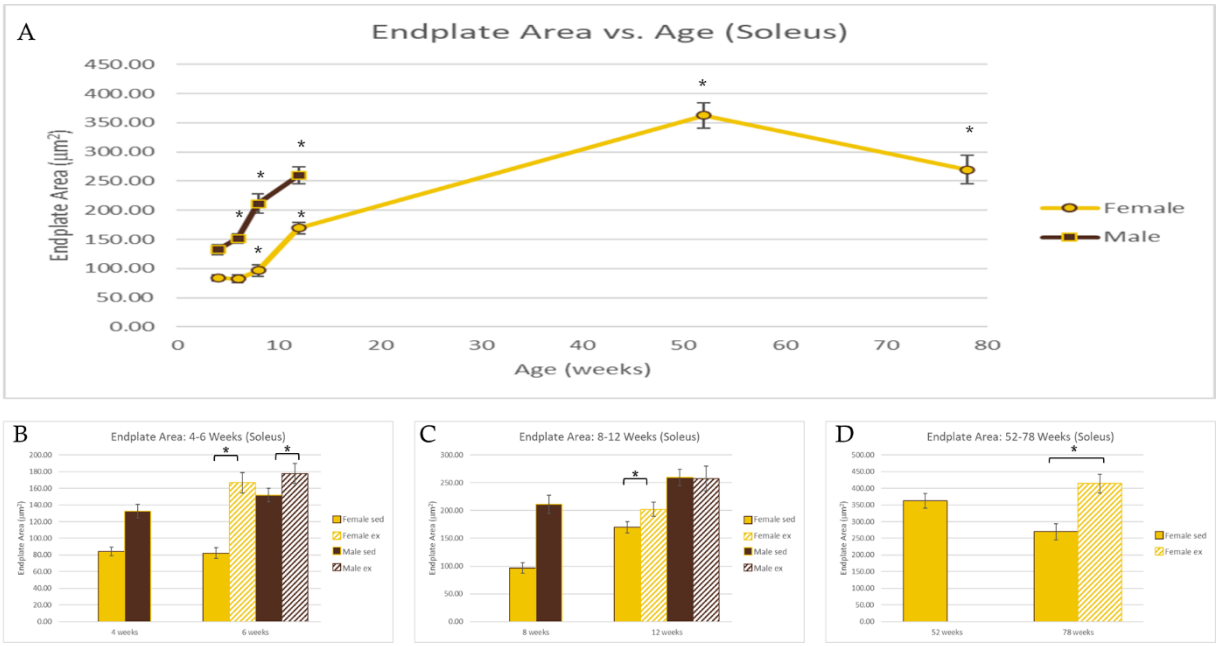


Figure 4: Effects of exercise on endplate area in SOL muscle of male and female rats. Acetylcholine receptors at the neuromuscular junction were visualized using α-bungarotoxin. Images were captured using Nikon Eclipse Confocal Microscope and were analyzed using the software ImageJ. Panel A: Changes in endplate area in SOL muscle with sedentary aging. The results show that there was an increase in endplate area in male sedentary SOL when comparing 4 week-old to all other time points. In female sedentary SOL, the average area of the endplate increased from 4 weeks to 52 weeks of age, with a decrease at 78 weeks. Panel B: Effects of exercise on SOL endplate area from ages 4-6 week-old. There was an increase in exercised 6-week-old males and females when compared to age-matched sedentary. Panel C: Effects of exercise on SOL endplate area from ages 8-12 week-old animals. Exercise increased endplate area in 12 week female SOL when compared to age-matched sedentary. Panel D: Effects of exercise on female endplate area from ages 52-78 weeks of age. Exercise increased endplate area at 78 weeks of age when compared to age-matched sedentary. Asterisk (*) indicates a significant difference ($p<0.05$).

Effects of Exercise on Endplate Dispersion

In males, exercise had no effect on levels of endplate dispersion when compared to the level of dispersion in the SOL (Figure 5) and PLA (Figure 7) of sedentary age-matched males. However, exercise had an effect on the level of endplate dispersion when compared to the endplate dispersion visualized in the SOL of age-matched sedentary females. At 6 weeks of age, the endplates were less dispersed in the exercised females compared to the level of dispersion seen in sedentary females of the same age (Figure 5). At 78 weeks of age, the endplates were also less dispersed in the exercised females compared to the more dispersed endplates visualized in the sedentary females. Exercise in 78-week-old females restored endplate dispersion (0.21±0.01) to levels observed in sedentary female rats at 52 weeks of age (0.21±0.01). The levels of endplate dispersion were higher in the PLA of 12-week-old, exercised females (0.43±0.02) compared to the level of dispersion in the PLA of 12-week-old sedentary females (0.35±0.01). As expected, the levels of endplate dispersion were also higher in the PLA of

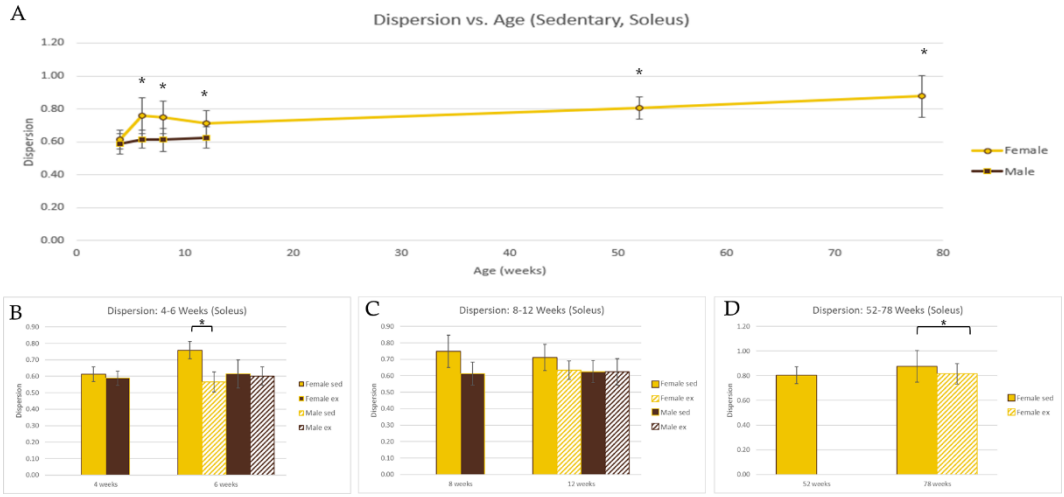


Figure 5: Effects of exercise on endplate dispersion in SOL muscle of male and female rats. The box is drawn so that the endplate is inscribed within the box. The bean is drawn so that it traces the perimeter of the endplate. The ratio between the two is an inverse metric of endplate dispersion. Having a lower bean-to-box ratio indicates a higher degree of dispersion. However, Y axis is just dispersion so a taller bar means a higher dispersion. Images were captured using Nikon Eclipse Confocal Microscope and were analyzed using the software ImageJ. Panel A: Changes in endplate dispersion in SOL muscle with aging. In sedentary aging in female, dispersion was significant when compared to 4-weeks of age across all ages. Panel B: Effects of exercise on SOL dispersion on 4-6 week-old male and females. Panel C: Effects of exercise on SOL dispersion for 8-12 week-old male and females. Panel D: Effects of exercise on SOL dispersion for 52-78 wee-old females. Exercise decreased endplate dispersion in SOL from 6- and 78-week-old female rats

Exercise had no impact on endplate dispersion in male SOL.

Asterisk (*) indicates a significant difference ($p<0.05$).

78-week-old, exercised females (0.41±0.01) when compared to the levels of endplate dispersion in the PLA of age-matched sedentary females (0.37±0.01). Furthermore, the levels of endplate dispersion in the PLA of 78-week-old exercised females (0.41±0.01) were similar to the levels of endplate dispersion seen in the PLA of 8-week-old sedentary females (0.42±0.02).

Effects of Exercise on Endplate Area

In male rats, exercise increased the area of endplates in SOL between 4 and 6 weeks of age, but not between 8 and 12 weeks of age (Figure 4). The endplate area in the PLA of 6-week-old, exercised males was larger (404.05±25.4) when compared to the endplate area in age-matched sedentary male rats (142.83±9.06). In female rats, exercise increased the area of endplates in SOL between 4 and 6 weeks of age, 8 and 12 weeks of age and 52 and 78 weeks of age (Figure 4). The endplate area in the PLA of 12-week-old, exercised females was larger (221.32±12.98) when compared to the endplate area in age-matched sedentary females (130.00±8.00).

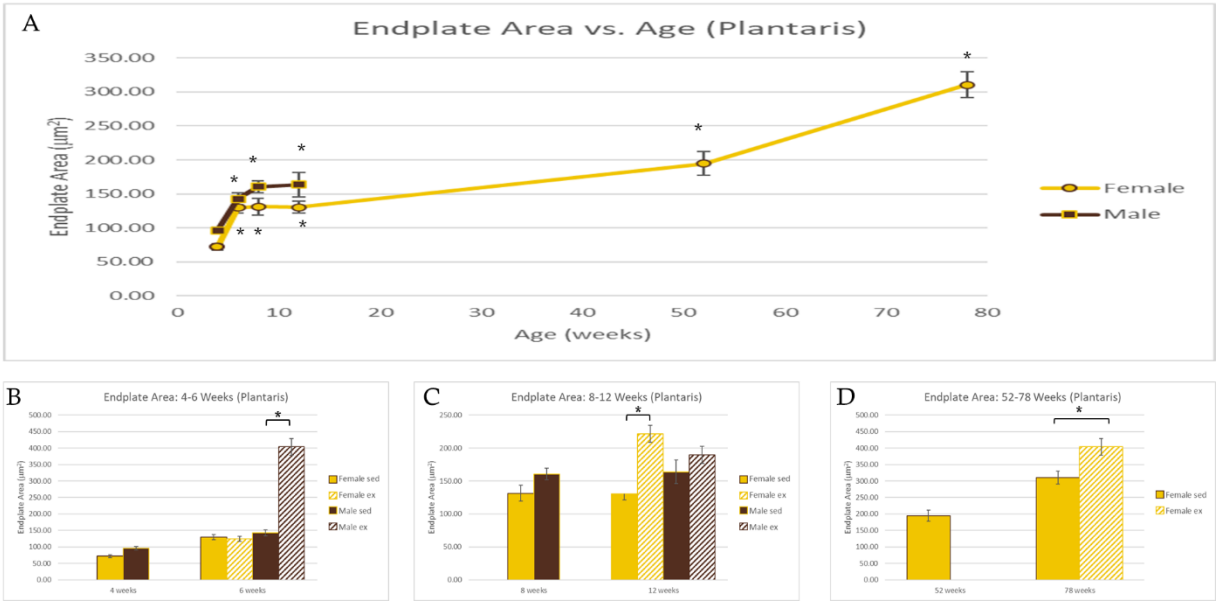


Figure 6: Effects of exercise on endplate area in PLA muscle of male and female rats. Tissues were bound with α-bungarotoxin staining of acetylcholine receptors in the neuromuscular junction to visualize endplates. Images captured by Nikon Eclipse Confocal and calculations and measurements were performed using the software ImageJ. Panel A: Changes in endplate area in PLA muscle with sedentary aging. There was an increase in male and female endplate area when comparing 4 weeks of age to all other time points. Panel B: Effects of exercise on PLA endplate area on 4-6 weeks of age. There was an increase in exercised 6-week-old males when compared to age-matched sedentary. Panel C: Effects of exercise on PLA endplate area in 8-12 week-old animals. In exercised 12-week-old females endplate area was higher when compared to age-matched sedentary. Panel D: Effects of exercise on PLA endplate area in 52-78 week-old females. Exercised 78-week-old females have higher endplate area when compared to age-matched sedentary. Asterisk (*) indicates a significant difference ($p<0.05$).

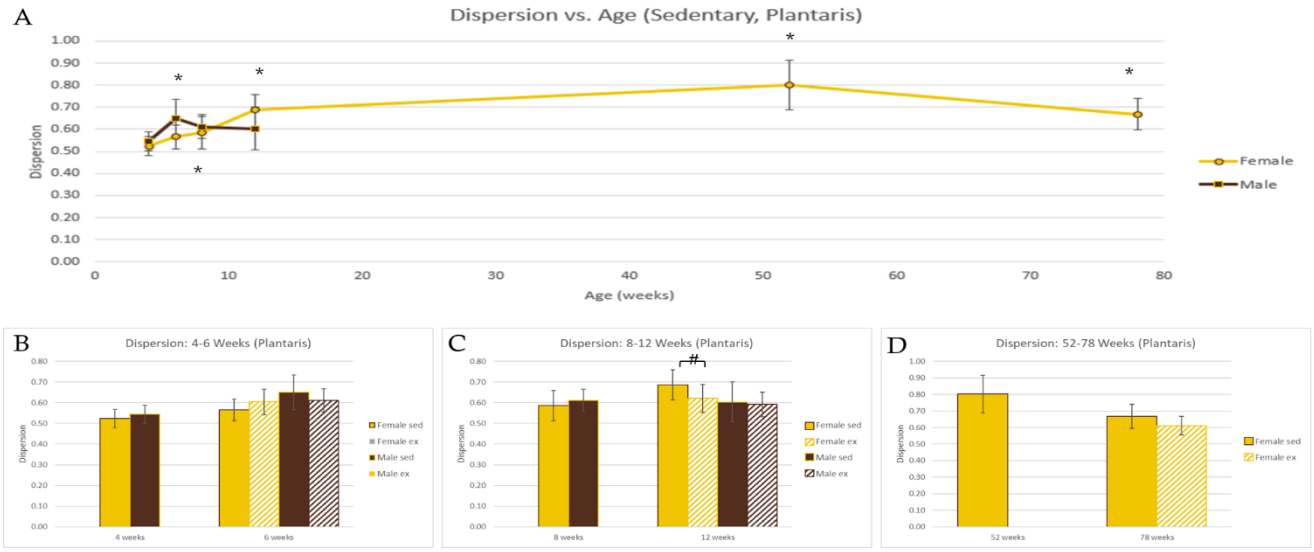


Figure 7: Effects of exercise on endplate dispersion in PLA muscle of male and female rats. The box is drawn so that the endplate is inscribed within the box. The bean is drawn so that it traces the perimeter of the endplate. The ratio between the two is an inverse metric of endplate dispersion. Having a lower bean-to-box ratio indicates a higher degree of dispersion. However, Y axis is just dispersion, so a taller bar means a higher dispersion. Images were captured using Nikon Eclipse Confocal Microscope and were analyzed using the software ImageJ. Asterisk (*) indicates a significant difference ($p<0.05$) when compared to 4 week-old. Panel A: Changes in endplate dispersion in PLA muscle with aging male and female rats. Sedentary male 6-week-old was the only significant age when compared to the 4-week control. There was not a significant change in female endplate dispersion from 4 week to 6-week of age but was significant in all other comparisons. Panel B: Effects of exercise on endplate dispersion in PLA in 4-6 week-old male and females. Panel C: Effects of exercise on endplate dispersion in 8-12 week-old male and females. Exercise decreased endplate dispersion in PLA in 12-week-old-female rats. Pound sign (#) indicates a significant difference ($p<0.05$) when comparing sedentary to exercise. Panel D: Effects of exercise on endplate dispersion in PLA in 52-78 week-old females.

Exercise had no impact on endplate dispersion in male PLA

GDNF Protein Content Correlates with Endplate Area in SOL Muscle from Male and Female Rats

Correlation statistics were run to determine if a relationship exists between GDNF protein content and endplate area. A relationship exists between GDNF protein content and endplate area in SOL of sedentary male and female rats. (Figure 8).

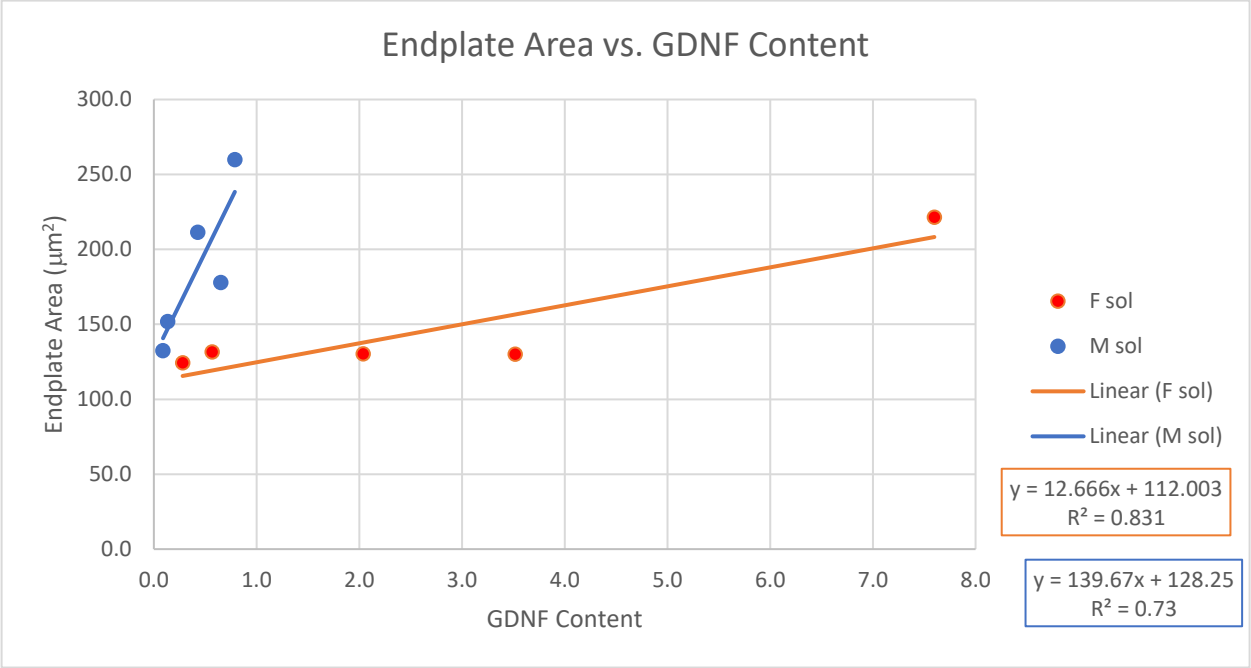


Figure 8: Correlation between GDNF levels and total endplate area in SOL muscle of male and female rats. Levels of GDNF (pg/ g of tissue weight) were positively correlated with endplate measurements for total area. Male SOL ($r=0.57$, $p<0.24$, $n=6$) and female SOL ($r=0.83$, $p<0.05$, $n=6$).

Discussion

The purpose of this study was to investigate the effects of voluntary exercise on GDNF protein content and endplate morphology in male and female rats at different ages. The results of our study show that exercise increased GDNF protein content for both males and females when compared to age-matched sedentary rats. The results also show that females have a higher concentration of GDNF protein content when compared to males of the same age. In females, exercise played a significant role in changes in endplate morphology in SOL. The changes in endplate morphology in SOL and the positive correlation observed with GDNF may suggest a role for GDNF in the changes observed.

Our results show that there are morphological changes in the NMJ in females with sedentary aging. Some of those changes include larger endplate area and increased degree of dispersion, which suggests neuromuscular junction deterioration with age (Campbell et al., 1973; Fling et al., 2009). Because there are fewer motor units as one ages (Campbell et al., 1973; Tomlinson & Irving, 1977) and older motor neurons show signs of deterioration (Hepple & Rice, 2016), it would likely mean that there is less communication with target muscles (Deschenes, 2011; Hepple & Rice, 2016). We observed such changes in the area of endplates of younger female rats as it was more dense but increased with age. We believe the area is smaller because their nervous system is not yet fully formed. At 12 weeks of age, we see more of the classic pretzel-like shape of endplates, when they are more sexually mature. Older female rats displayed the greatest endplate area and dispersion, suggesting that there are negative impacts of sedentary aging on the somatic nervous system.

With increased endplate dispersion observed during sedentary aging, we wondered if there were ways to mitigate those changes. It has been shown by (Valdez et al., 2010) that the increase in endplate dispersion was significantly attenuated by caloric restriction and exercise. Our results demonstrate that exercise helps maintain SOL endplate morphology and prevent dispersion in older females, whereas exercised 78-week-old females displayed dispersion similar to that seen in 52-week-old sedentary females. We saw similar trends in 78-week-old female PLA, where endplate dispersion was restored to levels seen in younger 8-week-old animals. This suggests exercise as a mechanism to slow or reverse the negative effect of aging on endplate structure.

As far as we know this is the first study to look at GDNF protein concentration in female rats. It was a novel result to see that females had higher levels of GDNF protein content than age-matched males, which supported our hypothesis. GDNF has also been shown to mitigate negative age-related changes in the nervous system ((Nguyen et al., 1998; Sariola & Saarma, 2003;). GDNF levels were below the detection limit for the ELISA in older sedentary male and female rats, suggesting that GDNF protein levels decrease with aging. Lower levels of GDNF in aging animals, could contribute to the morphological changes observed at the endplate. This may also suggest a need for GDNF expression in younger animals, to support the growing nervous system, and a smaller role for trophic factors in older animals. Higher levels of GDNF, as seen in the younger female rats, may also help explain why pre-menopausal females show lower incidences of neurological disease when compared to age-matched males (Hanamsagar & Bilbo, 2016).

Our results show that GDNF expression is different in muscle from females compared to males. We then asked what may be responsible for these differences. Different hormones have been shown to drive different sexes to move more (Chasland et al., 2021), however, we did not see evidence of that being a reason for differences between male and female in our study. Younger females had higher levels of GDNF when compared to young males, even though there was not a difference in the amount that they moved. Another possible explanation for the observed differences in GDNF levels between the sexes could be the different growth rates, both body weight and relative muscle weight. At 4 weeks of age there was no difference in body weight between males and females, despite levels of GDNF being higher in females. This suggests that differences in body weight or relative muscle weight are not the driving force for the different concentration of GDNF between the sexes.

Exercise is commonly used as an intervention for weight loss (Swift et al., 2013), and to reduce the loss of muscle mass and strength as one ages (Chen et al., 2017). Our results showed no effect of exercise on body mass in female rats at 4-6 weeks of age; however, exercise increased skeletal muscle GDNF content at this age. This suggests that exercise may not have a large impact on body mass until sexual maturity (after 12 weeks of age), yet still can increase skeletal muscle GDNF content in females. This was seen in young, sexually mature rats (12 weeks of age) as exercise did increase in body mass 12-week-old when compared to age-

matched males. We saw the highest concentration levels of GDNF with exercise, in SOL and PLA after puberty, when compared to pre-pubescent ages. This was most obvious at 12 weeks of age as exercised male rats had the highest overall concentration of GDNF protein content. It was at this age that males had more GDNF protein content than females, which had not been the case in pre-pubescent sedentary and exercised rats.

We are aware that in comparison to males of the same age group, females tend to have a lower proportion of fast-twitch muscle fibers (Haizlip et al., 2015). Among sedentary males, there was no significant difference in endplate dispersion within the PLA and SOL muscles. However, in females, endplate dispersion increased in both of these muscles. This discrepancy suggests that the dispersion of endplates in fast-twitch muscles may remain stable over time in males, but in females, it tends to increase with age. This potential rise in endplate dispersion, leading to reduced signaling to the target tissue, could possibly explain the diminished support of fast-twitch muscle as individuals age. Fast-twitch muscles are typically engaged during activities that involve exertion, such as heavy lifting or reacting swiftly to prevent a fall (Hepple & Rice, 2016; Rowan et al., 2011). This could explain the reasoning behind the greater vulnerability and heightened susceptibility to falls observed in the aging population.

Conclusion

Here, we studied differences in GDNF protein content between young female and male rats. We found that GDNF concentration in PLA and SOL muscle declines with sedentary aging for both sexes, but that GDNF concentrations were greater in sedentary females than in age-matched males. Higher levels of GDNF protein content in muscle from female rats could provide a possible explanation as to why they are more protected than males against disease with a neural component.

We also found that exercise increased levels of GDNF concentrations in muscle for both males and females. We know that GDNF is an important motoneuron protectant, which is why increased levels may help explain why exercise is so beneficial for neural tissues. We saw increased levels of GDNF due to exercise across all age groups studied, demonstrating that regardless of the age one starts exercising, there are positive effects on neural health.

Acknowledgements: Dr Sydney Sheltz-Kempf for all of her help with edits and proofreading and Dr Gaetan VanGyseghem for his help on statistical analysis.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CHAPTER III

VOLUNTARY EXERCISE INCREASES LEVELS OF ESTRADIOL *IN VIVO* AND GDNF PROTEIN CONTENT *IN VITRO* IN FEMALE RATS

ABSTRACT: Estrogen is a steroid hormone that plays various roles in the body. For females, it helps develop and maintain the reproductive system and female phenotypic characteristics. Also, estrogen has been shown to have "non-reproductive" effects and contribute to cognitive health, bone health, and cardiovascular system function. This is also evident in post-menopausal women, who have increased incidences of neurological and cardiovascular disease. Previous studies have shown that a decrease in strength and quality of skeletal muscle is related to decreased estrogen levels, which may contribute to higher levels of sarcopenia. While exercise has been shown to mitigate the rate of sarcopenia, it has also been shown to increase estrogen receptors (ER's) and levels of glial cell line-derived neurotrophic factor (GDNF) in skeletal muscle. Furthermore, it has been shown that GDNF and estrogen act through similar intracellular signaling pathways, and estrogen can enhance intracellular GDNF signaling. The goal of this study is to explore the effects of exercise on estrogen and GDNF and to explore the relationship between levels of estrogen and GDNF. Our hypothesis is that estrogen levels will be lower in sedentary females when compared to exercised rats. In addition, higher levels of GDNF will correlate with higher levels of estrogen. Trunk blood was collected from sedentary and exercised animals, centrifuged and serum was used for analysis. Enzyme-linked immunosorbent assay was used to quantify concentrations for estrogen and GDNF protein content. Different concentrations of estradiol were added to C2C12 murine skeletal muscle *in vitro* and analysis of GDNF protein concentration was performed on cultured cells. Levels of estrogen were significantly higher in exercised animals when compared to sedentary across all ages examined (4 weeks-78 weeks). There was a significant decrease in GDNF with the 5 nM and 100 nM concentration of estradiol

after 4 hours, but then a significant increase in GDNF with 5 nM after 48 hours. In conclusion, exercise does increase levels of both estrogen and GDNF *in vivo* and that there was a relationship between increased levels of estrogen and increased levels of GDNF.

Introduction

Estrogen is a hormone that helps develop and maintain the reproductive system and secondary sex characteristics in females (Huether and McCannce, 2019). There are three major endogenous estrogens: estrone (E1), estradiol (E2), and estriol (E3), with E2 being the most abundant and potent. While it is critical to the maintenance of normal reproductive and non-reproductive functions estrogen also may be an important factor contributing to sex differences observed in brain aging and neurodegeneration (Zarate, Stevnsner and Gredilla, 2017). Moreover, neuroprotective actions of E2 are apparent during aging and menopause. The decline in E2 is associated with mitochondrial dysfunction, neuroinflammation, synaptic decline, cognitive impairment, and increased risk of age-related disorders (Zarate, Stevnsner and Gredilla, 2017).

Women differ in how susceptible they are to disease depending on whether they are pre- or post-menopause, most likely due to the decrease in E2. For example, the higher prevalence and greater severity of Alzheimer's disease (AD) in women is possibly related to the postmenopausal reduction in E2 concentration (Tang et al., 1996; Brann et al., 2007; Li and Singh, 2014). The risk of developing Parkinson's disease (PD) is also influenced by E2, exhibiting a lower risk in premenopausal versus postmenopausal women. This may be explained in part by the estrogen-induced inhibition of microglial activation and neuroinflammation system leading to reduced oxidative stress, neuroinflammation, and neurodegeneration of dopaminergic neurons in murine models of PD (Rodriguez-Perez et al., 2010; Labandeira-Garcia et al., 2016). Taken together, data indicates that both aging and menopause are associated with increased neuroinflammation, which may also contribute to female and male sex differences in age-related neurological diseases such as AD and PD (Zarate et al., 2017).

Numerous studies have further shown that pre-menopausal women are protected against stroke relative to men. However, stroke incidence increases in women following menopause (Murphy, McCullough and Smith, 2004; Roquer, Campello and Gomis, 2003; Niewada et al., 2005; DiCarlo et al., 2003). Not only does the incidence increase in post-menopausal women, but worse outcomes are significantly higher including higher disability and fatality rates when compared to men (Roquer, Campello and Gomis, 2003; Niewada et al., 2005; DiCarlo et al., 2003; Hochner-Celnikier et al., 2005). These studies highlight differences in age and sexes in neurological disorders and further indicate that post-menopausal women are at a greater risk of developing neurological disease and degeneration.

The endocrine system plays a major role in cellular interactions, metabolism, and growth, which explains why changes in hormone levels affect many physiological processes. As women go through menopause, they exhibit an accelerated decline in muscle mass and strength (Calmels et al., 1995; Carville et al., 2006; Cooper et al., 2008; Greeves et al., 1999; Kurina et al., 2004; Samson et al., 2000; Skelton et al., 1999). This may be related to the considerable decline in estrogen levels, with an average of 80% estrogen loss during the first year of menopause (Phillips et al., 1993; Cauley et al., 1989; Vina et al., 2006). This has been shown to decrease muscle function (Sipila 2003; Lemoine et al., 2003). It has also been shown that lower rates of fall-related limb fractures in 75-year-old women along with increased muscle strength have been seen in women with higher endogenous estrogen levels (Sipila 2003). Lowe et al., (2010) showed that estrogens have many beneficial effects on muscle strength in postmenopausal women, along with age-related losses in muscle strength due to the declines in estrogen levels.

The beneficial actions of estrogen are mediated by estrogen receptors (ER). ERα and ERβ are encoded by ESR1 and ESR2 genes, respectively, and both of the ER have been found to be on skeletal muscle in humans and mammals (Wiik et al., 2009). Skeletal muscle is one of the largest tissues in mammals, responsible for movement, posture and exercise. Studies done by Velder et al (2012) showed the importance of both receptors in muscle regeneration after injury. This was done by ovariectomizing female rats and found that creatine kinase levels in the blood were elevated after muscle damage, when compared to control rats. It was found that ESR2 played a much larger role in muscle recovery than ESR1. However, ESR1 decreased ubiquitin-specific peptidase19 (estrogen agonist), consequently increasing muscle mass (Ogawa et al., 2015).

It has been shown that estrogen plays a significant role in the maintenance and strength of skeletal muscle. It has also been shown to play a neuroprotective effect in pre-menopausal women. Furthermore, estradiol may facilitate the release of neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1), and brain-derived neurotrophic factor (BDNF) to safeguard neurons and promote the repair of damaged neuronal circuits in pathological conditions (Yuan et al., 2019; Arevalo, Azcoitia, and Garcia-Segura, 2015).

GDNF

GDNF is a potent neurotrophic factor that plays a versatile role in many cell and tissue types, including skeletal muscle, Schwann cells and motor neuron axons, cell bodies and synapses (Henderson et al., 1994; Nguyen et al., 1998; Nosrat et al., 1996; Springer et al., 1994, 1995; Suter-Crazzolara and Unsicker, 1994; Suzuki et al., 1998; Trupp et al., 1995). GDNF has been shown to save somatic motor neurons from naturally occurring cell death (Oppenheim et

al., 2000), and axotomy-induced cell death (Oppenheim et al., 1995), and protects motor neurons from chronic degeneration (Corse et al., 1999). While the complete mechanism for the loss of skeletal muscle with age is unknown, it has been suggested that a decrease in neurotropic signaling could be a contributing factor (Bergman et al., 1999).

With aging, we know that there is a decrease in neurotrophic factor (Cintron-Colon and Spitsbergen, 2019). As one ages, one goes through a natural phenomenon called neural plasticity. That is where the neuron makes connections, retracts, makes connection and retracts with the target tissue. However, with aging the neuron stays retracted longer, which results in loss of connection and communication. That loss in communication is believed to be a factor in muscle atrophy, and ultimately sarcopenia. This is particularly high in cases involving the elderly population. So, we asked ourselves if there were ways to mitigate those losses.

Previous studies have shown that exercise increases glial cell line-derived neurotrophic factor (GDNF) protein concentrations in skeletal muscle (Wehrwein et al., 2002). Additionally, exercise increases levels of estrogen (Otag et al., 2016). With increases in both, we wanted to investigate if estrogen played a role in levels of GDNF. Given the observed neuroprotective and myoprotective properties attributed to estrogen and GDNF, we posited a mechanistic correlation between these factors.

Methods and Materials

Subjects

All animal experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council) and protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University. Male and female Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Female rats were acquired at 4 weeks of age, 8 weeks of age, and 52 weeks of age. Male rats were acquired at 4 weeks of age and 8 weeks of age. All rats were considered pre-puberty at 6 weeks of age and younger and adolescent at 8-12 weeks of age (Sengupta, 2013). Females at 52 and 78 weeks of age were considered to be aged as they are going through reproductive senescence. Animals were acquired and acclimated to their environment before testing began. Rats in each age group were randomly separated into a sedentary group, maintained in cages without access to running wheels (n=6), and an exercise group with access to running wheels (n=6). Animals were exposed to a 12-hour light/dark cycle and had access to food and water *ad libitum*.

Voluntary Exercise Protocol

Voluntary exercise protocols lasted for a two-week period, four-week period, or six-month period. All the animals completed the entire duration of the study. All rats were housed in clear polycarbonate living chambers (19" x 10.5" x 8"). The running wheels (Lafayette Instruments, Lafayette, IN) were attached to the living chambers and were freely accessible at all times throughout the study. Voluntary exercise was chosen as the training type because it has been shown that rats are internally motivated and do not need external stimuli to induce running (Legerlotz et

al., 2008; Sherwin, 1998). Sensors were placed on the running wheel where a computer recorded the distance run and running speed using software from Lafayette Instruments.

Tissue Collection and Processing

Following completion of exercise training protocols, exercised and sedentary rats were weighed and euthanized (CO_2 asphyxiation, followed by thoracotomy) and the predominately slow-twitch SOL and predominantly fast-twitch PLA muscles were removed.

The PLA and SOL muscles of the right side were frozen at resting length, stored at -80 °C, and later processed for analysis for endplate morphology. The PLA and SOL muscles from the left side were processed for GDNF protein content analysis via ELISA. Each muscle was weighed, snap fozen in liquid nitrogen, smashed into a fine powder, and homogenized with sample processing buffer (0.55 mol/L NaCl, 0.02 mol/L NaH_2PO_4, 0.08 mol/L Na_2HPO_4, 2 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L benzethonium chloride, 2 mmol/L benzamidine, 20 KIU/mL aprotinin, 0.5% bovine serum albumin (BSA), and 0.05% Tween-20). Homogenates were centrifuged and supernatants were collected and stored at -80 °C until ready for analysis.

Enzyme-Linked Immunosorbent Assays (ELISAs)

To determine the concentration of GDNF protein in SOL and PLA muscles ELISAs were run according to the manufacturer's (R&D System, Minneapolis, MN) specifications. Briefly, a 96-well plate was bound overnight with monoclonal anti-GDNF antibody (primary antibody) overnight. The plate was then washed and blocked with BSA. A series of standards were made in sample processing buffer and added to the top of the plate to create a standard curve. Study samples were then added to the remainder of the plate. After incubation, plates were washed and secondary

antibody (biotinylated anti-GDNF) was added and incubated overnight. The plate was washed, and horseradish peroxidase conjugated to streptavidin (Pierce, Rockford, IL) was added. The plate was washed again before adding tetramethylbenzidine color reagent (Sigma). The reaction was stopped using 0.1 M HCl, and absorbance was measured at 450 nm.

To determine the concentration of systemic estradiol protein *in vivo* ELISAs were run according to the manufacturer's (Calbiotech, El Cajon, CA) specifications. Standards, specimens and controls were plated on the 96-well coated plate. The working solution of estradiol biotin reagent was placed in each well. This was then incubated for 45 minutes. Estradiol enzyme reagent was added, and then incubated again for 45 minutes. The plate was washed three times with 1X wash buffer before TMB reagent was added. The reaction was stopped by adding the stop solution and gently mixed. The absorbance was measured at 450 nm.

Cell Culture

Mouse skeletal muscle cells (C2C12) and culture medium were obtained from the American Type Culture Collection (ATCC Manassas, VA). Culturing procedures were performed according to the ATTC protocols.

C2C12 myoblasts (undifferentiated skeletal muscle cells) were seeded on 100-mm plate (Cyto-one) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Mediatech, Manassas, VA) and 1% antibiotic-antimycotic (Invitrogen-GIBCO). Cells were incubated at 37ºC in a humidified chamber with 5% CO_2. Myoblasts were cultured after two days until about, $8.8x10^6$ cells/100 mm dish, 70% confluent. Once confluent cells were split 1:3 into new 100-mm plates. Cultured cells were maintained in the same medium and

incubator as described above. Differentiation of myoblasts to myotubes occurred by replacing growth medium with DMEM supplemented with 10% horse serum and 1% antibiotic-antimycotic. Medium was renewed every 1-2 days.

Estradiol Treatment

C2C12 cells were grown to myoblasts, as described above. β-estradiol (Sigma E2758-1G) (E_2) was added to myoblasts, once confluent. E_2 was added at 1 nM, 5 nM, 10 nM, 100 nM.

GDNF Content in Cultured Cells

GDNF protein content in each experiment was measured by enzyme-linked immunosorbent assay (ELISA). Both medium and cells were tested separately to determine GDNF protein concentration. For estradiol-muscle cultures, differing concentrations of E_2 was added to myoblasts and samples were collected every 4, 24 and 48 hours. For each experiment, cells were scraped and put in sample buffer, and were stored. To remove cells from dishes, culture medium was removed followed by washing with calcium-magnesium-free saline buffer. 1 mL of sample buffer (a mixture of phosphate buffered saline, 0.005% Tween-20, 0.5% bovine serum albumin, 0.1 mM benzethonium chloride, 2 mM benzamidine, 0.4 M NaCl, 2 mM EDTA and 164 µL/100mL aprotinin) was added to each culture dish containing cells. The cells were scraped from the dish using a cell lifter (Corning, NY). Cells were spun in a cold centrifuge and supernatant was removed and stored at -20 ºC.

Statistical Analysis

All data were reported as the mean ± standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's post-hoc test to assess statistical

significance between different groups. Differences were considered statistically significant at $p<0.05$. Where appropriate data was fit with a linear regression and correlation and variance were determined.

Results

In Vivo

The goal is to determine if there are higher levels of estrogen correlated with higher levels of GDNF following exercise. Estrogen and exercise have been shown to be a skeletal muscle protectant, but there is little known about the mechanism in which female sex hormone levels vary with exercise.

GDNF Concentration in Exercised Female Rats vs. Sedentary Female Rats

In 12-week-old female rats levels of GDNF increased after four weeks of voluntary exercise when compared to age-matched sedentary controls (Fig 9).

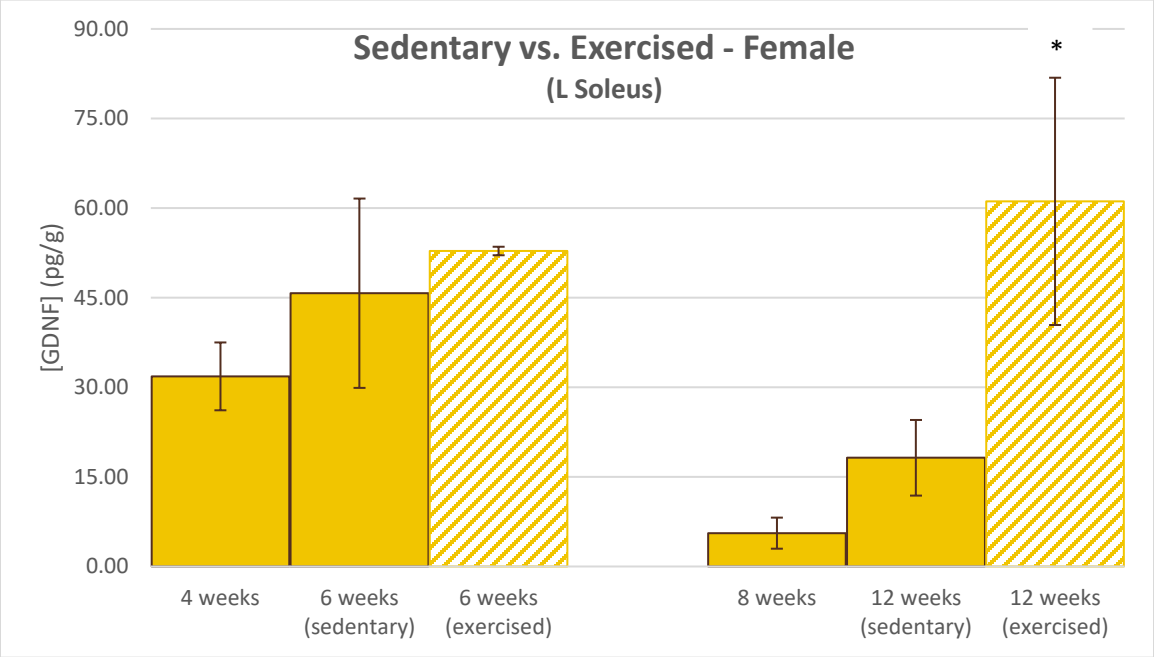


Figure 9: Effects of exercise on GDNF protein content in SOL muscle of female rats. GDNF protein concentration was determined by ELISA. GDNF protein concentration (pg/g tissue weight) is greater in 12-week-old exercised when compared to age-matched sedentary controls. Asterisk (*) indicates a significant difference ($p<0.05$) between exercise and sedentary rats of the same age.

Estradiol Concentration in Exercised vs. Sedentary

We compared serum estradiol concentration in skeletal muscle of female rats following exercise between different age groups, before and after puberty, and before and near the beginning of reproductive senescence (Fig 10).

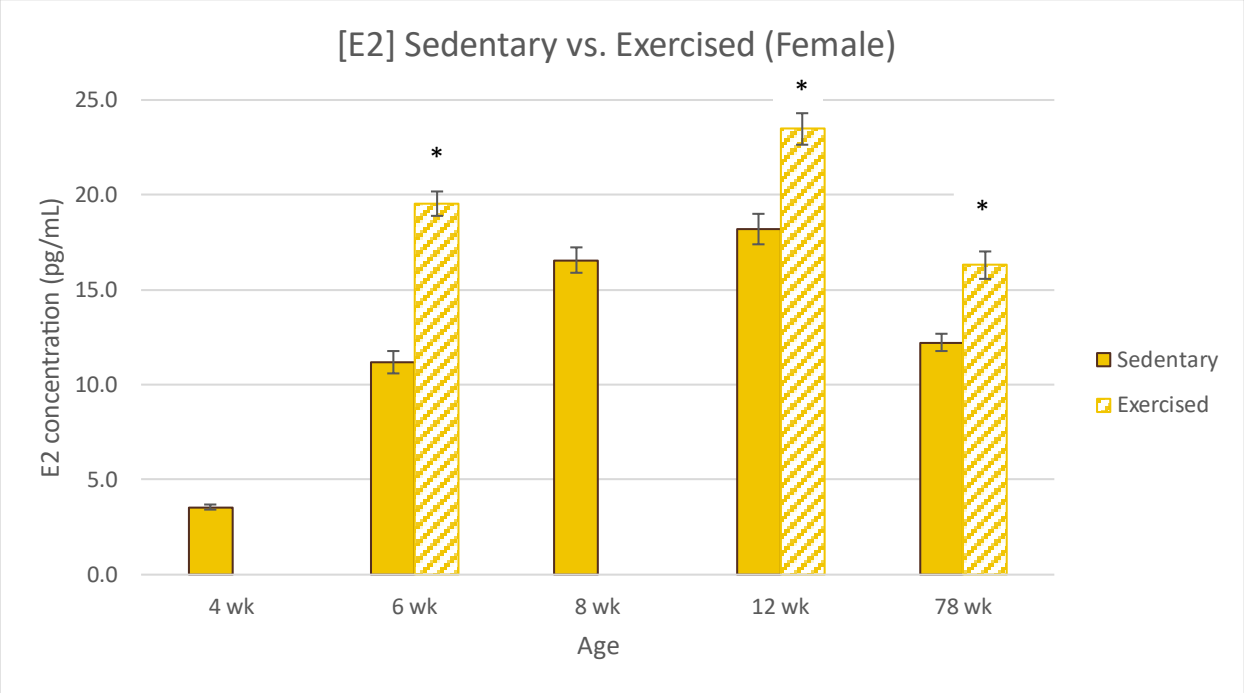


Figure 10: Effects of exercise on estradiol (E2) levels of female rats. E2 concentrations in serum was determined by ELISA. E2 concentrations (pg/mL) was greater in all exercised groups when compared to age-matched sedentary at 6-, 12- 78-week old rats. Asterisk (*) indicates a significant difference ($p<0.05$) between exercise and sedentary rats of the same age.

Estradiol levels increased with voluntary exercise across all ages, when compared to sedentary control.

In Vitro

Since we observed that exercise increased levels of GDNF and that levels of estrogen also increased with exercise, we needed to test if it the relationship between GDNF and estrogen

was correlation or causation. The way we investigated causation was by testing differing concentrations of estradiol on skeletal muscles *in vitro* and testing GDNF concentration.

Does Estrogen Affect GDNF Concentration in Culture

The goal of this experiment was to see if there was a causal relation between estrogen concentration and GDNF expression. We treated the skeletal muscle cell line, C2C12, with estrogen after they differentiated into myotubes. After they differentiated into myotubes we looked at how estrogen affects GDNF protein concentration (Kim, Qui and Kuang, 2020). Specifically, we examined changes in GDNF content both in cells as well as culture media to investigate the effect of E2 on GDNF secretion (Fig 11).

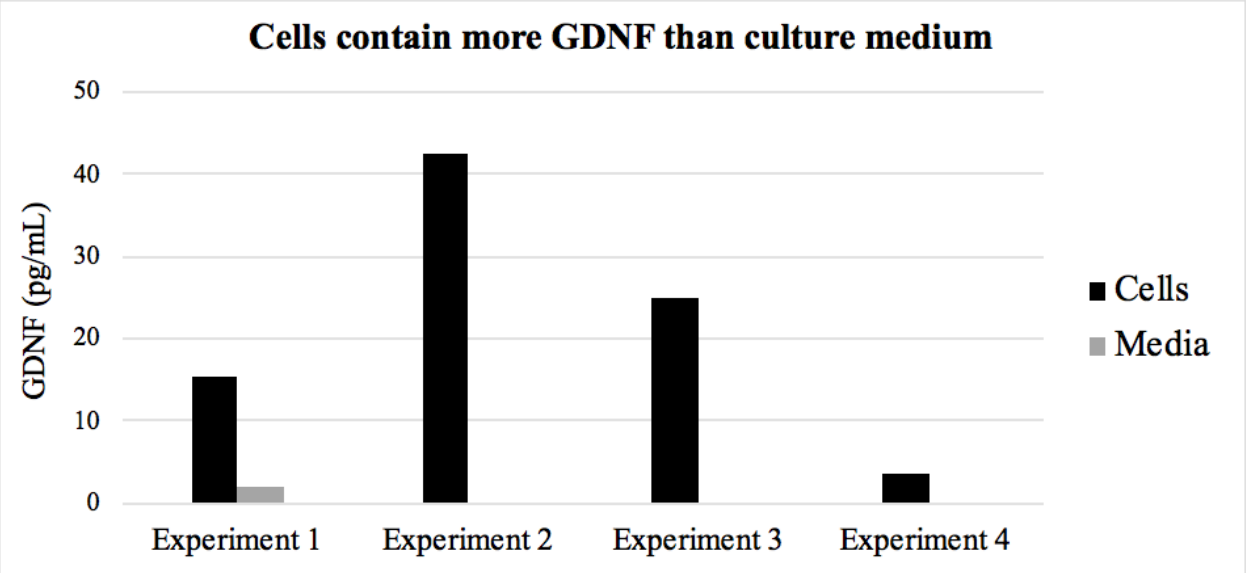


Figure 11: Cells in vitro contain more GDNF than medium. After 4 hours of treatment with E2, cells and media were tested using ELISA to quantify GDNF protein content. GDNF content was found to be in cells when compared to culture media.

Previous experiments have shown that skeletal muscle cells in culture produce GDNF but has also seen GDNF in media (Vianney et al., 2014) Cell and media samples were collected 4 hours after treatment and ELISA was used to quantify GDNF concentration.

The results (Figure 11) showed that the majority of GDNF produced is retained within cells and not found in incubating media. Due to the ELISA's indetectable GDNF levels in the media samples, the cells alone were further analyzed.

Different Length of Time that Estradiol was Administered to Cell Culture

After determining cell were to be analyzed for GDNF concentration the time in which the cells responded to the different estradiol concentrations was analyzed. The time points chosen were 4, 24, and 48 hours, providing a range of quick response (4 hours) to chronic exposure (48 hours).

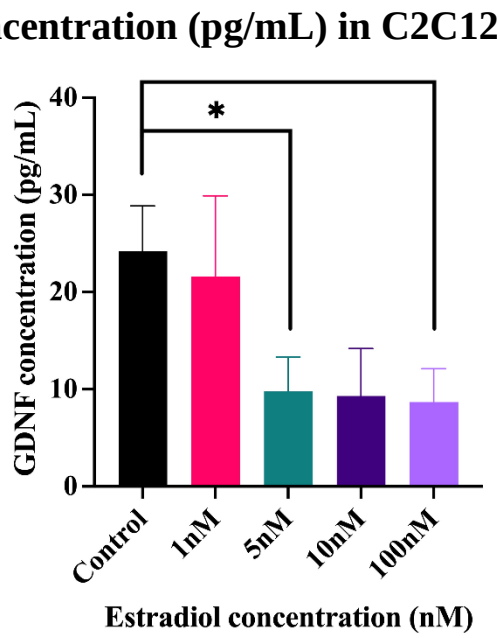


Figure 12: C2C12 myotubes in cell culture were treated with varying estradiol doses and samples were collected at four hours. GDNF protein concentrations were determined by ELISA. GDNF concentration decreased with the addition of 5 nM and 100 nM E2. Significance ($p<0.05$) is represented with an asterisk (*)

At 4 hours GDNF concentration decreased with the addition of 5 nM and 100 nM of E2 (Fig 12). The next time point of 24 hours and showed that across all concentrations there was no

change in GDNF concentration (Fig 13). However, at the last time point, 48 hours, GDNF concentration increased only with 5 nM E2 (Fig 14).

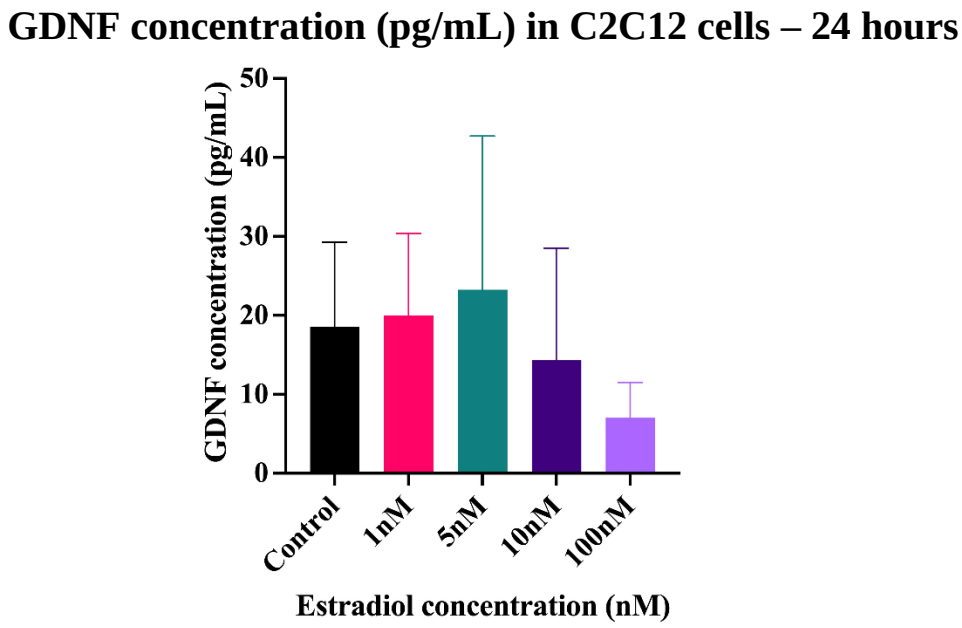


Figure 13: C2C12 myotubes in cell culture were treated with varying estradiol doses and samples were collected at 24 hours. GDNF protein concentrations were determined by ELISA. There was no change in GDNF concentration when compared to control at 24 hours.

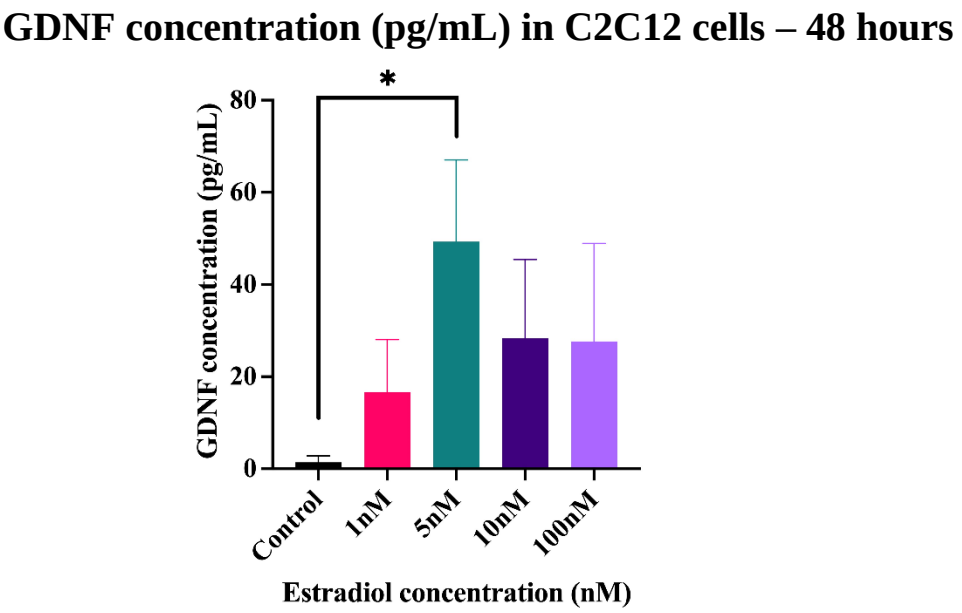


Figure 14: C2C12 myotubes in cell culture were treated with varying estradiol doses and samples were collected at 48 hours. GDNF protein concentrations were determined by ELISA. GDNF concentrations increased with the addition of 5 nM E2. Significance ($p<0.05$) is represented with an asterisk (*).

GDNF and Estrogen Correlation

While both GDNF and estrogen increased with exercise, there was no correlation between the levels of GDNF and estrogen that we observed in *in vivo* experiments.

Discussion

The purpose of this study was to investigate the effect of exercise on levels of estrogen. It is also to investigate the relationship between increased levels of estrogen and GDNF.

The results of this study suggest that the majority of GDNF produced is found within cells with little being released in medium in cells in culture. It also showed that estradiol treatment with 5nM and 100nM appeared to cause a decrease in GDNF production as a quick response but in a long-term response cells collected 48 hours after a 5 nM estradiol treatment produced an

increased amount of GDNF in comparison to the control. These results show that chronic estradiol treatments could increase GDNF production by skeletal muscle cells *in vitro*.

The results from this present study are consistent with those produced in a study investigating the effects of electrical stimulation on GDNF production (Vianney et al., 2014). First, both studies found that treated myotubes retained the GDNF they produced. Here, this was found specifically in cells sampled 4 hours after treatment, independent of the estradiol concentration added. These results suggest stored GDNF could be secreted based upon a system of demand. In an *in vivo* model, this could be due to a need for GDNF by spinal motor neurons. The *in vitro* model used here may not have allowed for this demand to be met, therefore no GDNF secretion was detected. Second, both studies showed differences in GDNF production with acute and chronic treatments. Acute treatments displayed inhibitory effects on GDNF production, whereas chronic treatments suggested stimulation of GDNF production in cell samples. Specifically in this study, these differences were seen in 5 nM and 100 nM estradiol treatments acutely, and 5 nM estradiol treatments alone chronically. These results suggest that estradiol exposure may cause changes in skeletal muscle cells that alters GDNF production, which changes from inhibition to stimulation over time.

The results of our study are also consistent with previous experiments where changes in GDNF content in skeletal muscle has been shown through exercise (Wehrwein et al., 2002). Our results showed that exercise showed a significant increase of GDNF content at 12-weeks of age, right around sexual maturity. While exercise showed trends towards an increase of GDNF content in pre-puberty rats, it was only at the age of sexual maturity that the effects of exercise played a significant role. This suggests that the physiological changes seen with puberty, such as

the increase of sex hormones, could play a role in the effectiveness of exercise on levels of GDNF.

Exercise has also been shown to increase levels of estrogen (Otag et al., 2016). In our study exercise increased levels of estradiol at all time points, but it was at the age of sexual maturity that we saw the highest increase in estradiol after exercise. We saw that levels of GDNF content were highest at this same age that estradiol levels were highest, following exercise. This result could suggest that the correlation between the highest concentration of estradiol plays a role in the highest levels of GDNF content.

As far as we know, there have not been any studies that have shown a mechanism for how GDNF may be a downstream effect of estrogen. Unfortunately, the results of our study does not shed any further light on that relationship. With the sample size from *in vitro* and *in vivo* being so small, it could play a role in our level of significance. With our data, it showed that there was no significant results from any age group showing that increased levels of estrogen increased levels of GDNF.

Conclusion

The data from these experiments have several implications for research for aging females and their relationship to levels of estrogen and GDNF protein concentration. In the past, studies looking at exercise and levels of GDNF have only been performed on male rats. This is a novel study, to our knowledge, looking at the relationship between estrogen and GDNF. The results from these experiments indicate that exercise does increase levels of estrogen and GDNF, there is no correlation between estrogen and GDNF.

CHAPTER IV

DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

The results from these studies have shown that exercise can help mitigate the negative effects seen on endplate morphology in aging male and female rats (Chapter 2). One explanation for these positive neuromuscular changes may be due to the beneficial effects of exercise on GDNF protein levels in the periphery. Higher levels of GDNF were observed in younger female rats when compared to age-matched male rats (Chapter 2), which are novel finding as these are the first studies to investigate the levels of GDNF protein content in female rats. With sedentary aging, we observed an increase in endplate area in both male and female PLA and SOL, two skeletal muscle groups with differing fiber phenotypes (Chapter 2), and in our oldest female rat group (52-78 weeks-old) we saw an increase in endplate dispersion in the SOL. In female rats, exercise positively impacted endplate morphology by increasing area and decreasing dispersion. Exercise increased levels of GDNF protein content in skeletal muscle of both males and females (Chapter 2). Exercise also increased levels of serum estradiol in both male and female rats (Chapter 3). Short-term exposure to 5 nM estradiol in skeletal muscle cells (C2C12) in culture decreased levels of GDNF, while chronic exposure of 5nM increased GDNF concentration (Chapter 3). Our research indicates that exercising could be good for the peripheral nervous system and endocrine system by counteracting the harmful effects of decreasing levels of GDNF content and estrogen that occurs with aging (menopause).

Discussion

In 1993 GDNF was discovered in glial cells (Lin et al.) and one year later, Henderson et al. (1994) found GDNF to be a potent survival factor for motor neurons. GDNF was later shown to be produced in skeletal muscle and to colocalize around endplates (Suzuki et al., 1998). To date studies examining GDNF expression and effects were only performed in male rats. Our project included female rats and has begun to fill in the gaps in our understanding of GDNF protein expression in female rats compared to male rats. Establishing this baseline knowledge also allows multiple variables to be examined, including exercise and hormonal changes which may impact GDNF protein expression differently in the two sexes. The importance of this novel information is that it could have therapeutic implications on how to better treat females who have disease with a neuromuscular or neuroendocrine component.

Differences in Endplate Morphology with Sedentary Aging

Our findings indicate that sedentary aging in both male and female rats lead to morphological changes in the NMJ. Larger endplate area and an increased degree of dispersion, indicative of NMJ deterioration with age, were observed and were previously noted by Campbell et al. (1973) and Fling et al. (2009). Since the aging process results in a reduced number of motor units (Campbell et al., 1973; Tomlinson & Irving, 1977) and older motor neurons show signs of deterioration (Hepple & Rice, 2016), it is likely that there are fewer connections and diminished communication with their target muscles and tissues (Deschenes, 2011 and Hepple & Rice, 2016). In younger male and female rats, we observed similar chronological changes in endplate area which included higher density. One explanation for these structural changes may be that the endplate area is smaller as the nervous system is not yet fully formed and is still being refined with development.

The results from our 4-6 weeks males and females we see endplate areas go from a small footprint with low complexity to endplate areas with more complexity and larger footprint of maturing animals from 8-12 weeks of age. This is normal with maturation and is seen as positive. However, the negative neurological effects that we often see with aging occurs when the increase in endplate area is coupled with greater endplate dispersion (Campbell et al., 1973; Fling et al., 2009). If increase in dispersion leads to failure to communication between nerve and muscle, this could contribute to a higher incidence of falls and frailty among the elderly. In our study involving both male and female rats, we showed that endplate area increased with age. In our older animals we began to see increases surrounding not only endplate area but also endplate dispersion. Older female rats displayed the greatest endplate area and dispersion, suggesting that there are negative impacts of sedentary aging on the somatic nervous system. However, in the male rats reaching sexual maturity, the endplate area increased while the level of dispersion within the NMJ remained stable.

Differences in Levels of GDNF Between Male and Females

We know that neurotrophic factors aid in the support, growth, and survival of neurons and that those levels decrease with aging. Results of previous studies in the laboratory show GDNF protein levels in male rat skeletal muscle decline with aging (Cintron-Colon and Spitsbergen, 2019). My results showed that young female rats have higher levels of GDNF compared to age-matched males, and GDNF levels declined with aging in both males and females. Differences observed at the NMJ between the sexes could be explained by the differing levels of GDNF.

GDNF has been shown to play an important role in both NMJ assembly and in maintaining the signaling between motor neurons and skeletal muscle (Keller Peck et al., 2001;

L. F. Lin et al., 1993). It supports pre- and postsynaptic structures during maturation and the synergy between GDNF and NMJ keeps the neuron happy, healthy and alive (L. F. Lin et al., 1993). However, the diminished levels of GDNF observed in aging animals could potentially contribute to the morphological alterations in the endplate, thereby affecting neural communication with muscle. Additionally, this may imply the necessity of heightened GDNF expression in younger animals to support the developing nervous system, but not in older animals. We saw this in our studies as the highest level of GDNF was in our 4-week old female animals. We then saw decreases with aging, suggesting that as the neuromuscular apparatus becomes established there is less need for GDNF, which is consistent with previous work done in Spitsbergen lab and others (McCullough et al., 2011; Nagano & Suzuki, 2003).

Interestingly, our young females (prior to reproductive senescence) had higher levels of GDNF when compared to our young males. However, when looking at the correlation between GDNF levels and endplate area, male SOL needed less GDNF to increase their endplate area. This was seen in Fig 8 as the slope of the correlation for males was steeper, showing that the correlation between having a larger endplate area required less GDNF content. This may suggest that males retrograde transport GDNF more quickly, which accounts for the lower levels of GDNF but increased endplate area. Female SOL required higher levels of GDNF to increase endplate area, which as shown by a flatter slope. One possible explanation could be that younger females are not as sensitive to GDNF or have fewer receptors for GDNF.

Recent studies have indicated that GDNF may be modulated by estradiol in the mid-brain (Bessa et al., 2015; Campos et al., 2012). Other studies have shown the downstream effects of estradiol on other neurotrophic factors, such as BDNF (brain-derived neurotrophic factor). Scharfman & MacLusky (2006) have suggested that estradiol can interact directly with BDNF by

inducing BDNF gene expression through turning on trkB and p75 (similar signaling pathways seen in both estradiol and BDNF). Because of the similarities between signaling pathways for GDNF and BDNF, this could be a possible explanation to why we see higher estrogen levels in our younger females and higher levels of GDNF levels.

In premenopausal females they have higher levels of estrogen and GDNF, which may contribute to the lower occurrence of neurological diseases when compared to age-matched males. However, the decrease in estrogen and GDNF levels observed during aging may explain why the severity and frequency of neurological diseases tend to rise following menopause.

Exercise to the Rescue

Previous work done in Spitsbergen lab has shown that exercise increased levels of GDNF protein in skeletal muscle in male rats (Wehrwein et al., 2002). The results of my studies showed similar increases in female rats. It also showed a difference in GDNF content in differing muscle fiber types, increase in slow-twitch but decrease in fast-twitch, similar to what was seen from Gyorkos in 2014.

GDNF protein content and endplate total area in the SOL both increased with run training which is consistent with previous findings in Spitsbergen lab (McCullough et al., 2011). It is possible that the total endplate area expanded due to changes in the presynaptic apparatus (Hill et al., 1991). The plasticity in this mechanism driven by activity, could potentially increase neurotransmitter stores (Stephens & Taylor, 1972), and enhance quantal storage and release (Dorlochter et al., 1991). As a result of these presynaptic changes, there may be corresponding adjustments in post-synaptic morphology, which lead to the increase in endplate area. That increase of postsynaptic endplate area increase has been previously shown following overexpression of GDNF (Keller Peck et al., 2001; Zwick et al., 2001). These factors may

possibly be an explanation to why young females had higher levels of GDNF than age-matched males. With the type of exercise administered (voluntary exercise) slow-twitch muscle would be recruited. We also know that females have a higher percentage of slow-twitch muscle fibers compared to males (Haizlip et al., 2015). Taken together, having more slow-twitch muscle fibers being recruited from exercise, could be why females had higher levels of GDNF than males.

While we did not see an increase of GDNF in fast-twitch muscle in my studies, that could be due to the lack of recruitment during the voluntary run exercise. However, we have seen an increase of GDNF in fast-twitch muscle in other exercise modalities, such as swim (Gyorkos & Spitsbergen, 2014). In that study, for swim training the need for higher recruitment, due to water resistance, of the extensor digitorum longus (a fast-twitch muscle) as compared to SOL (slow-twitch muscle) demonstrates that higher intensity exercise may result in higher levels of GDNF (Gyorkos &Spitsbergen, 2014).

We know that exercise has an overall beneficial effect leading to a lowering of the risk of certain diseases. And, with the observed differences seen in GDNF content in different muscle fiber types, this could play a role in protecting against sarcopenia. Sarcopenia has been shown to be muscle fiber-type specific as large motor units that innervate fast-twitch muscle are the most susceptible to denervation (Doherty et al., 1993; Edstrom et al., 2007; Frey et al., 2000). When denervation occurs in large motor units, adjacent motor units will sprout to the muscle fibers that are not innervated (Rich & Lichtman, 1989). That results in an increase in size of the remaining motor units, which could overwork that neuron, leading to even more denervation and ultimately rendering it non-functional (Gyorkos & Spitsbergen, 2014). In 2010, Deschenes suggested that if the loss of large motor units could be mitigated that the effects of aging due to sarcopenia may be delayed in the neuromuscular system. We have seen how exercise has led to increases in GDNF

in slow-twitch muscle, and that with certain high intensity exercises it has been shown to increase GDNF levels in fast-twitch muscle (Gyorkos & Spitsbergen, 2014). This suggests that exercise may play a pivotal role in slowing down the effects of aging in fast-twitch muscle fibers and the neuromuscular system. While we know that GDNF is a potent survival factor for motor neurons, and exercise increases levels of GDNF in skeletal muscle, further studies will be needed to elucidate mechanisms for how exercise increases concentrations of GDNF.

Exercise also increased estradiol levels in both males and females, and results from other studies have shown protective effects that estrogen plays in pre-menopausal women (Zarate, Stevnsner and Gredilla, 2017). Overall, exercise has positive effects on both physical and neurological well-being, rendering it a valuable regiment for individuals of all ages.

Conclusion

The results of our studies confirmed our hypotheses: The first being that with sedentary aging, NMJ area and endplate dispersion increased in both sexes. Our results also showed that prior to reproductive senescence, GDNF was higher in female rats than age-matched males. Lastly, we confirmed that exercise increased estrogen levels *in vivo* and that chronic exposure to estrogen *in vitro* increased levels of GDNF. Our work investigating GDNF levels in females will be crucial in laying the foundation for future experiments aimed at understanding more fully what is happening with females neurologically with aging. Our findings could help explain why pre-menopausal women are more protected against neurological diseases than males, and further illustrates the importance of estrogen and its protective effects.

Future Directions

Our findings indicate that voluntary exercise may serve as a potential strategy to counteract the aging-related effects on the somatic nervous system and enhance neural plasticity.

Considering that females possess a higher proportion of slow-twitch muscle fibers compared to males and that fast-twitch muscle fiber types deteriorate more rapidly with age, performing muscle fiber type staining could offer valuable insights into the impact of aging and exercise. This approach would also enable us to observe how the nervous system, via endplate size and complexity, may adapt to changes in muscle fiber types. Moreover, it could reveal the influence of various exercise modes on muscle fiber ratios and potentially how different motor units are innervated.

Muscle fiber type would pair wonderfully with previous research conducted in our laboratory that examined motor neuron cell bodies in the spinal cord. We have seen differences in the number and size of cell bodies with exercise and age and this would allow us to connect what is happening with the motor neurons in the spinal cord and muscle innervation. Not only that but it would help us establish connections between changes in motor neuron cell bodies and muscle innervation and make comparisons between the sexes. Furthermore, using q-PCR in the spinal cord and skeletal muscle to quantify the expression of GDNF mRNA would provide insights into GDNF production in these specific locations. While ELISA measurements reveal GDNF concentration, they do not provide information about local production. This analysis would enable us to determine whether GDNF is synthesized and retained within skeletal muscle or rapidly transported to the spinal cord. We could also run an *in situ* to see which muscle fiber type is producing GDNF mRNA with different exercise modalities. We could take it a step

further and conduct RNA-seq to identify the genes or pathways through which GDNF is regulated.

We briefly looked to see if there was an interaction between GDNF and the sex hormone estrogen. Potential future projects could include ovariectomizing female rats at a young age to look at NMJ and GDNF. This would allow us to look at potential effects of estrogen on a developing somatic nervous system. Another way to see if there is any interaction between GDNF and sex hormones would be to perform more studies in cell culture. In cell culture, adding different hormones, such as testosterone or progesterone, would allow us to see if there were increases or decreases with the level of GDNF production. We could even add a combination of hormones, such as progesterone with estrogen, to mimic more of what is happening *in vivo* and measure levels of GDNF.

REFERENCES

Aagaard, P., & Andersen, J. L. (1998). Correlation between contractile strength and myosin heavy chain isoform composition in human skeletal muscle. *Med Sci Sports Exerc*, *30*, 1217–1222.

Aagaard, P., Suetta, C., Caserotti, P., Magnusson, S. P., & Kjaer, M. (2010). Role of the nervous system in sarcopenia and muscle atrophy with aging: Strength training as a countermeasure. *Scandinavian Journal of Medicine and Science in Sports*, *20*(1), 49–64.

Acconcia, F., & Marino, M. (2011). The effects of 17b-estradiol in cancer are mediated by estrogen receptor signaling at the plasma membrane. *Front Physiol*, *2*, 30.

Airaksinen, M. S., & Saarma, M. (2002). The GDNF family: Signalling, biological functions and therapeutic value. *Nature Reviews Neuroscience*, *3*, 383–394.

Airaksinen, M., Titievsky, A., & Saarma, M. (1999). GDNF family neurotrophic factor signaling: Four masters, one servant? *Molecular and Cellular Neurosciences*, *13*(5), 313–325.

Amelink, G. J., & Bar, P. R. (1986). Exercise-induced muscle protein leakage in the rat: effects of hormonal manipulation. *J. Neurol Sci*, *76*, 61–68.

Amelink, G. J., Koot, R. W., & Erich, W. B. (1990). Sex-linked variation in creatine kinase release, and its dependence on oestradiol, can be demonstrated in an in-vitro rat skeletal muscle preparation. *Acta Physiol Scand*, *138*, 115–124.

Andonian, M. H., & Fahim, M. A. (1987). Effects of endurance exercise on the morphology of mouse neuromuscular junctions during ageing. *J. Neurocytol*, *16*, 589–599.

Arevalo, M. A., Azcoitia, I., & Garcia-Segura, L. M. (2015). The neuroprotective actions of oestradiol and oestrogen receptors. *Nat Rev Neurosci*, *16*(1), 17–29.

Azac-Fonseca, E., Avila-Rodriguez, M., Garcia-Segura, L. M., & Barreto, G. E. (2016). Regulation of astroglia by gonadal steroid hormones under physiological and pathological conditions. *Prog. Neurobiol.*, *144*, 5–26.

Baltgalvis, K. A., Greising, S. M., Warren, G. L., & Lowe, D. A. (2010). Estrogen regulates estrogen receptors and antioxidant gene expression in mouse skeletal muscle. *PLoS One*, *5*(4), 10164.

Bann, D., Hire, D., Manini, T., Cooper, R., Botoseneanu, A., McDermott, M. M., Pahor, M., Glynn, N. W., Fielding, R., King, A. C., Church, T., Ambrosius, W. T., & Gill, T. (2015). Light Intensity physical activity and sedentary behavior in relation to body mass index and grip strength in older adults: cross-sectional findings from the Lifestyle Interventions and Independence for Elders (LIFE) study. *PLoS One*, *10*, e0126063.

Bao, W., Sun, Y., Zhang, T., Zou, L., Wu, X., Wang, D., & Chen, Z. (2020). Exercise programs for muscle mass, muscle strength and physical performance in older adults with sarcopenia: a systematic review and meta-analysis. *Aging Dis*, *11*, 863–873.

Bar, P. R., Amelink, G. J., & Oldenburg, B. (1988). Prevention of exercise-induced muscle membrane damage by oestradiol. *Life Sci*, *42*, 2677–2688.

Baudet, C., Pozas, E., Adameyko, I., Andersson, E., Ericson, J., & Ernfors, P. (2008). Retrograde signaling onto Ret during motor nerve terminal maturation. *Journal of Neuroscience*, *28*(4), 963–975.

Berger, M. J., & Doherty, T. J. (2010). Sarcopenia: prevalence, mechanisms, and functional consequences. *Interdiscip. Top. Gerontol.*, *37*, 94–114.

Bergh, U., Thorstensson, A., Sjodin, B., Hulten, B., Piehl, K., & Karlsson, J. (1978). Maximal oxygen uptake and muscle fiber types in trained and untrained humans. *Med Sci Sports*, *10*, 151–154.

Bergman, E., Kullberg, S., Ming, Y., & Ulfhake, B. (1999). Upregulation of GFalpha-1 and c-ret in primary sensory neurons and spinal motoneurons of aged rats. *J. Neurol Sci*, *57*, 153–165.

Bergstrom, J., & Hultman, E. (1966). Muscle glycogen synthesis after exercise: An enhancing factor localized to the muscle cells in man. *Nature*, *210*, 309–310.

Bessa, A., Campos, F. L., Videira, R. A., Mendes-Oliveira, J., Bessa-Neto, D., & Baltazar, G. (2015). GPER: A New tool to protect dopaminergic neurons? *Biochim Biophys Acta-Mol Basis Dis.*, *1852*(10), 2035–2041.

Biering-Sorensen, B., Kristensen, I. B., Kjaer, M., & Biering-Sorensen, F. (2009). Muscle after spinal cord injury. *Muscle Nerve*, *40*(4), 499–519.

Brann, D. W., Dhandapani, K., Wakade, C., Mahesh, V. B., & Khan, M. M. (2007). Neurotrophic and neuroprotective actions of estrogen: basic mechanisms and clinical implications . *Steroids*, *72*, 381–405.

Buchman, A. S., Boyle, P. A., Yu, L., Shah, R. C., Wilson, R. S., & Bennett, D. A. (2012). Total daily physical activity and the risk of AD and cognitive decline in older adults. *Neurology*, *78*, 1323–1329.

Burke, R. E., Levine, D. N., Tsairis, P., & Zajac, F. E. (1973). Physiological types and hisochemical profiles in motor units of the cat gastrocnemius. *The Journal of Physiology*, *234*(3), 723–748.

Calmels, P., Vico, L., Alexandre, C., & Minaire, P. (1995). Cross-sectional study of muscle strength and bone mineral density in a population of 106 women between the ages of 44 and 87 years: relationship with age and menopause. *Eur. J. Appl. Physiol. Occup Physiol.*, *70*(2), 180–186.

Campbell, M. J., McComas, A. J., & Petito, F. (1973). Physiological changes in ageing muscles. *J. Neurol Neurosurg Psychiatry*, *36*, 173–183.

Campos, F. L., Cristovao, A. C., Rocha, S. M., Fonseca, C. P., & Baltazar, G. (2012). GDNF contributes to oestrogen-mediated protection of midbrain dopaminergic neurones. *J. Neuroendocrinol.*, *24*(11), 1386–1397.

Carville, S. F., Rutherford, O. M., & Newham, D. J. (2006). Power output, isometric strength and steadiness in the leg muscles of pre- and postmenopausal women; the effects of hormone replacement therapy. *Eur. J. Appl. Physiol.*, *96*(3), 292–298.

Casati, M., Costa, A. S., Capitanio, D., Ponzoni, L., Ferri, E., & Agostini, S. (2019). The biological foundations of sarcopenia: established and promising markers. *Front. Med.*, *6*, 184.

Cauley, J. A., Gutai, J. P., Kuller, L. H., LeDonne, D., & Powell, J. G. (1989). The epidemiology of serum sex hormones in postmenopausal women. *Am. J. Epidemiol.*, *129*(6), 1120–1131.

Centers for Disease Control and Prevention. (2022). *Benefits of Physical Activity*.

Chang, M., Jonsson, P. V., Snaedal, J., Bjornsson, S., Saczynski, J. S., & Aspelund, T. (2010). The effect of midlife physical activity on cognitive function among older adults: AGES-Reykajvik study. *J. Gerontol. Ser. A*, *65A*, 1369–1374.

Chang, Y. K., Labban, J. D., Gapin, J. I., & Etnier, J. L. (2012). The effects of acute exercise on cognitive performance: a meta-analysis. *Brain Res*, *1453*, 87–101.

Chasland, L., Yeap, B. B., Majorana, A. J., Chan, Y. X., Maslen, B. A., Cooke, B. R., Dembo, L., Naylor, L. H., & Green, D. J. (2021). Testosterone and exercise: effects on fitness, body composition, and strength in middle-to-older aged men with low-normal serum testosterone levels. *American Journal of Physiology-Heart and Circulatory Physiology*.

Chen, H. T., Chung, Y. C., Chen Y.J., Ho, S. Y., & Wu, H. J. (2017). Effects of different exercise on body composition, muscle strength, and IGF-1 in the elderly with sarcopenic obesity. . *J Am Geriatr Soc*, *65*, 827–832.

Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., & Humphries, C. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev*, *12*, 2499–2509.

Ciciliot, S., Rossi, A. C., Dyar, K. A., Blaauw, B., & Schiaffino, S. (2013). Muscle type and fiber type specificity in muscle wasting. *Int J Biochem Cell Biol.*, *45*(10), 2191–2199.

Cintron-Colon, A. F., & Spitsbergen, J. M. (2019). Effects of long-term exercise on GDNF expression and innervation in rat skeletal muscle. *FASEB Journal*.

Cisterna, B. A., Cardozo, C., & Saez, J. C. (2014). Neuronal involvement in muscular atrophy. *Front Cell Neurosci.*, *8*(405).

Clark, S., Rainville, J., Zhao, X., Katzenellenbogen, B. S., Pfaff, D., & Vasudevan, N. (2014). Estrogen receptor-mediated transcription involves the activation of multiple kinase pathways in neuroblastoma cells. *Journal of Steroid Biochemistry and Molecular Biology*, *139*, 45–53.

Clarke, B. L., & Khosla, S. (2010). Physiology of bone loss. *Radiol Clin North Am*, *48*(3), 483–495.

Clarkson, P. M., & Hubal, M. J. (2001). Are women less susceptible to exercise-induced muscle damage? *Curr Opin Clin Nutr Metab Care*, *4*(6), 527–531.

Cobianchi, S., Arbat-Plana, A., Lopez-Alvarez, V. M., & Navarro, X. (2017). Neuroprotective Effects of Exercise Treatments After Injury: The Dual Role of Neurotrophic Factors. *Curr Neuropharmacol*, *15*, 495–518.

Colom, B., Alcolea, M. P., Valle, A., Oliver, J., Roca, P., & Garcia-Palmer, F. J. (2007). Skeletal muscle of female rats exhibit higher mitochondrial mass and oxidative-phosphorylative capacities compared to males. *Cell Physiol Biochem.*, *19*, 205–212.

Cooke, P. S., Nanjappa, M. K., Ko, C., Prins, G. S., & Hess, R. A. (2017). Estrogens in Male Physiology . *Physiol Rev.*, *97*(3), 995–1043.

Cooper, R., Mishra, G., Clennell, S., Guralnik, J., & Kuh, D. (2008). Menopausal status and physical performance in midlife: findings from a British birth cohort study. *Menopause*, *15*(6), 1079–1085.

Corse, A. M., Bilak, M. M., Bilak, S. R., Lehar, M., Rothstein, J. D., & Kunel, R. W. (1999). Preclinical testing of neuroprotective neurotrophic factors in a model of chronic motor neuron degermation. *Neurobiol Dis*, *6*, 335–346.

Costantini, F. (2010). GDNF/RET signaling and renal branching morphogenesis: From mesenchymal signals to epithelial cell behaviors. *Organogenesis*, *6*, 252–262.

Costill, D. L., Daniels, J., Evans, W., Fink, W., Krahenbuhl, G., & Saltin, B. (1976). Skeletal muscle enzymes and fiber composition in male and female track athletes. *J Appl Physiol*, *40*, 149–154.

Couse, J. F., Lindzey, J., Grandien, K., Gustafsson, J. A., & Korach, K. S. (1997). Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild0type and ERalpha-knockout mouse. *Endocrinology*, *138*, 4613–4621.

Crossin, K. L., & Krushel, L. A. (2000). Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily. *Dev Dyn*, *218*, 260–279.

Cui, M. Y., Lin, Y., Sheng, J. Y., Zhang, X., & Cui, R. J. (2018). Exercise Intervention Associated with Cognitive Improvement in Alzheimer's Disease. *Neural Plast*, *9234105*.

Dalton, B. H., McNeil, C. J., Doherty, T. J., & Rice, C. L. (2008). Age-related reductions in the estimated numbers of motor units are minimal in the human soleus. *Muscle and Nerve*, *38*(3), 1108–1115.

De la Rosa, A., Olaso-Gonzalez, G., Arc-Chagnaud, C., Millan, F., Salvador-Pascual, A., Garcia-Lucerga, C., Blasco-Lafarga, C., Garcia-Dominguez, E., Carretero, A., Correas, A. G., Vina, J., & Gomez-Cabrera, M. C. (2020). Physical Exercise in the prevention and treatment of Alzheimer's disease. *J. Sport Health Sci.*, *9*(5), 394–404.

Delgado, B. J., & Lopez-Ojeda, W. (2022). Estrogen. *StatPearls*.

Deschenes, M. R. (2011). Motor unit and neuromuscular junction remodeling with aging. *Curr Aging Sci*, *4*, 209–220.

Deschenes, M. R., Judelson, D. A., Kraemer, W. J., Meskaitis, V. J., Volek, J. S., Nindl, B. C., Harman, F. S., & Deaver, D. R. (2000). Effects of resistance training on neuromuscular junction morphology. *Muscle and Nerve*, *23*(10), 1576–1581.

Deschenes, M. R., Maresh, C. M., Crivello, J. F., Armstrong, L. E., Kraemer, W. J., & Covault, J. (1993). The effects of exercise training of different intensities on neuromuscular junction morphology. *J. Neurocytol*, *22*, 603–615.

Deschenes, M. R., Tenny, K. A., & Wilson, M. H. (2006). Increased and decreased activity elicits specific morphological adaptions of the neuromuscular junction. *Neuroscience*, *137*, 1277–1283.

Deschenes, M. R., & Wilson, M. H. (2003). Age-related differences in synaptic plasticity following muscle unloading. *Journal of Neurobiology*, *57*(3), 246–256.

Di Carlo, A., Lamassa, M., Baldereschi, M., Pracucci, G., Basile, A. M., Wolfe, C. D., Giroud, M., Rudd, A., Ghetti, A., & Inzitari, D. (2003). Sex differences in the clinical presentations, resource use, and 3-month outcome of acute stroke in Europe: data from a multi-center multi-national hospital-based registry. *Stroke*, *34*, 1114–1119.

Doherty, T. J., Vandervoort, A. A., & Brown, W. F. (1993). Effects of ageing on the motor unit: a brief review. *Can J Appl Physiol.*, *18*, 331–358.

Dorlochter, M., Irintchev, A., Brinkers, M., & Wernig, A. (1991). Effects of enhanced activity on synaptic transmission in mouse extensor digitorum longus muscle. *Journal of Physiology*, *436*(1), 283–292.

Drey, M., Sieber, C. C., Degens, H., McPhee, J., Korhonen, M. T., Muller, K., Ganse, B., & Rittweger, J. (2016). Relation between muscle mass, motor units and type of training in master athletes. *Clin Physiol Funct Imaging*, *36*, 70–76.

Dworatzek, E., Mahmoodzadeh, S., Schubert, C., Westphal, C., Leber, J., & Kusch, A. (2014). Sex differences in exercise-induced physiological myocardial hypertrophy are modulated by oestrogen receptor beta. *Cardiovasc Res.*, *102*, 418–428.

Eccles, J. C., Eccles, R. M., Iggo, A., & Lundberg, A. (1960). Electrophysiological studies on gamma motoneurones. *Acta Physiol Scand*, *50*(1), 32–40.

Edstrom, E., Altun, M., Bergman, E., Johnson, H., Kullberg, S., Ramirez-Leon, V., & Ulfhake, B. (2007). Factors contributing to neuromuscular impairment and sarcopenia during aging. *Physiology and Behavior*, *92*(1–2), 129–135.

Eigenbrot, C., & Gerber, N. (1997). X-ray structure of glial cell-derived neurotrophic factor at 1.9 A resolution and implications for receptor binding. *Nat Struct Biol*, *4*, 435–438.

Einsiedel, L., & Luff, A. (1994). Activity and motor unit size in partially denervated rat medial gastrocnemius. *Journal of Applied Physiology*, *76*(6), 2663–2671.

Eketjall, S., Fainzilber, M., Murray-Rust, J., & Ibanez, C. F. (1999). Distinct structural elements in GDNF mediate binding to GRFalpha1 and activation of the GFRalpha1-c-Ret receptor complex. *EMBO J*, *18*, 5901–5910.

Encinas, M., Tansey, M. G., Tsui-Pierchala, B. A., Comella, J. X., Milbrandt, J., & Johnson, E. M. (2001). c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. *J Neurosci*, *21*, 1464–1472.

Engel, W. K. (1962). The essentiality of histo- and cyotchemical studies of skeletal muscle in the investigation of neuromuscular disease . *Neurology*, *12*, 778–784.

Enns, D. L., & Tiidus, P. M. (2010). The influence of estrogen on skeletal muscle: sex matters. *Spots Med.*, *40*(1), 41–58.

Esbjornsson, M., Sylven, C., Holm, I., & Jansson, E. (1993). Fast twitch fibres may predict anaerobic performance in both females and males. *Int J Sports Med*, *14*, 257–263.

Fahim, M. A. (1997). Endurance exercise modulates neuromuscular junction of C57BL/6NNia aging mice. *J Appl Physiol*, *83*, 59–66.

Faulkner, J. A., Larkin, L. M., Claflin, D. R., & Brooks, S. V. (2007). Age-related changes in the structure and function of skeletal muscles. *Clin. Exp. Pharmacol. Physiol.*, *34*, 1091–1096.

Feng, J., Zhang, G., Hu, X., Si, C. C., & Qin, X. (2013). Estrogen inhibits estrogen receptor a-mediated Rho-kinase expression in experimental autoimmune encephalomyelitis rats . *Synapse*, *67*(7), 399–406.

Feng, Y., & Gregor, P. (1997). Cloning of a novel member of the G protein-coupled receptor family related to peptide receptors. *Biochem Biophys Res Commun*, *231*, 651–654.

Fling, B. W., Knight, C. A., & Kamen, G. (2009). Relationships between motor unit size and recruitment threshold in older adults: implications for size principle. *Exp Brain Res*, *197*, 125–133.

Frey, D., Schneider, C., Xu, L., Borg, J., Spooren, W., & Caroni, P. (2000). Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *The Journal of Neuroscience*, *20*(7), 2534–2542.

Fry, A. C., Schilling, B. K., Staron, R. S., Hagerman, F. C., Hikida, R. S., & Thrush, J. T. (2003). Muscle fiber characteristics and performance correlates of male Olympic-style weightlifters. *J Strength Cond Res*, *17*, 746–754.

Fulco, C. S., Rock, P. B., Muza, S. R., Lammi, E., Cymerman, A., Butterfield, G., Moore, L. G., Braun, B., & Lewis, S. F. (1999). Slower fatigue and faster recovery of the adductor pollicis muscle in women matched for strength with men. *Acta Physiol Scand*, *167*, 233–239.

Gallagher, P., Trappe, S., Harber, M., Creer, A., Mazzetti, S., Trappe, T., Alkner, B., & Tesch, P. (2005). Effects of 84-days of bedrest and resistance training on single muscle fibre myosin heavy chain distribution in human vastus lateralis and soleus muscles. *Acta Physiol Scand*, *185*(1), 61–69.

Gao, Y., Arfat, Y., Wang, H., & Goswami, N. (2018). Muscle Atrophy Induced by Mechanical Unloading: Mechanisms and Potential Countermeasures. *Front Physiol*, *9*(235).

Gardiner, P., Michel, R., & Iadeluca, G. (1984). Previous exercise training influences functional sprouting of rat hindlimb motoneurons in response to partial denervation. *Neuroscience Letters*, *45*(2), 123–127.

Glenmark, B., Nilsson, M., Gao, H., Gustafsson, J.-A., Dahlmna-Wright, K., & Westerblad, H. (2004). Difference in skeletal muscle function in males vs females: role of estrogen receptor-B. *American Journal of Physiology-Endocrinology and Metabolism*, *287*(6).

Gomez Pinilla, F., Ying, Z., Roy, R., Molteni, R., & Edgerton, V. R. (2002). Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. *Journal of Neurophysiology*, *88*(5), 2187–2195.

Goodwin, V. A., Richards, S. H., Taylor, R. S., Taylor, A. H., & Campbell, J. L. (2008). The effectiveness of exercise interventions for people with parkinson's disease: A systematic review and meta-analysis. *Movement Disorders*, *23*(5), 631–640.

Green, H. J., Fraser, I. G., & Ranney, D. A. (1984). Male and female differences in enzyme activities of energy metabolism in vastus lateralis muscle. *J Neurol Sci*, *65*, 323–331.

Greeves, J. P., Cable, N. T., Reilly, T., & Kingsland, C. (1999). Changes in muscle strength in women following the menopause; a longitudinal assessment of the efficacy of hormone replacement therapy. *Clin Sci (Lond)*, *97*(1), 79–84.

Greising, S. M., Baltgalvis, K. A., Kosir, A. M., Moran, A. L., Warren, G. L., & Lowe, D. A. (2011). Estradiol's beneficial effect on murine muscle function is independent of muscle activity. *J. Appl. Physiol*, *110*(1), 109–115.

Greising, S. M., Baltgalvis, K. A., Lowe, D. A., & Warren, G. L. (2009). Hormone therapy and skeletal muscle strength: a meta-analysis. *J. Gerontol A Biol Sci Med Sci.*, *64*(10), 1071–1081.

Grimby, G., Brogerg, C., Krotkiewska, I., & Krotkiewski, M. (1976). Muscle fiber composition in patients with traumatic cord lesion. *Scand J Rehabil Med.*, *8*(1), 37–42.

Gruner, J. A., & Altman, J. (1980). Swimming in the rat: Analysis of locomotor performance in comparison to stepping. *Experimental Brain Research*, *40*(4), 371–382.

Gustafsson, J. A. (2003). What pharmacologists can learn from recent advances in estrogen signalling. *Trends Pharmacol Sci*, *24*, 479–485.

Gustafsson, T., & Ulfhake, B. (2021). Sarcopenia: What Is the Origin of This Aging-Induced Disorder? *Front Genet*, *12*, 1–16.

Gyorkos, A. M., & Spitsbergen, J. M. (2014). GDNF content and NMJ morphology are altered in recruited muscles following high-speed and resistance wheel training. *Physiological*, *25*(2).

Haas, E., Bhattacharya, I., Brailoui, E., Damjanovic, M., Brailoui, G. C., Gao, X., Mueller-Guerre, L., Marjon, N. A., Gut, A., Minotti, R., & Meyer, M. R. (2009). Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity . *Circulation Res*, *104*, 288–291.

Haase, G., Dessaud, E., Garces, A., de Bovis, B., Birling M.C., Filippi, P., Schmalbruch, H., Arber, S., & deLapeyriere, O. (2002). GDNF acts through PEA3 to regulate cell body motor positioning and muscle innervation of specific motor neuron pools. *Neuron*, *35*, 893–905.

Haizlip, K. M., Harrison, B. C., & Leinwand, L. A. (2015). Sex-Based Differences in Skeletal Muscle Kinetics and Fiber-Type Composition. *American Physiological Society*, *30*(1).

Hakkinen, K. (1993). Neuromuscular fatigue and recovery in male and female athletes during heavy resistance exericse. *Int J Sports Med*, *14*, 53–59.

Hamilton, K. J., Hewitt, S. C., Arao, Y., & Korach, K. S. (2017). Estrogen Hormone Biology. *Curr Top Dev Biol.*, *125*, 109–146.

Hanamsagar, R., & Bilbo, S. (2016). Sex differences in neurodevelopmental and neurogenerative disorders: Focus on microglial function and neuroinflammation during development. *J. Steroid Biochem Mol Biol.*, *160*, 127–133.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simpson, L. C., Moffet, B., Vandlen, R. A., Koliatsos, V. E., & Rosenthal, A. (1994). GDNF- a potent survival factor for motoneurons present in peripheral-nerve and muscle. *Science*, *266*, 1062–1064.

Hepple, R. T., & Rice, C. L. (2016). Innervation and neuromuscular control in ageing skeletal muscle. *J. Physiol*, *594*, 1965–1978.

Hewitt, S. C., Deroo, B. J., Hansen, K., Collin, J., Grissom, S., Afshari, C. A., & Korach, K. S. (2003). Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. *Molecular Endocrinology*, *17*, 2070–2083.

Hewitt, S. C., Winuthayanon, W., & Korach, K. S. (2016). What's new in estrogen receptor action in the female reproductive tract. *Journal of Molecular Endocrinology*, *56*, 55–71.

Hickey, M. S., Carey, J. O., Azevedo, J. L., Houmard, J. A., Pories, W. J., Israel, R. G., & Dohm, G. L. (1995). Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans. *Am J Physiol-Endocrinol Metab*, *268*(3), E453–E457.

Hill, R. R., Robbins, N., & Fang, Z. P. (1991). Plasticity of presynaptic and postsynaptic elements of neuromuscular junctions repeatedly observed in living adult mice. *Journal of Neurocytology*, *20*(.3), 165–182.

Hochner-Celnikier, D., Manor, O., Garbi, B., & Chajek-Shaul, T. (2005). Gender gap in cerebrovascular accidents: comparison of the extent, severity, and risk factors in men and women aged 45-65. *International Journal of Fertility and Women's Medicine*, *50*(3), 122–128.

Huether, S. E., & McCance, K. L. (2019). *Understanding Pathophysiology*.

Hunter, S. K., Pereira, H. M., & Keenan, K. G. (2016). The aging neuromuscular system and motor performance. *J. Appl. Physiol*, *121*(4).

Jacob, J. M. (1998). Lumbar Motor Neuron Size and Number Is Affected by Age in Male F344 Rats. *Mech Ageing Dev*, *106*, 205–216.

Janssen, I., Shepherd, D. S., Katzmarzyk, P. T., & Roubenoff, R. (2004). The healthcare costs of sarcopenia in the United States. *J. Am Geriatr Soc*, *52*, 80–85.

Johnson, H., Hokfelt, T., & Ulfake, B. (1999). Expression of p75NTR, trkB, and trkC in nonmanipulated and axotomized motoneurons of aged rats. *Mol Brain Res*, *69*, 21–34.

Kander, M. C., Cui, Y., & Liu, Z. (2017). Gender difference in oxidative stress: a new look at the mechanisms for cardiovascular diseases. *J. Cell Mol Med*, *21*, 1024–1032.

Kawamura, Y., O'Brien, P., Okazaki, H., & Dyck, P. J. (1977). Lumbar motoneurons of man II: the number and diameter distribution of large- and intermediate-diameter cytons in "motoneuron columns'' of spinal cord." *J. Neuropathol. Exp. Neurol.*, *36*, 861–870.

Keller Peck, C. R., Feng, G., Sane, J. R., Yan, Q., Lichtman, J. W., & Snyder, W. D. (2001). Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. *The Journal of Neuroscience*, *21*(16), 6136–6146.

Kelly, M. J., & Levin, E. R. (2001). Rapid actions of plasma membrane estrogen receptors. *Trends in Endocrinology and Metabolism:TEM*, *12*, 152–156.

Kendall, B., & Eston, R. (2002). Exercise-induced muscle damage and the potential protective role of estrogen. *Sports Med.*, *32*(2), 103–123.

Khan, M. M., Wakade, C., de Sevilla, L., & Brann, D. W. (2015). Selective estrogen receptor modulators (SERMS) enhance neurogenesis and spine density following focal cerebral ischemia. *J Steroid Biochem Mol Biol*, *146*, 38–47.

Kim, M., & Kim, D. J. (2018). A novel molecular target for the prevention of osteosarcoma chemoresistance. *Int J Mol Sci*, *19*, 1–15.

Kim, S. E., & Rhee, J. H. (2015). A case of 17 alpha-hydroxylase deficiency. *Clin Expt Reprod Med*, *42*, 72–76.

Klein-Hitpass, L., Tsai, S. Y., Greene, G. L., Clark, J. H., Tsai, M. J., & O'Malley, B. W. (1989). Specific binding of estrogen receptor to the estrogen response element. *Mol Cell Biol*, *9*, 43–49.

Komi, P. V., & Karlsson, J. (1978). Skeletal muscle fibre types, enzyme activities and physical performance in young males and females. *Acta Physiol Scand*, *103*, 210–218.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Hilsson, S., & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA*, *93*, 5925–5930.

Kumar, A., Banerjee, A., Singh, D., Thaku, G., Kasarpalkar, N., Gavali, S., Gadkar, S., Madan, T., Mahale, S. D., Balasinor, N. H., & Sachdeva, G. (2018). Estradiol: A Stoid with Multiple Facets. *Thieme*.

Kumar, V., Green, S., Staub, A., & Chambon, P. (1986). Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor. *EMBO*, *5*, 2231–2236.

Kurina, L. M., Gulati, M., & Everson-Rose, S. A. (2004). The effect of menopause on grip and pinch strength: results from Chicago, Illinois, site of the Study of Women's Health Across the Nation. *Am. J. Epidemiol.*, *160*(5), 484–491.

Labandeira-Garcia, J. L., Rodriguez-Perez, A. I., Valenzuela, R., Costa-Besada, M. A., & Guerra, M. J. (2016). Menopause and Parkinson's disease. Interaction between estrogens and brain renin-angiotensin system in dopaminergic degeneration. *Front. Neuroendocrinol.*, *43*, 44–59.

Labhart, A. (2012). *Clinical Endocrinology: Theory and Practice*. Springer Science and Business Media.

Lagranha, C. J., Deschamps, A., Aponte, A., Steenbergen, C., & Murphy, E. (2010). Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circulation Res*, *106*, 1681–1691.

Larson, E. B., Wang, L., Bowen, J. D., McCormick, W. C., Teri, L., & Crane, P. (2006). Exercise is associated with reduced risk for incident dementia among persons 65 years of age and older. *Ann. Intern. Med*, *144*, 73–81.

Laurent, C. M., Green, J. M., Bishop, P. A., Sjokvist, J., Schumacker, R. E., Richardson, M. T., & Curtner-Smith, M. (2010). Effect of gender on fatigue and recovery following maximal intensity repeated sprint performance. *J Sports Med Phys Fitness*, *50*(3), 243–253.

Lee, R. H., & Heckman, C. J. (1998). Bistability in Spinal Motoneurons In Vivo: Systematic Variations in Persistent Inward Currents. *Journal of Neurophysiology*, *80*(2), 583–593.

Legerlotz, K. B., Elliott, B., Guillemin, B., & Smith, H. K. (2008). Voluntary resistance running wheel activity pattern and skeletal muscle growth in rats. *Exp. Physiol*, *93*, 754–762.

Leitner, M. L., Molliver, D. C., Osborne, P. A., Vejsada, R., Golden, J. P., Lampe, P. A., Kato, A. C., Milbrandt, J., & Johnson, E. J. (1999). Analysis of the retrograde transport of glial cell line-derived neurotrophic factor (GDNF), neurturin, and persephin suggests that in vivo signaling for the GDNF family is GRFa coreceptor-specific. *J Neurosci*, *19*, 9322–9331.

Lemoine, S., Granier, P., Tiffoche, C., Berthon, P. M., Rannou-Bekono, F., Thieulant, M. L., Carre, F., & Delamarche, P. (2002). Effect of Endurance Training On Oestrogen Receptor Alpha Transcripts in Rat Skeletal Muscle. *Acta Physiol Scand*, *174*, 283–289.

Lemoine, S., Granier, P., Tiffoche, C., Rannou-Bekono, F., Thieulant, M. L., & Delamarche, P. (2003). Estrogen receptor alpha mRNA in human skeletal muscles. *Med Sci Sports Exerc*, *35*(3), 439–443.

Levin, E. R. (2015). Extranuclear Steroid Receptors Are Essential for Steroid Hormone Actions. *Annual Review of Medicine*, *66*(66), 271–280.

Lewis, K., Livsey, L., Naughton, R. J., & Burton, K. (2020). Exercise and dementia: what should we be recommending? *Qual. Ageing Older Adults*, *21*, 109–127.

Lexell, J. (1993). Ageing and human muscle: observations from Sweden. *Can J Appl Physiol.*, *18*, 2–18.

Lexell, J. (1995). Human aging, muscle mass, and fiber type composition. *J Gerontol A Biol Sci Med Sci.*, *50(Special Issue)*, 11–16.

Li, L., Wu, W., Lin, L. F. H., Lei, M., Oppenheim, R. W., & Houenou, L. J. (1995). Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. *Proc Natl Acad Sci USA*, *92*, 9771–9775.

Li, R., & Singh, M. (2014). Sex differences in cognitive impairment and Alzheimer's disease . *Front. Neuroendocrinol.*, *35*, 385–403.

Lie, D. C., & Weis, J. (1998). GDNF expression is increased in denervated human skeletal muscle. *Neurosci Lett*, *250*, 87–90.

Liljedahl, M. E., Holm, I., Sylven, C., & Jansson, E. (1996). Different responses of skeletal muscle following sprint training in men and women. *Eur J Appl Physiol Occup Physiol*, *74*, 375–383.

Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., & Wu, Z. (2002). Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature*, *418*, 797–801.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S., & Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopamingeric neurons. *Science*, *260*, 1130–1132.

Lin, L. H., Zhang, T. J., Collins, F., & Armes, L. G. (1994). Purification and Initial Characterization of Rat B49 Glial Cell Line-Derived Neurotrophic Factor. *J Neurochem*, *63*, 758–768.

Linnamo, V., Hakkinen, K., & Komi, P. V. (1998). Neuromuscular fatigue and recovery in maximal compared to explosive strength loading. *Eur J Appl Physiol Occup Physiol*, *77*, 176–181.

Longcope, C. (1998). Androgen metabolism and the menopause. *Semin Reprod Endocrinol.*, *16*(2), 111–115.

Love, F. M., Son, Y. J., & Thompson, W. J. (2003). Activity alters muscle reinnervation and terminal sprouting by reducing the number of Schwann cell pathways that grow to link synaptic sites. *J Neurobiol*, *54*, 566–576.

Lowe, D. A., Baltgalvis, K. A., & Greising, S. M. (2010). Mechanisms behind estrogen's beneficial effect on muscle strength in females. *Exerc Sport Sci Rev.*, *38*(2), 61–67.

Luconi, M., Muratori, M., Forti, G., & Baldi, E. (1999). Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. *J. Clin Endocrinol. Metabol.*, *84*, 1670–1678.

MacNeil, L. G., Baker, S. K., Stevic, I., & Tarnopolsky, M. A. (2011). 17beta-estradiol attenuates exercise-induced neutrophil infiltration in men. *Am J Physiol Regul Integr Comp Physiol*, *300*(6), 1443–1451.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., & Chambon, P. (1995). The nuclear receptor superfamily: the second decade. *Cell*, *83*, 835–839.

Marcell, T. J. (2003). Sarcopenia: Causes, Consequences, and Preventions. *The Journals of Gerontology: Series A*, *58*(10), 911–916.

Marieb, E. (2013). *Anatomy and Physiology*. Benjamin-Cummings.

Marini, J. F., Pons, F., Leger, J., Loffreda, N., Anoal, M., Chevallay, M., Fardeau, M., & Leger, J. J. (1991). Expression of myosin heavy chain isoforms in Duchenne muscular dystrophy patients and carriers. *Neuromuscul Disord.*, *1*(6), 397–409.

McCullough, M. J., Gyorkos, A. M., & Spitsbergen, J. M. (2013). Short-term exercise increases GDNF protein levels in the spinal cord of young and old rats. *Neuroscience*, *240*, 258–268.

McCullough, M. J., Peplinski, N. G., Kinnell, K. R., & Spitsbergen, J. M. (2011). Glial cell line-derived neurotrophic factor (GDNF) protein content in rat skeletal muscle is altered by increased physical activity in vivo and in vitro. *Neuroscience*, *174*, 234–244.

Meeuwsen, I. B., Samson, M. M., & Verhaar, H. J. (2000). Evaluation of the applicability of HRT as a preservative of muscle strength in women. *Maturitas* , *36*(1), 49–61.

Meng, X., Lindahl, M., Hyvonen, M., Parvinen, M., de Rooij, D., Hess, M., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J., Westphal, H., Saarma, M., & Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, *80*(287), 1489–1493.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K., & Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature*, *382*(6586), 76–79.

Moran, A. L., Nelson, S. A., Landisch, R. M., Warren, G. L., & Lowe, D. A. (2007). Estradiol replacement reverses ovariectomy-induced muscle contractile and myosin dysfunction in mature female mice. *J. Appl. Physiol*, *102*(4), 1387–1393.

Morcuende, S., Munoz-Hernandez, R., Benitez-Temino, B., Pastor, A. M., & de la Cruz, R. R. (2013). Neuroprotective effects of NGF, BDNF, NT-3 and GDNF on axotomized extraocular motoneurons in neonatal rats. *Neuroscience*, *250*, 31–48.

Mrowczynski, W. (2019). Health benefits of endurance training: implications of the brain-derived neurotrophic factor-a systematic review. *Neural Plast*, *2019*, 5413067.

Murgia, M., Serrano, A., Calabria, E., Pallafacchina, G., & Lono, T. (2000). Ras is involved in nerve-activity-dependent regulation of muscle genes . *Nat Cell Bio*, *2*, 142–147.

Murphy, S. J., McCullough, L. D., & Smith, J. M. (2004). Stroke in the female: a role of biological sex and estrogen. *ILAR J.*, *45*, 147–159.

Naftolin, F., Ryan, K. J., & Petro, Z. (1971). Aromatization of androstenedione by limbic system tissue from human foetuses. *J. Endocrinol*, *51*(4), 795–796.

Nagano, M., & Suzuki, H. (2003). Quantitative analyses of expression of GDNF and neurotrophins during postnatal development in rat skeletal muscles . *Neuroscience Research*, *45*(4), 391–399.

Nation, D. A., Hong, S., Jak, A. J., Delano-Wood, L., Mills, P. J., Bondi, M. W., & Dimsdale, J. E. (2011). Stress, exercise, and alzheimer's disease: A neurovasulcar pathway. *Medical Hypotheses*, *76*(6), 847–854.

National Institute of Health. (n.d.). *Mental Health Benefits of Exercise and Physical Activity*.

Naya, F. J., Mercer, B., Shelton, J., Richardson, J. A., & Williams, R. S. (2000). Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *J Biol Chem*, *275*, 4545–4548.

Needham, D. M. (1926). Red and white muscle. *Physiol Rev.*, *6*, 1–27.

Nguyen, Q. T., Parsadanian, A. S., Snider, W. D., & Lichtman, J. W. (1998). Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science*, *279*(5357), 1725–1729.

Niewada, M., Kobayashi, A., Sandercock, P. A., Kaminiski, B., & Czlonkowska, A. (2005). Influence of gender on baseline features and clinical outcomes among 17,370 patients with confirmed ischemic stroke in the international stroke trial. *Neuropeidemiology*, *24*, 123–128.

Nilsson, S., Makela, S., & Treuter, E. (2001). Mechanisms of estrogen action. *Physiol Rev.*, *81*(4), 1535–1565.

Nilwik, R., Snijders, T., Leenders, M., Groen, B. B., van Kranenburg, J., Verdijk, L. B., & van Loon, L. J. (2013). The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. *Exp. Gerontol*, *48*(5), 492–498.

Nosrat, C. A., Tomac, A., Lindquist, E., Lindskog, S., Humpei, C., Stromberg, I., Ebendal, T., Hoffer, B. J., & Olson, L. (1996). Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. *Cell Tissue Res*, *286*, 191–207.

Oberbach, A., Bossenz, Y., Lehmann, S., Niebauer, J., Adams, V., Paschke, R., Schon, M. R., Bluher, M., & Punkt, K. (2006). Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. *Diabetes Care*, *29*(4), 895–900.

Oh-hashi, K., Ito, M., Tanaka, T., Hirata, Y., & Kiuchi, K. (2009). Biosynthesis, processing, and secretion of glial cell line-derived neurotrophic factor in astroglial cells. *Mol Cell Biochem*, *323*, 1–7.

O'Malley, M. W. (1967). In vitro hormonal induction of a specific protein (avidin) in chick oviduct. *Biochem*, *6*, 2546–2551.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annu Rev Neurosci*, *14*, 453–501.

Oppenheim, R. W., Houenou, L. J., Johnson, J. E., Lin, L. F., Li, L., Lo, A. C., Newsome, A. L., Prevette, D. M., & Wang, S. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature*, *373*, 344–346.

Oppenheim, R. W., Houenou, L. J., Parsadanian, A. S., Prevette, D., Snider, W. D., & Shen, L. (2000). Glial cell line-derived neurotrophic factor and developing mammalian motoneurons: regulation of programmed cell death among motoneuron subtypes. *J. Neurosci*, *20*, 5001–5011.

Palasz, E., Niewiadomski, W., Gasiorowska, A., Wysocka, A., Stepniewska, A., & Niewiadomska, G. (2019). Exercise-Induced Neuroprotection and Recovery of Motor function in Animal Models of Parkinson's Disease. *Front. Neurol.*, *10*.

Paratcha, G., Ledda, F., & Ibanez, C. F. (2003). The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell*, *113*, 867–879.

Parkash, V., Leppanen, V.-M., Virtanen, H., Jurvansuu, J. M., Bespalov, M. M., Sidorova, Y. A., Runeberg-Roos, P., Saarma, M., & Goldman, A. (2008). The structure of the glial cell line-derived neurotrophic factor-coreceptor complex: insights into RET signaling and heparin binding. *Biol Chem*, *283*, 35164–35172.

Pedemonte, M., Sandri, C., Schiaffino, S., & Minetti, C. (1999). Early decrease of IIx myosin heavy chain transcripts in duchenne muscular dystropyy. *Biochem Biophys Res Commun*, *255*(2), 466–469.

Pedram, A., Razandi, M., Evinger, A. J., Lee, E., & Levin, E. R. (2009). Estrogen inhibits ATR signaling to cell cycle checkpoints and DNA repair. *Mol Biol Cell*, *20*, 3374–3389.

Peterziel, H., Unsicker, K., & Krieglstein, K. (2001). Molecular mechanisms underlying the cooperative effect of glial cell line-derived neurotrophic factor and transforming growth factor-B on neurons. *Soc. Neurosci. Abstr.*, *27*, 364.

Phillips, S. K., Rook, K. M., Siddle, N. C., Bruce, S. A., & Woledge, R. C. (1993). Muscle weakness in women occurs at an earlier age than in men, but strength is preserved by hormone replacement therapy. *Clin Sci (Lond)*, *84*, 95–98.

Piccinini, E., Kalkkinen, N., Saarma, M., & Runeberg-Roos, P. (2013). Glial cell line-derived neurotrophic factor: Characterization of mammalian posttranslational modifications. *Ann Med*, *45*, 66–73.

Pisani, V., Panico, M. B., Terracciano, C., Bonifazi, E., Meola, G., Novelli, G., Bernardi, G., Angelini, C., & Massa, R. (2008). Preferential central nucleation of type 2 myofibers is an invariable feature of myotonic dystrophy type 2. *Muscle Nerve*, *38*(5), 1405–1411.

Poteryaev, D., Titievsky, A., Sun, Y. F., Thomas-Crusells, J., Lindahl, M., Billaud, M., & Saarma, M. (1999). GDNF triggers a novel ret-independent src kinase family-coupled signaling via a GPI-linked GDNF receptor a1. *FEBS Letters*, *463*(1), 63–66.

Prokai, L., & Simpkins, J. W. (2007). Structure-nongenomic neuroprotection relationship of estrogens and estrogen-derived compounds. *Pharmacol Ther*, *114*, 1–2.

Prossnitz, E. R., Arterburn, J. B., & Sklar, L. A. (2007). GPR30: A G protein-coupled receptor for estrogen. *Molecular and Cellular Endocrinology*, 265; 138–266; 142.

Prossnitz, E. R., & Barton, M. (2011). The G-Protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol*, *7*, 715–726.

Purves, W. K., Orians, G. H., Heller, H. C., & Sadava, D. (1998). *Life, The Science of Biology, 5th Edition* (Sinauer Associates, Ed.).

Real, C. C., Garcia, P. C., & Britto, L. R. G. (2017). Neuroinflammation Markers Involved in the Dopaminergic Damage of the 6-OHDA Parkinson's Disease Model. *J. Mol. Neurosci., 63*, 36–49.

Regan, J. C., & Partridge, L. (2013). Gender and longevity: why do men die earlier than women? Comparative and experimental evidence. *Best Pract. Res. Clin. Endocrinol. Metab., 27*, 467–479.

Rettberg, J. R., Yao, J., & Brinton, R. D. (2014). A master regulator of bioenergetic systems in the brain and body. *Front. Neuroendocrinol., 35*(1), 8–30.

Rich, M., & Lichtman, J. W. (1989). Motor nerve terminal loss from degenerating muscle fibers. *Neuron*, *3*(6), 677–688.

Rind, H. B., & von Bartheld, C. S. (2002). Anterograde axonal transport of internalized GDNF in sensory and motor neurons. *Neuroreport*, *13*, 659–664.

Rodriguez-Perez, A. I., Valenzuela, R., Villar-Cheda, B., Guerra, M. J., Lanciego, J. L., & Labandeira-Garcia, J. L. (2010). Estrogen and angiotensin interaction in the substantia nigra. Relevance to postmenopausal Parkinson's disease. *Exp. Neurol., 224*, 517–526.

Rolland, Y., Pillard, F., Klapouszczak, A., Reynish, E., Thomas, D., & Andrieu, S. (2007). Exercise program for nursing home residents with Alzheimer's disease: a 1-year randomized, controlled trial. *J. Am Geriatr Soc*, *55*, 158–165.

Ronn, L. C., Berezin, V., & Bock, E. (2000). The neural cell adhesion molecule in synaptic plasticity and ageing. *Int J Dev Neurosci*, *18*, 193–199.

Roquer, J., Campello, A. R., & Gomis, M. (2003). Sex differences in first-ever acute stroke. *Stroke*, *34*, 1581–1585.

Rowan, S. L., Purves-Smith, F. M., Solbak, N. M., & Hepple, R. T. (2011). Accumulation of severely atrophic myofibers marks the acceleration of sarcopenia in slow and fast twitch muscles. *Exp. Gerontol*, *46*, 660–669.

Roy, R. R., Hutchinson, D. L., Pierotti, D. J., Hodgson, J. A., & Edgerton, V. R. (1991). EMG patterns of rat ankle extensors and flexors during treadmill locomotion and swimming. *Journal of Applied Physiology*, *70*(6), 2522–2529.

Rudolf, R., Khan, M. M., Labeit, S., & Deschenes, M. R. (2014). Degeneration of Neuromuscular Junction in Age and Dystrophy. *Front Aging Neurosci., 6*, 99.

Russell, F. D., Koishi, K., Jiang, Y., & McLennan, I. S. (2000). Anterograde axonal transport of glial cell line-derived neurotrophic factor and its receptors in rat hypoglossal nerve. *Neuroscience, 97*, 575–580.

Ruven, C., Badea, S. R., Wong, W. M., & Wu, W. (2018). Combination treatment with exogenous GDNF and fetal spinal cord cells results in better motoneuron survival and functional recovery after avulsion injury with delayed root reimplantation. *J Neuropathol Exp Neurol*, *77*, 325–343.

Ryan, K. J., Naftolin, F., Reddy, V., Flores, F., & Petro, Z. (1972). Estrogen formation in the brain. *Am J Obstet Gynecol*, *114*(4), 454–460.

Samson, M. M., Meeuwsen, I. B., Crowe, A., Dessens, J. A., Duursma, S. A., & Verhaar, H. J. (2000). Relationships between physical performance measures, age, height and body weight in healthy adults. *Age Ageing* , *29*(3), 235–242.

Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R., Whitty, A., Pepinsky, R., & Cate, R. (1997). Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. *Proc Natl Acad Sci USA*, *94*, 6238–6243.

Saraceno, G. E., Gellini, M. J., Garcia-Segura, L. M., & Capani, F. (2018). Estradiol actives PI3k/Akt/GSK3 pathway under chronic neurodegenerative conditions triggered by perinatal asphyxia. *Front Pharmacol*, *9*, 335.

Sariola, H., & Saarma, M. (2003). Novel functions and signaling pathways for GDNF. *J Cell Sci*, *116*, 3855–3862.

Sartori, R., Romanello, V., & Sandri, M. (2021). Mechanisms of muscle atrophy and hypertrophy: implications in health and disease. *Nat Commun*, *12*(330).

Schachner, M. (1997). Neural recognition molecules and synaptic plasticity. *Curr Opin Cell Biol*, *9*, 627–634.

Scharfman, H. E., & MacLusky, N. J. (2006). Estrogen and brain-derived neurotrophic factor (BDNF) in hippocampus: complexity of steroid hormone-growth factor interactions in the adult CNS. *Front. Neuroendocrinol.*, *27*(4), 415–435.

Schinder, A. F., & Poo, M. (2000). The neurotrophin hypothesis for synaptic plasticity. *Trends in Neuroscience*, *23*(12), 639–645.

Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. *International Journal of Preventive Medicine*, *4*, 624–630.

Sherwin, C. M. (1998). Voluntary wheel running: a review and novel interpretation. *Anim. Behav.*, *56*(11).

Silvian, L., Jin, P., Carmillo, P., Boriack-Sjodin, P. A., Pelletier, C., Rushe, M., Gong, B., Sah, D., Pepinsky, B., & Rossomando, A. (2006). Artemin crystal structure reveals insights into heparan sulfate binding. *Biochemistry*, *45*, 6801–6812.

Simoneau, J. A., & Bouchard, C. (1989). Human variation in skeletal muscle fibre-type proportion and enzyme activities. *Am J Physiol*, *257*, 567–572.

Simpkins, J. W., Yang, S. H., Sarkar, S. N., & Pearce, V. (2008). Estrogen actions on mitochondria-physiological and pathological implications. *Mol. Cell. Endocrinol.*, *290*, 51–59.

Simpkins, J. W., Yi, K. D., Yang, S.-H., & Dykens, J. A. (2010). Mitochondrial mechanisms of estrogen neuroprotection. *BBA-GEN Subj*, *1800*(10), 1113–1120.

Sipila, S. (2003). Body composition and muscle performance during menopause and hormone replacement therapy. *J. Endocrinol Invest.*, *26*(9), 893–901.

Sitnick, M., Foley, A. M., Brown, M., & Spangenburg, E. E. (2006). Ovariectomy prevents the recovery of atrophied gastrocnemius skeletal muscle mass. *J. Appl. Physiol*, *100*(1), 286–293.

Skelton, D. A., Phillips, S. K., Bruce, S. A., Naylor, C. H., & Woledge, R. C. (1999). Hormone replacement therapy increases isometric muscle strength of adductor pollicis in post-menopausal women. *Clin Sci (Lond)*, *96*, 357–364.

Soltysik, K., & Czekaj, P. (2013). Membrane estrogen receptors - is it an alternative way of estrogen action? *Journal of Physiology and Pharmacology*, *64*(2), 129–142.

Springer, J. E., Mu, X., Bergmann, L. W., & Trojanowski, J. Q. (1994). Expression of Gdnf messenger-Rna in rat and human nervous-tissue. *Exp Neurol*, *127*, 167–170.

Springer, J. E., Seeburger, J. L., Jin, H. E., Gabrea, A., Blankenhorn, E. P., & Bergman, L. W. (1995). CDNA sequence and differential mRNA regulation of two forms of glial cell line-derived neurotrophic factor in Schwann cells and rat skeletal muscle. *Exp. Neurol.*, *131*, 47–52.

Stanga, S., Boido, M., & Kienlen-Campard, P. (2020). How to Build and to Protect the Neuromuscular Junction: The Role of the Glial Cell Line-Derived Neurotrophic Factor. *Int J Mol Sci*, *22*, 136. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7794999/

Staron, R. S., Hagerman, F. C., Hikida, R. S., Murray, T. F., Hostler, D. P., Crill, M. T., Ragg, K. E., & Kumika, T. (2000). Fiber Tyle Composition of the Vastus Lateralis Muscle of Young Men and Women. *Journal of Histochemistry and Cytochemistry*, *48*(5), 623–629.

Stephens, J. A., & Taylor, A. (1972). Fatigue of maintained voluntary muscle contraction in man. *Journal of Physiology*, *220*(1), 1–18.

Stifani, N. (2014). Motor neurons and the generation of spinal motor neuron diversity. *Frontiers in Cellular Neuroscience*, *8*.

Sullivan, S. M., & Pittman, R. N. (1987). Relationship between mitochondrial volume density and capillarity in hamster muscles. *Am J Physiol*, *252*, H149–H155.

Sung, Y.-H., Kim, S.-C., Hong, H.-P., Park, C.-Y., Shin, M.-S., Kim, C.-J., Seo, J.-H., Kim, D.-Y., Kim, D.-J., & Cho, H.-J. (2012). Treadmill exercise ameliorates dopaminergic neuronal

loss through suppressing microglial activation in Parkinson's disease mice. *Life Sciences*, *91*(25–26), 1309–1316.

Suter-Crazzolara, C., & Unsicker, K. (1994). GDNF is Expressed in Two Forms in Many Tissues outside the CNS. *Neuroreport*, *5*(18), 2486–2488.

Suzuki, H., Hase, A., Miyata, Y., Arahata, K., & Akazawa, C. (1998). Prominent expression of glial cell line-derived neurotrophic factor in human skeletal muscle. *J. Comp Neurol*, *402*(3), 303–312.

Swift, D. L., Johannsen, N. M., Lavie, C. J., Earnest, C. P., & Church, T. S. (2013). The Role of Exercise and Physical Activity in Weight Loss and Maintenance. *Prog Cardiovasc Dis*, *56*(4), 441–447.

Tajsharghi, H. (2008). Thick and thin filament gene mutations in striated muscle diseases. *Int J Mol Sci*, *9*(7), 1259–1275.

Talbot, J., & Maves, L. (2016). Skeletal muscle fiber type: using insights from muscle development biology to dissect targets for susceptibility and resistance to muscle disease. *Wiley Interdiscip Rev Dev Biol*, *5*(4), 518–534.

Tang, M. X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., & Gurland, B. (1996). Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. *Lancet*, *348*, 429–432.

Tanner, C. J., Barakat, H. A., Dohm, G. L., Pories, W. J., MacDonald, K. G., Cunningham, P. R., Swanson, M. S., & Houmard, J. A. (2002). Muscle fiber type is associated with obesity and weight loss. *Am J Physiol-Endocrinol Metab*, *282*(6), E1191–E1196.

Tansey, M. G., Baloh, R. H., Milbrandt, J., & Johnson Jr., E. M. (2000). GFRa-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron*, *25*(3), 611–623.

Taylor, A. M., Blurton-Jones, M., Rhee, S. W., Cribbs, D. H., Cotman, C. W., & Jeon, N. L. (2005). A microfluidic culture platform for CNS axonal injury, regeneration and transport. *Nat. Methods*, *2*, 599–605.

Tiidus, P. M. (2001). Oestrogen and sex influence on muscle damage and inflammation: evidence from animal models. *Curr Opin Clin Nutr Metab Care*, *4*(6), 509–513.

Tomanek, R. J., & Lung, D. D. (1974). Degeneration of different types of skeletal muscle fibres. II. Immobilization. *J. Anat.*, *118*, 531–541.

Tomlinson, B. E., & Irving, D. (1977). The numbers of limb motor neurons in the human lumbosacral cord throughout life. *J. Neuro Sci*, *34*, 213–219.

Trappe, S. W., Costill, D. L., Vukovich, M. D., Jones, J., & Melham, T. (1996). Aging among elite distance runners. *J Appl Physiol*, *80*, 285–290.

Trupp, M., Belluardo, N., Funakoshi, H., & Ibanez, C. F. (1997). Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *J Neurosci*, *17*, 3554–3567.

Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Arenas, E., & Ibanez, C. E. (1995). Peripheral expression and biological-activities of Gdnf, a new neurotrophic factor for avian and mammalian peripheral neurons. *J. Cell Biol*, *130*, 137–148.

Trupp, M., Scott, R., Whittemore, S. R., & Ibanez, C. F. (1999). Ret-dependent and -independent mechanisms of GDNF signaling in neuronal cells. *J. Biol Chem*, *274*, 208885–208894.

Valdez, G., Tapia, J. C., Kang, H., Clemenson, G. D., Gage, F. H., Lichtman, J. W., & Sanes, J. R. (2010). Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. *PNAS*, *107*(33), 14863–14868.

Vandenput, L., & Ohlsson, C. (2009). Estrogens as regulators of bone health in men. *Nat Rev Endocrinol*, *5*, 437–443.

Veiga, S., Melcangi, R. C., Doncarlos, L. L., Garcia-Segura, L. M., & Azcoitia, I. (2004). Sex hormones and brain aging. *Exp. Gerontol*, *39*, 1623–1631.

Vianney, J.-M., Miller, D. A., & Spitsbergen, J. M. (2014). Effects of acetylcholine and electrical stimulation on glial cell line-derived neurotrophic factor production in skeletal muscle cells. *Brain Res*, *1588*, 47–54.

Vihola, A., Bassez, G., Meola, G., Zhang, S., Haapasalo, H., & Paetau, A. (2003). Histopathological differences of myotonic dystrophy type 1 (DM1) and PROM/DM2. *Neurology*, *60*(11), 1854–1857.

Vina, J., Sastre, J., Pallardo, F. V., Gambini, J., & Borras, C. (2006). Role of mitochondrial oxidative stress to explain the different longevity between genders: protective effect of estrogens. *Free Radic Res*, *40*(12), 1359–1365.

Wang, X., & Poo, M. (1997). Potentiation of developing synapses by postsynaptic release of neurotrophin-4 . *Neuron*, *19*(4), 825–835.

Watsona, C. S., Norfleet, A. M., Pappas, T. C., & Gametchu, B. (1999). Rapid actions of estrogens in GH 3/B6 pituitary tumor cells via a plasma membrane version of estrogen receptor-a. *Steroids*, *64*, 5–13.

Webster, C., Silberstein, L., Hays, A. P., & Blau, H. M. (1988). Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. *Cell*, *52*(4), 503–513.

Wehrwein, E. A., Roskelley, E. M., & Spitsbergen, J. M. (2002). GDNF is regulated in an activity-dependent manner in rat skeletal muscle. *Muscle Nerve*, *26*, 206–211.

Weihua, Z., Andersson, S., Cheng, G., Simpson, E. R., Warner, M., & Gustafsson, J. A. (2003). Update on estrogen signaling. *FEBS Lett*, *546*, 17–24.

Whitehead, S. A., & Nussey, S. (2001). *Endocrinology: an integrated approach*. Oxford: BIOS: Taylor and Francis .

Widrick, J. J., Stelzer, J. E., Shoepe, T. C., & Garner, D. P. (2002). Functional properties of human muscle fibers after short-term resistance exercise training. *Am J Physiol*, *283*, R408–R416.

Wiik, A., Ekman, M., Johansson, O., Jansson, E., & Esbjornsson, M. (2009). Expression of both oestrogen receptor alpha and beta in human skeletal muscle tissue. *Histochem Cell Biol.*, *131*(2), 181–189.

Wiik, A., Glenmark, B., Ekman, M., Esbjornsson-Liljedahl, M., Johansson, O., Bodin, K., Enmark, E., & Jansson, E. (2003). Oestrogen receptor Beta is expressed in adult human skeletal muscle both at the mRNA and protein level. *Acta Physiol Scand*, *179*, 381–387.

Wiik, A., Gustafsson, T., Esbjornsson, M., Johannson, O., Ekman, M., Sundberg, C. J., & Jansson, E. (2005). Expression of Oestrogen Receptor Alpha and Beta is higher in Skeletal Muscle of Highly Endurance-Trained Than of Moderately Active Men. *Acta. Physiol. Scand.*, *184*, 105–112.

Wise, P. M., Dubal, D. B., Wilson, M. E., Rau, S. W., Bottner, M., & Rosewell, K. L. (2001). Estradiol is a protective factor in the adult and aging brain: understanding of mechanisms derived from in vivo and in vitro studies. *Brain Res Brain Res Rev.*, *37*(1–3), 313–319.

Wu, C., Cui, B., He, L., Chen, L., & Mobley, W. C. (2009). The coming of age of axonal neurotrophin signaling endosomes . *J Proteomics*, *72*, 46–55.

Wu, H., Kanatous, S. B., Thurmond, F. A., Gallardo, T., & Isotani, E. (2002). Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science*, *296*, 349–352.

Yamamoto, M., Sobue, G., Yamamoto, K., Terao, S., & Mitsuma, T. (1996). Expression of glial cell line-derived growth factor mRNA in the spinal cord and muscle in amyotrophic lateral sclerosis. *Neurosci Lett*, *204*, 117–120.

Yan, Q., Matheson, C., & Lopez, O. T. (1995). In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature*, *373*, 341–344.

Yu, T., Scully, S., Yu, Y., Fox, G. M., Jing, S., & Zhou, R. (1998). Expression of GDNF family receptor components during development: implications in the mechanisms of interaction. *J Neurosci*, *18*, 4684–4696.

Yuan, L. J., Wang, X. W., Wang, H. T., Zhang, M., Sun, J. W., & Chen, W. F. (2019). G protein-coupled estrogen receptor is involved in the neuroprotective effect of IGF-1 against MPTP/MPP(+)-induced dopaminergic neuronal injury. *J Steroid Biochem Mol Biol*, *192*, 105384.

Zahavi, E. E., Ionescu, A., Gluska, S., Gradus, T., Ben-Yaakov, K., & Perlson, E. (2015). A compartmentalized microfluidic neuromuscular co-culture system reveals spatial aspects of GDNF functions. *J Cell Sci*, *128*, 1241–1252.

Zahavi, E. E., Maimon, R., & Perlson, E. (2017). Spatial-specific functions in retrograde neuronal signaling . *Traffic*, *18*, 415–424.

Zarate, S., Stevnsner, T., & Gredilla, R. (2017). Role of Estrogen and Other Sex Hormones in Brain Aging Neuroprotection and DNA repair. *Front Aging Neurosci.*, *9*, 430.

Zhao, C., Veltri, K., Li, S., Bain, J. R., & Fahnestock, M. (2004). NGF, BDNF, NT-3, and GDNF mRNA expression in rat skeletal muscle following denervation and sensory protection. *J Neurotrauma*, *21*, 1468–1478.

Zhu, B., Pennack, J. A., McQuilton, P., Forero, M. G., Mizuguchi, K., Sutcliffe, B., Gu, C. J., Fenton, J. C., & Hidaldo, A. (2008). Drosophila neurotrophins reveal a common mechanism for nervous system formation. *PLoS Biol*, *6*, 2476–2495.

Zwick, M., Teng, L., Mu, X., Springer, J. E., & Davis, B. M. (2001). Overexpression of GDNF induces and maintains hyperinnervation of muscle fibers and multiple endplate formation. *Experimental Neurology*, *171*(2), 342–350.

APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

Date: May 17, 2022

To: John Spitsbergen, Principal Investigator

From: Cindy Linn, Chair

Re: IACUC Protocol Number 22-05-02

Your protocol titled "The Effect of Age and Exercise and Gender on Target-Derived Neurotrophic Factor Expression and Innervation of Heart, Vascular Tissues and Skeletal Muscle of Rat." has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: **May 16, 2023**

251 W. Walwood Hall, Kalamazoo, MI 49008-5456
PHONE: (269) 387-8293, FAX: (269) 387-8276