4-2013

Exercise Alters Glial Cell Line-Derived Neurotrophic Factor (GDNF) Protein Content in the Spinal Cord

Monica Janine McCullough
Western Michigan University, monicajmccullough@gmail.com

Follow this and additional works at: https://scholarworks.wmich.edu/dissertations

Part of the Exercise Physiology Commons, and the Molecular and Cellular Neuroscience Commons

Recommended Citation
https://scholarworks.wmich.edu/dissertations/151
EXERCISE ALTERS GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) PROTEIN CONTENT IN THE SPINAL CORD

by

Monica J. McCullough

A dissertation submitted to the Graduate College in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Biological Sciences
Western Michigan University
April 2013

Doctoral Committee:

John M. Spitsbergen, Ph.D., Chair
Christine Byrd-Jacobs, Ph.D.
Cindy Linn, Ph.D.
Damon Miller, Ph.D.
EXERCISE ALTERS GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) PROTEIN CONTENT IN THE SPINAL CORD

Monica Janine McCullough, Ph.D.
Western Michigan University, 2013

Neurotrophic factors may play a role in exercise-induced neuroprotective effects; however, it is not known if exercise mediates changes in glial cell line-derived neurotrophic factor (GDNF) protein levels in the spinal cord. The aim of the studies described herein was to determine if exercise alters GDNF protein expression in the lumbar spinal cord of healthy and diseased animals. The lumbar spinal cord was analyzed from adult rats aged 6-, 12-, 18- and 24-months, and from transgenic amyotrophic lateral sclerosis (ALS) mice and wild-type mice aged 3 months. Animals had undergone either forced wheel running, voluntary wheel running or swimming for either 2- or 4-weeks or 6 months in duration. GDNF protein was quantified via enzyme-linked immunosorbent assay and Western blot. Immunohistochemical analysis localized GDNF in choline acetyltransferase (ChAT)-positive motor neurons and cell body areas were measured. Results indicate that short-term exercise (2 weeks) increases GDNF protein content in the rodent lumbar spinal cord. Chronic exercise (4 weeks to 6 months) has no effect on GDNF protein content in the rodent lumbar spinal cord. All exercise protocols increased the size of ChAT-positive motor neuron cell body areas of healthy, non-diseased animals. Treatment with neutralizing antibodies for GDNF in the transgenic ALS animals blocked the beneficial effects of exercise on motor neurons. These results suggest that short-term exercise elicits an increase in neurotrophic factors
via an activity-dependent relationship that occurs with GDNF protein expression in spinal cord. Understanding how neurotrophic factors are regulated by physical activity is crucial for maintaining a healthy motor nervous system and for developing therapies for individuals with compromised motor nervous systems, e.g. due to aging or disease.
ACKNOWLEDGMENTS

I would like to begin by acknowledging the excellent guidance and mentoring that I have received during these past years from Dr. John Spitsbergen. I could not have asked for a better mentor and am grateful for all the opportunities that I've had because of him.

I would also like to thank my committee members, Dr. Christine Byrd-Jacobs, Dr. Cindy Linn and Dr. Damon Miller, who have taken the time out of their busy schedules to help me succeed as a teacher and a researcher.

I would like to thank all of my family and friends for their constant support and for always being positive. Most importantly, I would like to thank my wonderful husband, Matt, for his continued support during this process, and for enduring all my piles of articles in every room of our house. He has been so supportive and patient with me, and I could not have asked for a better husband and friend.

Monica Janine McCullough
TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................................................................................. ii

LIST OF TABLES .......................................................................................................................... xi

LIST OF FIGURES ....................................................................................................................... xii

LIST OF ABBREVIATIONS ........................................................................................................ xiv

CHAPTER

I. INTRODUCTION .................................................................................................................. 1

   Spinal cord ............................................................................................................................ 1

   Motor neurons ....................................................................................................................... 4

   Development of motor neurons ........................................................................................... 5

   Neurotrophic factors ........................................................................................................... 6

   GDNF .................................................................................................................................... 7

   GDNF signaling .................................................................................................................... 8

   GDNF distribution ................................................................................................................. 10

   GDNF is essential for motor neurons .................................................................................. 11

   Exercise alters neurotrophic factors in the motor nervous system ...................................... 14

II. EXPERIMENTAL DESIGN ...................................................................................................'18
## Table of Contents - Continued

### CHAPTER

Specific aims ........................................................................................................ 18

Rationale and development .............................................................................. 20

- Murine animals as model organisms for studying neurotrophic factors 20
- Optimal time for euthanasia after the last bout of exercise 22
- Duration of exercise ............................................................................... 28
- Spinal cord selection ............................................................................... 32

- GDNF protein expression in the spinal cord following exercise ........ 35
- Differences between Fischer 344 and Sprague-Dawley rats ............... 40
- Mouse studies .................................................................................. 44

### III. SHORT-TERM EXERCISE INCREASES GDNF PROTEIN LEVELS IN THE SPINAL CORD OF YOUNG AND OLD RATS 47

- Introduction ......................................................................................... 47
- Experimental procedures ...................................................................... 49
- Subjects ............................................................................................ 49
- Training protocol ............................................................................... 49
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue processing</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>GDNF protein quantification</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Western blot</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Immunohistochemistry</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Measurement of motor neuron cell body size</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Statistical analysis</td>
<td>54</td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Short-term exercise increases GDNF protein in the lumbar spinal cord of 6-month-old rats</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Short-term exercise increases GDNF protein in the spinal cord of 24-month-old rats</td>
<td>61</td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Motor neuron size increases at the same time as GDNF levels following short term exercise</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Low-intensity exercise is a potent stimulus for enhancing neurotrophic factor levels</td>
<td>68</td>
</tr>
</tbody>
</table>
CHAPTER

Punctate immunoreactivity for GDNF is altered with exercise and age.......................................................... 69

Molecular weight of GDNF changes with age....................... 70

GDNF transport following exercise.................................... 71

Exercise stressors................................................................. 72

Conclusion........................................................................... 73

IV. CHRONIC EXERCISE DOES NOT ALTER GDNF PROTEIN CONTENT IN ADULT RAT SPINAL CORD..................................... 74

Introduction...................................................................................... 74

Materials and methods..................................................................... 77

Experimental design...................................................................... 77

Tissue processing........................................................................... 78

GDNF protein quantification...................................................... 78

Western blot............................................................................. 79

Immunohistochemistry............................................................ 80

Measurement of motor neuron cell body size......................... 81
Table of Contents - Continued

CHAPTER

Statistical analysis................................................................. 81

Results......................................................................................... 82

Four weeks of voluntary running.......................................... 82

Six months of voluntary running.......................................... 88

Discussion..................................................................................... 92

Aging increases molecular weight of GDNF in the spinal cord................................................................. 93

Chronic exercise does not increase neurotrophic factor protein levels.............................................................. 93

Hypertrophy of motor neurons with chronic exercise..... 96

Conclusion..................................................................................... 97

V. INVOLUNTARY EXERCISE INCREASES GDNF PROTEIN CONTENT IN THE LUMBAR SPINAL CORD OF WILD TYPE MICE................................................................. 98

Introduction..................................................................................... 98

Materials and methods.............................................................. 99

Training protocol........................................................................ 99

Tissue processing........................................................................ 100

GDNF protein quantification...................................................... 101
### Table of Contents - Continued

**CHAPTER**

Western blot

Immunohistochemistry

Motor neuron cell body area analysis

Statistical analysis

Results

2 weeks of low-intensity forced running increases GDNF protein content in the mouse spinal cord

Discussion

Expression of GDNF protein increases with 2 weeks of exercise

Motor neuron size increases at the same time as GDNF levels following short term exercise

Conclusion

**VI. GDNF PROTEIN IS ALTERED IN A TRANSGENIC MOUSE MODEL OF RAPID MOTOR NERVOUS SYSTEM DEGENERATION**

Introduction

Materials and methods

Subjects
Table of Contents - Continued

CHAPTER

Low-intensity training protocol........................................... 118

Blocking of circulating GDNF............................................. 119

Tissue processing.................................................................. 119

GDNF protein quantification............................................... 120

Western blot......................................................................... 121

Immunohistochemistry and histological examination....... 122

Statistical analysis............................................................... 123

Results.............................................................................................. 124

Behavioral analysis.............................................................. 124

GDNF protein content.......................................................... 125

Western blot analysis........................................................... 126

Immunohistochemistry and analysis................................... 129

Discussion........................................................................................ 135

Long-term exercise does not alter GDNF protein content in the spinal cord of ALS mice......................... 136

Increased molecular weight of GDNF occur at the same time as motor neuron disruption....................... 137
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GDNF treatment abolishes the neuroprotective effects of exercise</td>
<td>139</td>
</tr>
<tr>
<td>Enhancing GDNF signaling in compromised motor neurons</td>
<td>140</td>
</tr>
<tr>
<td>Conclusion</td>
<td>142</td>
</tr>
<tr>
<td>VII. DISCUSSION AND CONCLUSIONS</td>
<td>143</td>
</tr>
<tr>
<td>Control levels of GDNF from rodents undergoing senescence</td>
<td>144</td>
</tr>
<tr>
<td>GDNF protein content is not modulated by all forms of exercise</td>
<td>147</td>
</tr>
<tr>
<td>Conclusion</td>
<td>149</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>151</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>184</td>
</tr>
<tr>
<td>Institutional Animal Care and Use Committee (IACUC) Forms</td>
<td>184</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1. Exercise parameters and GDNF protein expression following 2 weeks of exercise.............................................................. 56

2. Densitometry analysis of Western blot of ALS mice................................. 129
LIST OF FIGURES

1. GDNF protein content is increased in the extensor hallucis longus following 2 weeks of exercise and 72 hours of rest............................................. 26

2. GDNF protein content in the diaphragm does not change following 2 weeks of exercise.................................................................................. 27

3. GDNF protein content is increased in the extensor hallucis longus following 2 weeks of exercise................................................................. 30

4. GDNF protein content in the diaphragm is increased following 6 weeks of exercise......................................................................................... 31

5. Motor neuron measurements collected from Lamina XI of the lumbar spinal cord...................................................................................... 34

6. GDNF protein content in the spinal cord is not altered with 4 weeks of exercise.......................................................................................... 38

7. GDNF is localized in ChAT-positive motor neurons in the lumbar spinal cord............................................................................................ 39

8. Different rat strains do not affect GDNF protein content in the spinal cord................................................................................................. 41

9. GDNF protein content is not altered in the spinal cord following 6 weeks of exercise.................................................................................. 43

10. GDNF is expressed within ChAT-positive cells in the mouse lumbar spinal cord......................................................................................... 46

11. GDNF protein expression is increased in the lumbar spinal cord following 2 weeks of exercise in 6-month-old rats........................................ 59

12. GDNF protein localization within ChAT-positive motor neurons in the lumbar spinal cord in 6-month-old rats.......................................... 60
List of Figures - Continued

13. Histogram analysis of 6-month-old ChAT-positive motor neuron cell body area........................................................................................................ 60
14. GDNF protein expression is increased in the lumbar spinal cord following 2 weeks of exercise in 24-month-old rats.............................................. 63
15. GDNF protein localization within ChAT-positive motor neurons in the lumbar spinal cord in 24-month-old rats .................................................. 64
16. Histogram analysis of 24-month-old ChAT-positive motor neuron cell body area........................................................................................................ 66
17. 4 weeks of exercise does not alter GDNF protein content in the adult spinal cord.................................................................................................. 83
18. Negative correlation between GDNF protein content in the spinal cord versus total distance run by each animal......................................................... 85
19. GDNF immunoreactivity is localized to motor neurons in 12-month-old control and exercised animals ........................................................................ 86
20. Histogram analysis of ChAT-positive motor neurons of control and exercised animals .................................................................................................. 87
21. 6 months of voluntary running does not alter GDNF protein content in the rat spinal cord.......................................................................................... 89
22. GDNF in the spinal cord of 18-month-old control and exercised rats........ 90
23. Histogram analysis of the 18-month-old motor neurons............................... 91
24. GDNF protein content is increased in the mouse spinal cord following 2 weeks of involuntary running ........................................................................ 105
List of Figures - Continued

25. Western blot of GDNF in mouse spinal cord.............................................. 106
26. Exercise increases GDNF immunolabeling in ChAT-positive cells............ 108
27. Body weights of the ALS mice................................................................ 125
28. GDNF protein content in lumbar spinal cord of ALS mice...................... 127
29. Western blot analysis of the lumbar spinal cord of ALS mice............... 128
30. Immunohistochemistry with no primary antibodies in the ALS mouse spinal cord........................................................................................................ 131
31. Immunohistochemistry in the lumbar spinal cord of ALS mice............. 132
32. Detection of neutralizing antibodies in ALS spinal cords....................... 133
33. Cell counts of ChAT-positive motor neurons......................................... 134
LIST OF ABBREVIATIONS

ALS - Amyotrophic lateral sclerosis
ARTN- Artemin
BDNF - Brain derived neurotrophic factor
CNTF - Ciliary neurotrophic factor
ELISA - Enzyme-linked immunosorbent assay
GDNF - Glial cell line-derived neurotrophic factor
GFRα-1 - Glial cell line-derived neurotrophic family receptor alpha 1
LIF - Leukemia inhibitory factor
NGF - Nerve growth factor
NT-3 - Neurotrophin-3
NT-4 - Neurotrophin-4
NTRN - Neurturin
PBS - Phosphate buffered saline
RET - Rearranged after transfection
PSPN - Persephin
CHAPTER I

INTRODUCTION

The broad goal of our laboratory is to better understand what role neurotrophic factors play in the communication between neurons and the target tissues they innervate. The aim of the current study herein is to determine the role that physical activity plays in altering glial cell line-derived neurotrophic factor (GDNF) protein expression and what subsequent changes occur in the motor nervous system.

Spinal cord

The spinal cord is the main pathway for transmission of information between the brain, peripheral nervous system and muscles. It controls the voluntary muscles of the limbs and trunk, and it receives sensory information from these regions controlling most of the viscera and blood vessels of the thorax, abdomen and pelvis. The spinal cord is a continuous cylinder of nervous tissue that is enclosed by a tube of cerebrospinal fluid, where the fluid is incased by the spinal meninges between the pia and arachnoid mater. The human spinal cord is on average 45cm in length for males and 42-43cm in length for females, and is divided by different segments. In humans it consists of 31 segments, whereas the rodent spinal cord consists of 34 segments. Rodent spinal cords contain 8 cervical segments (C1-C8), 13 thoracic segments (T1-T13), 6 lumbar segments (L1-L6),
4 sacral segments (S1-S4) and 3 coccygeal segments (Co1-Co3) whereas the human spinal cord contains 8 cervical segments (C1-C8), 12 thoracic segments (T1-T12), 5 lumbar segments (L1-L5), 5 sacral segments (S1-S5) and 1 coccygeal segment (Co1). Most mammals possess a cervical (brachial) enlargement that extends from C5-T1 and a lumbosacral enlargement that extends from L2-S2 in humans and L2-L6 in rodents. The caudal end of the spinal cord narrows to form the conus medullaris, where the spinal nerves of the lumbar, sacral and coccygeal nerves form a bundle called the cauda equina, as it resembles a horse's tail. The dorsal and ventral sides of the spinal cord contain rootlets that bundle together to form the dorsal root and the ventral root, respectively. Humans have 6-8 dorsal and ventral rootlets on each side and rodents have 15 dorsal and ventral rootlets on each side. The dorsal root contains sensory (afferent) fibers from the skin, subcutaneous and deep tissues, and viscera along with a few efferent fibers. Each dorsal root contains an ovoid swelling known as the dorsal root ganglion that contains the cell bodies of afferent neurons. The ventral root contains motor (somatic efferent) fibers and presynaptic autonomic fibers (Watson et al., 2009).

The spinal cord consists of white matter and gray matter. The white matter of the spinal cord is organized mainly into longitudinally myelinated running axons and glial cells. The white matter is divided into three columns by the gray matter, the dorsal, lateral and ventral columns. The white matter surrounds the gray matter except for where the dorsal horn touches the margin of the spinal cord. The ventral surface of the spinal cord contains a deep longitudinal fissure in the midline, termed the ventral median
fissure, that extends about one-third of the rostrocaudal length. The ventral median fissure contains blood vessels that reach the center of the gray matter. The dorsal column of the white matter is composed primarily of the central processes and synapses onto interneurons of the dorsal root ganglion cells that form the main pathways for conveying skin sensation and proprioception from the limbs and trunk. The dorsal column is largest in the rostral cervical spinal cord and smallest in the sacral segments because fibers are added in a caudorostral fashion. The largest propriospinal pathways connect the cervical and lumbosacral enlargements to coordinate limb movements (Watson et al., 2009).

The dorsally projecting arms of the butterfly-shaped gray matter are the dorsal horns, and the ventrally projecting arms are the ventral horns. The intermediate gray matter contains interneurons that connect the dorsal and ventral horns and contains the central canal which is where the cerebrospinal fluid is housed. The thoracic and upper lumbar segments of the spinal cord contain an additional area of the intermediate gray matter (intermediolateral horn) that contains cells originating from the autonomic nervous system, such as the preganglionic sympathetic neurons. The gray matter is composed of mostly multipolar neuronal cell bodies that vary in size, dendrites, axons and glial cells. The gray matter is divided into 10 regions, Laminas I through X, that are based on their cytoarchitecture. Lamina IX is located at the base of the ventral horn and contains the alpha (α) motor neurons that supply the extrafusal skeletal muscle fibers and the gamma (γ) motor neurons that supply the intrafusal fibers in muscle spindles.
**Motor neurons**

Motor neurons represent <0.0005% of all neurons in the human central nervous system. They have relatively large cell bodies and their target tissues of skeletal muscles are easily identifiable for physiological studies. Due to these properties, motor neurons have provided physiologists with much of the information about the basic physiological functions of neuronal populations (Kernell, 2004).

There are two functional groups of motor neurons, the visceral (autonomic) motor neurons that innervate smooth muscle and glands, and the somatic motor neurons that innervate skeletal muscle. As mentioned earlier, autonomic motor neurons are found in the intermediolateral horn of the spinal cord and are divided into sympathetic motor neurons and parasympathetic motor neurons. These motor neurons range from intermediate- to small-sized perikarya because they have less metabolic demands compared to α-motor neurons. Sympathetic preganglionic motor neurons are found in the thoracic and upper lumbar segments of the spinal cord and innervate the sweat glands and vasodilators to smooth muscle of the blood vessels. Parasympathetic preganglionic motor neurons are located in the sacral spinal cord segments and innervate to the pelvic viscera and hindgut. Somatic motor neurons are found in the ventral horn of the gray matter and the majority of these are α-motor neurons that innervate the extrafusal skeletal muscle fibers. These multipolar neurons are among the largest neurons in the nervous system due to their high metabolic demands. γ-motor neurons represent a smaller proportion of motor neurons and innervate the intrafusal fibers within muscle spindles.
Approximately 30% of the total number of neurons in Lamina IX are γ-motor neurons (Burke et al., 1977). A third group, the beta (β) motor neurons, represents a very small proportion of motor neurons and are found predominantly innervating distal muscles of the limbs that send axon branches within the muscle to extrafusal and intrafusal muscle fibers (Kernell, 2004).

**Development of motor neurons**

Initially, neurons of both the peripheral and central nervous systems are overproduced and subsequently degenerate until half or less of the original members survive. Soon after the motor neurons innervate their target skeletal muscle, approximately 50% of motor neurons begin to die and the survivors continue on their path to differentiation. In humans, most motor neuron degeneration occurs between 12 and 16 weeks post conception (Forger and Breedlove, 1987). This naturally occurring cell death process for motor neurons is dependent on interactions with the periphery. The survival of embryonic motor neurons is critically dependent on the presence of skeletal muscle tissues (Hamburger, 1975). The addition of embryonic skeletal muscle extracts during the period of naturally occurring cell death decreases the number of degenerating motor neurons and increases their survival. Neurons degenerate and die during normal embryonic development due to competition of limited amounts of target-derived trophic factors (Oppenheim, 1989) and this event appears to be the main reason for death of motor neurons. Motor neurons themselves produce growth and trophic factors that
mediate interactions between motor neurons and Schwann cells (Carroll et al., 1997) and between motor neurons and muscle fibers (Fischbach and Rosen, 1997).

**Neurotrophic factors**

Neurotrophic factors are small proteins that regulate the development, survival, function and plasticity of neurons in both the central and peripheral nervous systems (Henderson, 1996; Gould and Oppenheim, 2006). Most neurotrophic factors are target-derived and are taken up at the synaptic terminals where they reach the neuronal cell body through a retrograde axonal transport system (Taniuchi et al., 1988). Motor neurons are also known to express neurotrophic factors that can undergo anterograde transport to target tissues in the periphery. Receptors of neurotrophic factors are also detected in motor neurons of the lumbar spinal cord, but their expression and that of the ligand are not confined only to these cellular populations (Yan et al., 1993). When neurotrophic factors reach the neuronal cell bodies, they exert their neuroprotective effects, such as enhancing growth and survival (Purves et al., 1988).

Neurotrophic factors can be classified into several subgroups, including the neurotrophin family, which consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), the neurocytokine family, which includes ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and interleukins, and the glial cell line-derived neurotrophic factor (GDNF) family, which includes GDNF, neurturin (NTRN), artemin (ARTN), and persephin (PSPN).
GDNF

GDNF was isolated and purified from B49 midbrain cell cultures of rostral mesencephalic tegmenta of embryonic Sprague-Dawley rats and was discovered for its ability to promote dopamine uptake (Lin et al., 1993). GDNF is a distant member of the transforming growth factor-β (TGF-β) superfamily, as GDNF contains the seven conserved cysteine residues in the same relative spacing as all other members; however, the mature GDNF protein shares less than 20% homology of the amino acid sequence of the superfamily (Lin et al., 1993). Ligands of the TGF-β superfamily can prevent developmental neuronal death, promote the survival of axotomized motor neurons (Iwasaki et al., 1997) and mature motor neurons (Oorschot, 1998), yet they do not provide trophic activity for neurons (Sauer and Bjorklund, 1995; Maxwell et al., 1996; Ho et al., 2000). GDNF is a secretory protein that is a disulfide-bonded homodimer where it has been shown to have a molecular mass between 32 to 42kDa on non-reducing sodium dodecyl sulfate (SDS) gels. GDNF has a molecular weight of 18 to 22kDa on reduced SDS gels and is considered to be the monomeric mature, glycosylated form. The recombinant unglycosylated monomer of GDNF (also referred to as recombinant human GDNF (rhGDNF)) has an apparent molecular weight of 15kDa and the unglycosylated monomer does not appear to affect the bioactivity of GDNF (Lin et al., 1993, 1994). The amino acid sequence of rat GDNF is 93% identical to that in humans (Lin et al., 1993). The GDNF gene encodes a 211 amino acid precursor protein that is processed and secreted as a mature protein (glycosylated, disulfide-linked homodimer) of 134 amino acids with two N-linked glycosylation sites in the mature protein (Lin et al., 1993, 1994).
About 25% of the molecular mass of glycosylated GDNF is due to N-linked carbohydrates (Lin et al., 1994). The NH₂ terminus of GDNF is not critical for trophic activity (Xu et al., 1998). The mature homodimer of GDNF is considered to be the biologically active protein and can initiate various cell signaling pathways.

**GDNF signaling**

The signaling pathway of the GDNF family ligands utilizes a two-component receptor system where the transmembrane receptor tyrosine kinase c-Ret acts as a common signaling receptor with each of the different receptors of the glial cell line-derived family receptors alpha (GFRα) family (Nomoto et al., 1998; Takahashi, 2001; Airaksinen and Saarma, 2002). The receptors are anchored to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchored co-receptor that binds ligands with high affinity to their receptor complex. The preferential receptor/ligand complex for the GDNF family consists of: GFRα1 binding to GDNF, GFRα2 binding to NRTN, GFRα3 binding to ARTN and GFRα4 binding to PSPN. There is crosstalk between the ligands and the other receptors, where they bind at low affinity, for example GDNF can bind to GFRα2 and GFRα3 (Cacalano et al., 1998; Baloh et al., 2000; Airaksinen and Saarma, 2002). The ultimate signaling complex for GDNF includes 2 copies of GFRα1 and 2 copies of rearranged after transfection (Ret), where a GFRα1 molecule binds to a GDNF epitope and then an additional GFRα1 binds to an epitope formed by both GDNF and the first GFRα1 (Eigenbrot and Gerber, 1997). Upon GDNF stimulation, GFRα1 recruits Ret into lipid rafts, resulting in dimerization and tyrosine phosphorylation of Ret (Vega et al., 1996), then activates several downstream intracellular signaling pathways. The
pathways that are activated include: the mitogen-activated protein kinase (MAPK) pathway, which is involved in neurite outgrowth, neuronal survival, and migration of Schwann cells (Airaksinen and Saarma, 2002); the phosphoinositide 3-kinase (PI3K) pathway, which is vital for neuronal survival and neurite outgrowth (Van Weering and Bo, 1997; Pong et al., 1998; Soler et al., 1999; Besset et al., 2000; Jin et al., 2002; Ugarte et al., 2003); the Akt pathway, which is important for neuron survival effects of several neurotrophic factors by promoting X-linked inhibitor of apoptosis protein (XIAP) and inhibiting pro-apoptotic members of the bcl2 family of transcription factors (Brunet et al., 1999, 2001; Dan et al., 2004). GDNF requires inhibitors of apoptosis proteins (IAPs) for its protective effects on motor neurons, where inhibition of XIAP or neuronal apoptosis inhibitor protein (NAIP) activity prevents neuroprotective effects of GDNF (Perrelet et al., 2002); and the phospholipase Cγ (PLC-γ) pathway, which is involved in regulation of intracellular levels of Ca^{2+} ions and plays a neuroprotective and survival role for developing motor neurons and dopaminergic neurons (Fu et al., 2012); and the Src-family kinase pathway, which stimulates neurite outgrowth and neuronal survival (Airaksinen and Saarma, 2002). While the GDNF-Ret signal has been found to participate in the organization and maturation of neuromuscular synapses (Baudet et al., 2008), GDNF can signal independently of Ret (Pozas, 2005). In Ret-deficient cells, the GDNF-GFRα1/Src complex forms first, activating the subsequent phosphorylation of PLCγ and MAPK (Poteryaev et al., 1999). In the absence of Ret, GDNF can also induce c-met proto-oncogene phosphorylation to partially restore ureteric morphogenesis (Popsueva et al., 2003). Furthermore, GDNF stimulates the fast internalization of GFRα1
in neuroblastoma cells and hippocampal neurons lacking Ret expression (Vieira and Vieira, 2003).

**GDNF distribution**

GDNF is widely distributed in the central nervous system, including the cortex, hippocampus, cerebellum, striatum, spinal cord, hypothalamus, midbrain, and in the peripheral nervous system, including Schwann cells, skeletal muscle fibers, kidneys, and peripheral neurons. GDNF is produced by motor neurons, oligodendrocytes, and Schwann cells in the spinal cord (Henderson et al., 1994; Yamamoto et al., 1996; Russell et al., 2000; Rind and von Bartheld, 2002) and by skeletal muscle (Yamamoto et al., 1996). GDNF mRNA is present in somatic motor neurons of the ventral horn of the spinal cord as detected by *in situ* hybridization (Pochon et al., 1997), but not in the dorsal root ganglion (Hammarberg et al., 1996; Nosrat et al., 1996; Golden et al., 1999; Widenfalk et al., 1999). Only motor neurons expressing islet-1, Ret, and GFRα1 are responsive to GDNF (Oppenheim et al., 2000). Motor neurons transport GDNF in a receptor-mediated fashion where GDNF can bind to receptors in nerve terminals and be transported from target tissues to the cell bodies of the motor neurons (Yan et al., 1995). Evidence for retrograde transport of GDNF has been demonstrated in nerve injury studies, where GDNF expression is upregulated at the distal part of injured nerves, in skeletal muscles, and in Schwann cells (Naveilhan et al., 1997; Frostick et al., 1998; Lie and Weiss, 1998). Whereas glial-derived GDNF, including that produced in Schwann cells and satellite cells, is taken up by motor neurons and transported anterogradely along the axons for release from terminals (Rind and von Bartheld, 2002; Russell et al., 2000;
von Bartheld et al., 2001). It has been suggested that both endogenous and exogenous neurotrophic factors can avoid the degradation pathway after internalization where they can retain their biological activity after transcytosis (von Bartheld et al., 2001; Rind and von Bartheld, 2002). It has also been suggested that only a subpopulation of GDNF-receptor bearing dorsal root ganglion neurons anterogradely transport glial-derived GDNF and others may transport GDNF exclusively in a retrograde fashion to their cell bodies (Rind and von Bartheld, 2002). GDNF may have more target neurons than any other neurotrophic factor (Bohn, 2004).

**GDNF is essential for motor neurons**

GDNF is the most potent survival factor for motor neurons (Henderson et al., 1994) and may be involved in the maintenance of cell body size and the cholinergic phenotype of motor neurons (Ulfflake and Bergman, 2000). GDNF mRNA in the ventral horn of the spinal cord is found in large neurons in lamina IX (Zhou et al., 2008). The primary GDNF receptors, GFRα1 and Ret, are also found in spinal motor neurons (Golden et al., 1999; Garces et al., 2000), and in skeletal muscle (Yang and Nelson, 2004). Heterozygous GDNF knockout mice lack 22% of their lumbar motor neurons (Moore et al., 1996), and GFRα-1 knockout mice lack 24% of their lumbar motor neurons (Cacalano et al., 1998). GFRα-2 knockout mice do not seem to have a survival role for motor neurons (Garces et al., 2000), suggesting a survival role for motor neurons that is mediated by the GDNF/GFRα-1 complex. Deprivation of neurotrophic factors is an established trigger for apoptosis of neurons. Prevention of apoptosis of mature neurons
has been demonstrated by the addition of exogenous neurotrophic factors (Henderson et al., 1993; Oppenheim et al., 1995; Lee et al., 2000). Neurotrophic factors may prevent apoptosis by activating kinases, such as MAPK and the serine-threonine kinase Akt, and transcription factors, such as cyclic adenosine monophosphate (AMP) response element-binding protein (CREB) (Mattson and Meffert, 2006; Riccio et al., 1999; Nicole et al., 2001). In cultured motor neurons, GDNF was found to be 75-, 650-, and 2500-fold more potent in preventing programmed cell death than BDNF, CNTF, and LIF, respectively (Henderson et al., 1994). A role for motor neuron protection from death has been demonstrated in vivo by local administration of neurotrophic factors following sciatic nerve transection, where BDNF rescues 77%, NT-3 and NGF rescues 65% of motor neurons as compared to only 52% survival of control motor neurons (Yan et al., 1993). Interestingly, the only neurotrophic factor that was found to elicit 100% motor neuron survival following axotomy was GDNF as compared to only 6% survival of motor neurons in axotomy-induced, untreated controls (Yan et al., 1995). Furthermore, administration of exogenous GDNF prevented death of nearly all facial motor neurons in neonatal rats (Henderson et al., 1994) and prevented death and atrophy of nearly 100% of axotomy-induced motor neurons in chick embryos (Oppenheim et al., 1995).

GDNF also plays a chemoattractant role in axon path finding during development. Studies have shown that GDNF acts with ephrinA/ephrinA receptor, the complex that induces motor neuron repulsion, to ensure the correct connection of motor axons with target tissues (Kramer et al., 2006; Dudanova et al., 2010). Over production of muscle-
derived GDNF leads to motor neuron sprouting (Nguyen et al., 1998), and astrocyte-derived GDNF increases the survival of motor neurons following the period of programmed cell death during development (Zhao et al., 2004). Overexpression of GDNF in developing muscle shows an increase in survival of nearly all motor neuron populations during naturally occurring cell death compared to GDNF-deficient embryos (Oppenheim et al., 2000), where the survival of motor neurons during programmed cell death depends on GDNF secreted specifically by the target tissue of skeletal muscle (Angka et al., 2008). Recently, it has been reported that γ-motor neurons express higher levels of GFRα-1 compared to α-motor neurons in the early postnatal period, suggesting that early γ-motor neurons depend on muscle spindle-derived GDNF (Shneider et al., 2009; Kanning et al., 2010). These studies demonstrate that developing and injured motor neurons depend on access to GDNF for survival, and possibly more from muscle-derived GDNF rather than centrally-derived GDNF.

GDNF has been suggested to regulate the physiological function of developing and/or adult motor neurons (Wang et al., 2001; Martin-Caraballo and Dryer, 2002). Mature motor neurons obtain trophic support from various types of cells, including Schwann cells, skeletal muscle fibers and other neurons (Nishi, 1994; Oppenheim, 1996), and may resist death by autocrine production of neurotrophic factors and by increasing their ability to respond to these factors. In aging rats there appears to be selective loss of the large α-motor neurons that innervate the hindlimb skeletal muscles first (Ishihara et al., 1987; Ishihara and Araki, 1988). Aging is marked by loss of neuronal connections,
axon dystrophy, myelin disturbances, and neuronal atrophy in certain cellular populations (Ulphake et al., 2000). A greater prevalence of axon lesions are present in ventral roots of the lumbar spinal cord and peripheral nerves of aged individuals (Van Steenis and Kroes, 1971; Burek et al., 1976). Among old individuals, a large majority of motor neurons are present but are not connected to an intact set of target muscle cells. During the initial phase of muscle denervation, the motor neurons that are still connected to the muscle may try to compensate by collateral reinnervation (Edstrom and Larsson, 1987; Larsson, 1995) possibly through interactions with the terminal Schwann cells. Effects of aging on motor neurons, such as neuronal dropout, is greater in hindlimb motor neurons than in forelimb motor neurons (Hashizume and Kanda, 1990). There is an upregulation of GFRα-1 and Ret in aged spinal motor neurons (Bergman et al., 1999); however, γ-motor neurons express higher levels of Ret than α-motor neurons (Jongen et al., 2007). Maintenance of high levels of the GDNF receptors through adulthood in motor neurons (Zhang and Huang, 2006) may reflect a functional role for GDNF after spinal cord injury, aging, and disease. Following nerve injury, GDNF expression is increased in surrounding Schwann cells, in peripheral nerves, and in skeletal muscle, and GFRα-1 is upregulated in Schwann cells and motor neurons, and Ret is upregulated in motor neurons and in dorsal root ganglion neurons (Naveilhan et al., 1997).

**Exercise alters neurotrophic factors in the motor nervous system**

Increased physical activity has been well established to alter the structure and function of the neuromuscular apparatus. Exercise increases the size and degree of
branching of motor nerve terminals at the neuromuscular junction (Andonian and Fahim, 1987), increases total area of both pre- and postsynaptic elements (Deschenes et al., 1993), and increases quantal content of acetylcholine release (Dorlochter et al., 1991). Endurance exercise elicits hypertrophy of nerve terminals and increases neurotransmitter release (Fahim, 1997). Furthermore, exercise has been found to have beneficial effects for individuals with neurodegenerative diseases and injury. Increased physical activity extends the lifespan of mouse mutants with Cu/Zn Superoxide Dismutase-1 (SOD1)-associated motor neuron disease (Kirkinezos et al., 2003; Kaspar et al., 2005), spinal muscular atrophy (Grondard et al., 2005), and progressive motor neuronopathy (Ferrer-Alcon et al., 2008). Exercise has been also shown to offer significant improvement in spinal cord injury models (Hutchinson et al., 2004; Dupont-Versteegden et al., 2004; Engesser-Cesar et al., 2005, 2007). A role for neurotrophic factors has been proposed as contributing to exercise-induced changes in the nervous system (Wehrwein et al., 2002; Adlard and Cotman, 2004; McCullough et al., 2011). Neurotrophic factors, such as BDNF, IGF-1, and vascular endothelial growth factor (Trejo et al., 2001; Fabel et al., 2003; Wu et al., 2008), have also been suggested to play a role in exercise-mediated neuroprotective effects, where levels of expression of BDNF and NT-3 are increased in the spinal cord and skeletal muscle following both involuntary and voluntary exercise in skeletal muscle (Gómez-Pinilla et al., 2001, 2002). A relationship between neurotrophic factors and physical activity has been demonstrated with concentrations of NT-4 in skeletal muscle changing in proportion to the intensity of exercise (Funakoshi et al., 1995). We have previously shown that 4 weeks of treadmill training and 2 weeks of
forced wheel running can increase GDNF protein content in rat skeletal muscle, while decreases in GDNF content are observed with hindlimb suspension (Wehrwein et al., 2002; McCullough et al., 2011).

Changes in motor neuron structure and function caused by GDNF resemble the changes observed with altered physical activity. Increased expression of GDNF in developing skeletal muscle leads to increased axonal branching and increased motor unit size (Nguyen et al., 1998; Zwick et al., 2001), while treatment with exogenous GDNF causes continuous synaptic remodeling at the neuromuscular junction (Keller-Peck et al., 2001) and prevents motor neuron degeneration following axotomy (Oppenheim et al., 1995). Choline acetyltransferase (ChAT) activity of embryonic motor neurons is increased by GDNF (Zurn et al., 1994). GDNF rescues somatic motor neurons from natural occurring cell death (Oppenheim et al., 2000), rescues motor neurons from axotomy-induced cell death (Oppenheim et al., 1995), slows the loss of motor neurons in mice exhibiting progressive motor neuropathy (Sagot et al., 1996), and protects motor neurons from chronic degeneration (Corse et al., 1999). Alterations in GDNF expression have been observed in skeletal muscle from humans with amyotrophic lateral sclerosis (Yamamoto et al., 1996), polymyositis, and Duchenne type muscular dystrophy (Suzuki et al., 1998). While independent researchers have found similar beneficial effects following exercise with exogenous treatment with GDNF, no one has been able to link the two together. One of the goals of our studies is to determine if the beneficial effects of exercise for the motor nervous system may be in part driven by changes in GDNF
levels. The long-term goals of these studies was to identify if exercise increases GDNF protein expression, including GDNF protein content and localization. If GDNF is an activity-dependent neurotrophic factor, then this may be one mechanism to help reverse degenerative changes in motor nervous system function due to aging, injury and disease. The purpose of these studies described in the following dissertation were to determine how GDNF protein expression is altered in the spinal cord of rodents following exercise, aging, and disease.
CHAPTER II

EXPERIMENTAL DESIGN

Specific aims

These studies were aimed to determine if an activity-dependent relationship occurs with GDNF and the physiological implications for how the motor nervous system changes with aging, injury and disease. The studies described herein are based on the following hypotheses:

1. Exercise alters GDNF protein expression in the spinal cord of aging animals. To determine how GDNF protein expression is altered during the aging process and how various levels of exercise can affect GDNF protein expression:
   a. GDNF protein expression was examined from the lumbar spinal cord of adult rats at various ages.
   b. Adult rats underwent exercise training for short-term and long-term durations and GDNF protein expression was analyzed from the lumbar spinal cord.
   c. GDNF protein expression was analyzed from the lumbar spinal cord of adult rats that underwent low-intensity and high-intensity exercise.

2. Exercise alters GDNF protein expression in the spinal cord of a transgenic animal model of rapid motor nervous system degeneration. Using a disease model of rapid aging:
a. GDNF protein expression was examined from the lumbar spinal cord of transgenic amyotrophic lateral sclerosis (ALS) mice and from age-matched wild-type C57BL/6 mice.

b. Mice underwent low-intensity exercise and GDNF protein expression was analyzed from the lumbar spinal cord of transgenic ALS mice and wild-type C57BL/6 mice.

3. **Beneficial effects of exercise on the motor nervous system are blocked by treatment with anti-GDNF in a transgenic animal model of rapid motor nervous system degeneration.** A causative role for GDNF in nervous system plasticity with exercise in an advanced model of aging was investigated:

   a. Transgenic ALS mice were treated with anti-GDNF and underwent low-intensity exercise where GDNF protein expression was analyzed from the lumbar spinal cord.

   b. Neurodegenerative changes were analyzed from the lumbar spinal cord of transgenic ALS mice treated with anti-GDNF that had undergone low intensity exercise.

The results of these experiments provided insight into intrinsic mechanisms by which exercise can regulate neurotrophic factor levels in the spinal cord.
Rationale and development

The purpose of this section is to explain the rationale and development of the experimental design for the studies described herein. The methods of all the following experiments are described in complete detail in the following chapters.

Murine animals as model organisms for studying neurotrophic factors

Rodents are considered to be excellent candidates for biological research. Rats and mice that are non-transgenic, are inexpensive and easily attainable. Humans, rats, and mice have about the same number of genes and about 99% of the DNA is shared. Almost all known human genes that are associated with diseases have counterparts in the rat genome and appear highly conserved through mammalian evolution, confirming that the rat is an excellent model for many areas of research. Humans have 23 pairs of chromosomes, rats have 21 pairs of chromosomes and mice have 20 pairs of chromosomes. Chromosomes from all three organisms are related to each other by about 280 large regions of sequence similarity. Rats possess some genes that are not found in the mouse, such as those involved in pheromone production, immunity, and breakdown of proteins (Rat Genome Sequencing Project Consortium, 2004). Another advantage of rodents for biological studies are the use and availability of transgenic and knockout animals to inhibit or over-express a gene that displays similar characteristics of human diseases and conditions. For example, transgenic mice of ALS have been extensively studied since 1994 (Gurney et al., 1994). Since mice are smaller in size than rats, performing treatment studies with antibody injections against GDNF are less expensive
than performing similar studies in the rat, which would require a larger overall dosage of treatment per body weight.

Both mice and rats have a short life-span as compared to humans, living on average 2-3 years. They provide excellent models to study changes associated with aging without having to wait a long duration for the animal to reach an appropriate age for studies to begin. Male subjects were chosen for all studies as changes in monthly hormone levels of estrogen and progesterone would not have to be factored in, since some of the benefits of exercise on neurotrophic factors in females appear to be dependent on estrogen levels (Berchtold et al., 2001). Also, female rats have disruption of their sex hormones, similar to menopause in human females, from 15-18 months of age, and this variable may affect neurotrophic factor protein expression with the aging studies. Many neurotrophic factors, including BDNF, NGF, and GDNF have been quantified from various rat and mouse tissues. Furthermore, GDNF was originally purified from B49 midbrain cell cultures of Sprague-Dawley rat embryos (Lin et al., 1993), making Sprague-Dawley rats an excellent organism to study GDNF protein expression.

Finally, the anatomy of the spinal cord for rats and mice differ in comparison to humans, including a difference in spinal cord segments. Rodents have had their hind limb motor neurons identified by horseradish peroxidase retrograde labeling (McHanwell and Biscoe, 1981) and both rats and mice have relatively large motor neurons that are well documented in regards to topographic position of motor neuron groups supplying
skeletal muscles (Watson et al., 2009). Since neurotrophic factors are found in many neuronal populations in the spinal cord, examining changes in GDNF protein expression of the rodent spinal cord following varying levels of physical activity can be applied to the intrinsic mechanisms for how neurotrophic factors are regulated with physical activity in humans.

**Optimal time for euthanasia after the last bout of exercise**

One area of interest for exercise studies is to determine the optimal time after the last bout of exercise to euthanize the animals and measure protein expression. Researchers have found that there are differences in mRNA levels of neurotrophic factors in skeletal muscle and the spinal cord that are dependent on time of sacrifice after the last bout of exercise (Gomez-Pinilla et al., 2001). Preliminary studies have found altered GDNF protein content 24 hours after the last bout of exercise; however, euthanization immediately after the last bout of exercise did not alter GDNF protein content in rat skeletal muscle (Wehrwein et al., 2002; Peplinski, 2007). The aim of this study was to determine if GDNF protein levels are dependent on longer durations of time of sacrifice after the last bout of exercise. GDNF protein content was examined from skeletal muscles 24 hours, 72 hours and 1 week after the last bout of exercise.

Seventeen-week-old male Fischer 344 rats (Charles River, Portage, MI, USA) were selected because 17-week-old animals have a relatively stable distribution of skeletal muscle fiber types (Maltin et al., 1989). Animals were exercised for 2 weeks in individually housed running wheels (Lafayette Instruments, Lafayette, IN) that were
maintained at a speed of 10m/min for 45 min/day for 5 consecutive days with 2 days of rest. At the completion of the exercise training, one group was euthanized 24 hours after the last bout of exercise (n = 4), 72 hours after the last bout of exercise (n = 4), 1 week after the last bout of exercise (n = 4), and a final group was used as age-matched sedentary controls (n = 8).

The extensor hallucis longus muscle and the diaphragm muscles were removed and frozen on dry ice. The extensor hallucis longus muscle was chosen for this study as it is a predominantly fast-twitch hindlimb muscle that extends the hallux bone (the big toe). Its composition is ~59% Type IIa fibers (fast oxidative glycolytic) and ~41% Type IIb fibers (fast glycolytic) (Burkholder et al., 1994). The diaphragm was selected as it is a continuously contracting muscle and its composition is ~42% Type I fibers (slow oxidative), ~25% Type IIa fibers and ~33% Type IIb fibers (Kilarski and Sjostrom, 1990). To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin, and 0.05% Tween-20) was added and the mixture was homogenized on ice. Samples were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C.

GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA). Ninety-six well plates were incubated overnight at room temperature in a humidified chamber with a monoclonal antibody raised against GDNF (R&D Systems,
Minneapolis, MN). The following day, plates were rinsed with wash buffer and blocked with phosphate buffered saline containing 1% bovine serum albumin and 5% sucrose for 1 hour at room temperature. Plates were rinsed with wash buffer and the GDNF standard (R&D Systems) or tissue supernatants were added to the wells. For each assay, a standard curve was calculated from the known GDNF standard concentration, ranging from 1000 – 2pg/ml. Following a 2 hour incubation at room temperature, the plates were washed then incubated with biotinylated anti-GDNF secondary antibody (R&D Systems) for 2 hours at room temperature. The plates were washed again and coated with β-galactosidase conjugated to streptavidin (Molecular Probes, Eugene, OR) for 20 minutes at room temperature. The plates had a final wash and chlorophenol red-β-D-galactopyranoside (CPRG) substrate was added (in phosphate buffered saline + bovine serum albumin) and incubated until the color had developed.

Data were displayed as mean ± the standard error of the mean (SEM) for all variables. GDNF protein values are expressed as pg GDNF/mg of wet tissue weight. Data were analyzed using a one-way ANOVA and Tukey’s post-hoc comparison to test for differences between groups, where p values ≤ 0.05 were considered as statistically significant.

GDNF protein content was significantly increased in the exercised extensor hallucis longus following 72 hours of rest (1.6 ± 0.3pg GDNF/mg tissue weight) as compared to the age-matched sedentary controls (0.8 ± 0.2pg GDNF/mg tissue weight) (Figure 1). There was no change in GDNF protein content in the extensor hallucis longus
following 24 hours or 1 week of rest (0.6 ± 0.2 and 1.1 ± 0.4pg GDNF/mg tissue weight, respectively) as compared to controls. No differences were observed in GDNF protein content in the extensor hallucis longus muscle among the exercise groups and any duration of rest.

No change in GDNF protein content was detected in the diaphragm following 2 weeks of exercise following any duration of rest (Figure 2). There was a trend toward an increase of GDNF protein content following exercise and 72 hours of rest. Interestingly, about a 25-fold increase was observed in GDNF protein concentration in the diaphragm as compared to the extensor hallucis longus muscle. Although there was no significant effect of exercise on GDNF protein content in the diaphragm, these results suggest that there may be an activity-dependent relationship for GDNF protein content. In that target tissues which are subject to continuous activity, including the diaphragm and postural muscles, may express more baseline levels of GDNF protein. Based on the preliminary results from this study, all animals were euthanized 72 hours after the last bout of exercise for future studies.
GDNF protein content is increased in the extensor hallucis longus following 2 weeks of exercise and 72 hours of rest.

**Figure 1.** GDNF protein content in the extensor hallucis longus muscle increased after 2 weeks of forced wheel running and 72 hours of rest. The extensor hallucis longus muscle was removed from control (n=8) and exercised 17-week-old rats (n=12) that had different periods of rest after the last bout of exercise. The exercised animals were placed in either 24 hours of rest after the last bout of exercise (n=4), 72 hours of rest after the last bout of exercise (n=4), or 1 week of rest after the last bout of exercise (n=4). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM. Asterisk (*) indicates significance (p≤0.05).
GDNF protein content in the diaphragm does not change following 2 weeks of exercise.

**Figure 2.** GDNF protein content in the diaphragm muscle is not altered after 2 weeks of forced wheel running. The diaphragm was removed from control (n=8) and exercised 17-week-old animals (n=12) that had different periods of rest after the last bout of exercise. The exercised animals were placed in either 24 hours of rest after the last bout of exercise (n=4), 72 hours of rest after the last bout of exercise (n=4), or 1 week of rest after the last bout of exercise (n=4). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM.
**Duration of exercise**

Another task was to determine what duration of exercise best elicits neurotrophic factor expression (Gomez-Pinilla et al., 2001; Molteni et al., 2002; Ying et al., 2003; Cuppinf et al., 2007). We have previously shown that GDNF protein content is increased in the soleus but decreased in the extensor digitorum longus muscle of adult rats following 2 weeks of exercise (McCullough et al., 2011). Following 4 weeks of exercise GDNF protein content is increased in the gastrocnemius, pectoralis and soleus muscles of young rats (Wehrwsein et al., 2002). Based on results from these studies, studies were conducted to examine GDNF protein content in skeletal muscles following increasing durations of exercise.

Adult (17-week-old) male Fischer 344 rats had undergone either 2 (n = 4), 4 (n = 6), or 6 weeks (n = 6) of exercise in individually housed running wheels (Lafayette Instruments) at a speed of 10m/min for 45min/day for 5 consecutive days and 2 days of rest. Additional groups were kept as age-matched sedentary controls for each duration of exercise (n = 8, n = 6, n = 6, respectively).

At the completion of the exercise training, animals were euthanized 72 hours after the last bout of exercise. To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH$_2$PO$_4$, 0.08 M Na$_2$HPO$_4$, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin, and 0.05% Tween-20) was added and was homogenized on ice. Samples
were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C. GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA) as described earlier. Data are displayed as mean ± the standard error of the mean (SEM) for all variables. GDNF protein values are expressed as pg GDNF/mg of wet tissue weight. Data were analyzed using a one-way ANOVA and Tukey’s post-hoc comparison to test for differences between groups, where p values ≤ 0.05 were considered as statistically significant.

All of the control extensor hallucis longus tissues were pooled together as there was no statistical difference among the control groups (p>0.05). GDNF protein content was increased in the extensor hallucis longus muscle following 2 weeks of exercise as compared to controls (Figure 3). A strong trend toward an increase in GDNF protein content was observed following 6 weeks of exercise (p = 0.06) as compared to controls.

All of the control diaphragm tissues were pooled together as there was no statistical difference among the groups (p>0.05). GDNF protein content was increased in the diaphragm following 6 weeks of exercise as compared to controls (Figure 4). A trend toward an increase of GDNF protein content following 2 and 4 weeks of exercise was observed. Together, with the findings of the extensor hallucis longus and the diaphragm, these results possibly suggest that increased levels of physical activity may increase GDNF protein in skeletal muscles.
GDNF protein content is increased in the extensor hallucis longus following 2 weeks of exercise

Figure 3. GDNF protein content is increased in the extensor hallucis longus muscle after 2 weeks of forced wheel running. The extensor hallucis longus muscle was removed from control (n=8) and exercised 17-week-old animals (n=12) that had undergone different durations of running. The exercised animals were placed in either 2 weeks of exercise (n=4), 4 weeks of exercise (n=6), or 6 weeks of exercise (n=6). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM. Asterisk (*) indicates significance (p≤0.05).
GDNF protein content in the diaphragm is increased following 6 weeks of forced wheel running. The diaphragm was removed from control (n=8) and exercised 17-week-old animals (n=12) that had undergone different durations of exercise. The exercised animals were placed in either 2 weeks of exercise (n=4), 4 weeks of exercise (n=6), or 6 weeks of exercise (n=6). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM. Asterisk (*) indicates significance (p≤0.05).

**Figure 4.** GDNF protein content in the diaphragm muscle is increased after 6 weeks of exercise.
Spinal cord selection

Our lab has provided evidence that GDNF protein content is altered in healthy rat skeletal muscle following increased levels of physical activity (Wehrwein et al., 2002; McCullough et al., 2011). To our knowledge, these are the first reports of altered GDNF protein levels in skeletal muscles from non-injured, non-diseased, and non-treated animals. These results may suggest an activity-dependent relationship for GDNF protein content in the peripheral nervous system. While alterations in GDNF protein levels have been observed in skeletal muscle following exercise, little is still known as to what changes in GDNF protein occur at the level of the spinal cord following exercise. Since GDNF is still considered to be one, if not the, most potent survival factors for motor neurons (Henderson et al., 1994; Oppenheim et al., 1995), it is important to understand how GDNF protein expression is regulated at the level of the spinal cord by physical activity. By examining changes of GDNF protein expression that occur in the spinal cord following exercise, we will gain insight to the consequences for altered expression for GDNF in the motor nervous system.

To ensure that GDNF protein content could be measured with the similar methodologies as with skeletal muscle, the lumbar spinal cord was removed from young rats to analyze GDNF protein expression for content and localization. The lumbar spinal cord region was chosen to examine changes in GDNF protein expression as the motor neurons from this region that innervate the hindlimb muscles are located in this region in Lamina IX (Figure 5). In order to minimize the number of animals used for each study,
different regions of the lumbar spinal cord were selected to quantify and visualize GDNF protein. The lumbar spinal cord region of L1 – L3 was chosen for quantification of GDNF protein since these motor neurons innervate to the muscles of the quadriceps, gluteus, adductor muscles, flexor muscles and extensor muscles, including the extensor hallucis longus and extensor digitorum longus, and the soleus (Nicolopoulous-Stournaras and Iles, 1983). Others have also published that the lumbar enlargement (L2-L6 segments) of the rat spinal cord was utilized for quantification of GDNF protein content as measured via an ELISA (Tokumine et al., 2003). The lumbar spinal cord region of L4 - L5 was chosen to localize GDNF protein with immunohistochemistry as these motor neurons innervate the muscles of the hamstrings, adductor muscles, flexor muscles, extensor muscles including the extensor hallucis longus and extensor digitorum longus, gastrocnemius and the soleus (Nicolopoulous-Stournaras and Iles, 1983). Others have used immunohistochemical techniques to localize GDNF in this region of the lumbar spinal cord (Tokumine et al., 2003).
Motor neuron measurements collected from Lamina XI of the lumbar spinal cord

Figure 5. A diagram representing motor neuron cell bodies that were gathered and measured from Lamina IX of the rat lumbar spinal cord (adopted from Jacob, 1998).
GDNF protein expression in the spinal cord following exercise

To determine how GDNF protein expression is altered in the spinal cord following exercise, 1-month-old male Sprague-Dawley rats underwent 4 weeks of running. The rats were assigned to a sedentary control group at the beginning of the study (n=3), a sedentary control group at the end of the study (n=3) or an exercise group (n=6). Animals from the exercise group were individually housed in cages that were attached to running wheels (Lafayette Instruments, Lafayette, IN) and had free access to the running wheels where the running variables, such as time, duration and distance, were recorded with the activity wheel monitoring system (Lafayette Instruments). Voluntary running was chosen as it allows the animals to choose their own desired duration and intensity of running as well as the time in which they want to run (i.e. nocturnal running). Voluntary running is also a better representation of how humans prefer to exercise, at one’s will, instead of being forced to exercise by negative reinforcement. The animals had exercised for a total duration of 4 weeks because previous results found this duration increases GDNF protein content in skeletal muscle of young animals (Wehrwein et al., 2002). At the conclusion of all experiments, lumbar spinal cord sections (L1–L3) were removed and frozen on dry ice. To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH$_2$PO$_4$, 0.08 M Na$_2$HPO$_4$, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin, and 0.05% Tween-20) was added and was homogenized on ice. Samples
were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C. GDNF protein content was measured using an ELISA as mentioned earlier.

Lumbar spinal cord sections (L4–L5) were fixed in 4% paraformaldehyde overnight at 4°C and then washed in fresh phosphate buffered saline (Oppenheim et al., 2000). Tissues were embedded in O.C.T. compound mounting medium, cut into 40µm transverse sections on a cryotome (Widenfalk et al., 2001), and thaw mounted onto Histobond® slides (VWR International, Bridgeport, NJ). Slides were incubated for 30 min at room temperature with 0.1% triton X-100 and 10% donkey serum to increase the permeability and decrease non-specific binding. Slides were incubated overnight at 4°C with primary antibodies (1:200) of rabbit anti-GDNF (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-ChAT (Millipore, Temecula, CA) in phosphate buffered saline containing 1% bovine serum albumin and 0.1% triton X-100. Slides were then incubated with secondary antibodies (1:500) of donkey anti-mouse conjugated to Alexafluor 568 and donkey anti-rabbit conjugated to Alexafluor 488, for 2 hours at room temperature. Negative control slides had primary antibodies omitted. Slides were viewed with a Zeiss LSM 510 laser scanning confocal microscope and images were examined with the Zeiss LSM 5 Image Examiner program.

All data values are reported as mean ± the standard error of the mean (SEM). GDNF protein values are expressed as pg GDNF/mg of wet tissue weight. Data were analyzed using a one-way ANOVA and Tukey’s post-hoc comparison to test for
differences between groups, where p values ≤ 0.05 were considered as statistically significant.

The exercised animals averaged a distance of 3.4km per day during the course of the study, and peaked at a speed of 63m/min. As expected, the majority of the running activity was conducted during the night. Following 4 weeks of voluntary running, there was a trend toward a decrease in GDNF protein content in the lumbar spinal cord (79.5 ± 11.7pg GDNF/mg tissue weight) as compared to age-matched sedentary controls (93.9 ± 9.7pg GDNF/mg tissue weight) or compared to the beginning-of-study sedentary controls (135.5 ± 43.0pg GDNF/mg tissue weight) (Figure 6). Interestingly, a trend toward a decrease in GDNF protein content in the lumbar spinal cord was observed with advancing age in the control animals.

We were able to visualize both GDNF and ChAT in the spinal cord tissues from all the animals (Figure 7B-D). When the primary antibodies were omitted, no immunoreactivity was detected within the lumbar spinal cord (Figure 7A).
GDNF protein content in the spinal cord is not altered with 4 weeks of exercise.

**Figure 6.** GDNF protein content in the spinal cord does not change after 4 weeks of exercise. The lumbar spinal cord (L1-L3) was removed from 1- and 2-month-old controls (n = 3 for each group) and exercised 2-month-old animals (n = 6). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM.
GDNF is localized in ChAT-positive motor neurons in the lumbar spinal cord

Figure 7. GDNF protein localization in ChAT-positive motor neurons in the spinal cord. Representative images from (A) a 2-month-old animal where primary antibodies were omitted, (B) a 1-month-old control animal, (C) a 2-month-old sedentary control animals, and (D) a 2-month-old exercised animal. Sections were exposed to primary antibodies against GDNF (green) and ChAT (red). Scale bar represents 20µm².
Differences between Fischer 344 and Sprague-Dawley rats

The Fischer 344 rat and the Sprague-Dawley rat are commonly used for aging studies; however, little is known about any differences of GDNF protein content in various tissues between these two strains. We have previously shown that there is no difference in GDNF protein content in the soleus or extensor digitorum longus hindlimb skeletal muscles between age-matched Fischer 344 rats and Sprague-Dawley rats (McCullough et al., 2011). To determine if there is a difference of GDNF protein content in the lumbar spinal cord among the two strains of rat, 3-month-old animals were examined.

The lumbar spinal cord section (L1–L3) was removed from 3-month-old Fischer 344 rats (n=3) and Sprague-Dawley rats (n=3) and frozen on dry ice. To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin, and 0.05% Tween-20) was added and was homogenized on ice. Samples were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C. GDNF protein content was measured using an ELISA as mentioned earlier.

No difference in GDNF protein content (p = 0.57) was observed in the lumbar spinal cord between the Fischer 344 rats (49.2 ± 22.3pg GDNF/mg tissue weight) and the Sprague-Dawley rats (32.0 ± 16.2pg GDNF/mg tissue weight) (Figure 8).
Different rat strains do not possess different levels of GDNF protein content in the spinal cord.

**Figure 8.** GDNF protein content in the lumbar spinal cord is not significantly different in Fischer 344 rats as compared to Sprague-Dawley rats. The lumbar spinal cord (L1-L3) was removed from 3-month-old control Fischer 344 rats (n = 3) and 3-month-old control Sprague-Dawley rats (n = 3). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM.
To determine if exercise affects GDNF protein content in the lumbar spinal cord from both strains, animals underwent 6 weeks of wheel running. Three-month-old Fischer 344 rats and Sprague-Dawley rats were individually housed in cages with continuous access to running wheels (Lafayette Instruments) where running parameters were recorded (Lafayette Instruments). An additional group of age-matched sedentary controls were maintained with each strain. GDNF protein content was measured via an ELISA as mentioned above.

Six weeks of voluntary exercise did not alter GDNF protein content in the Fischer 344 rats (95.6 ± 21.6pg GDNF/mg tissue weight) as compared to their age-matched sedentary controls (p = 0.21; Figure 9a). Voluntary exercise did not alter GDNF protein content in the Sprague Dawley rats (72.9 ± 14.4pg GDNF/mg tissue weight) as compared to their age-matched sedentary controls (p = 0.13; Figure 9b). Since no difference was observed in GDNF protein content in skeletal muscle or in the lumbar spinal cord between the two strains of rat, all future studies were conducted with male Sprague-Dawley rats as they were more accessible.
GDNF protein content is not altered in the spinal cord following 6 weeks of exercise

**Figure 9.** GDNF protein content is not altered following 6 weeks of voluntary running in the lumbar spinal cord of (A) Fischer 344 rats or in (B) Sprague-Dawley rats. The lumbar spinal cord (L1-L3) was removed from (A) 3-month-old control Fischer 344 rats (n = 3) and exercised 3-month-old Fischer 344 rats (n = 3) and from (B) 3-month-old control Sprague-Dawley rats (n = 3) and exercised 3-month-old Sprague-Dawley rats (n = 3). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM.
Mouse studies

To examine alterations in GDNF protein expression in transgenic mouse models of rapid motor nervous system degeneration, analyses on control mice was performed first. To verify that GDNF protein expression could be measured in the mouse with the same methodologies as in the rat, 3-month-old C57Bl/6 wild-type control mice were examined.

The lumbar spinal cord section (L1–L3) was removed from 3-month-old C57Bl/6 wild-type control mice (n=3) and frozen on dry ice. To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin, and 0.05% Tween-20) was added and was homogenized on ice. Samples were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C. GDNF protein content was measured using an ELISA as mentioned earlier.

Lumbar spinal cord sections (L4–L5) were fixed in 4% paraformaldehyde overnight at 4°C and then washed in fresh phosphate buffered saline (Oppenheim et al., 2000). Tissues were embedded in O.C.T. compound mounting medium, cut into 40µm transverse sections on a cryotome (Widenfalk et al., 2001), and thaw mounted onto Histobond® slides (VWR International, Bridgeport, NJ). Slides were incubated for 30 min at room temperature with 0.1% triton X-100 and 10% donkey serum to increase the permeability and decrease non-specific binding. Slides were incubated overnight at 4°C.
with primary antibodies (1:200) of rabbit anti-GDNF (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-ChAT (Millipore, Temecula, CA) in phosphate buffered saline containing 1% bovine serum albumin and 0.1% triton X-100. Slides were then incubated with secondary antibodies (1:500) of donkey anti-mouse conjugated to Alexafluor 568 and donkey anti-rabbit conjugated to Alexafluor 488, for 2 hours at room temperature. Negative control slides had primary antibodies omitted. Slides were viewed with a Zeiss LSM 510 laser scanning confocal microscope and images were examined with the Zeiss LSM 5 Image Examiner program.

The mouse lumbar spinal cord had 0.4 ± 0.2 pg GDNF/mg of tissue weight. Immunohistochemical analysis was successful in immunolabeling the mouse spinal cord for GDNF and ChAT (Figure 10). Based on these preliminary results in the wild-type mouse, similar methodologies were performed for studies in transgenic mice with ALS.
GDNF is expressed within ChAT-positive cells in the mouse lumbar spinal cord

Figure 10. The lumbar spinal cord (L4-L5) was removed from 3-month-old C57BL/6 mice. Sections were exposed to primary antibodies against GDNF (green) and ChAT (red). Slides were washed in PBS and incubated with donkey anti-mouse 568 and donkey anti-rabbit 488 (1:500) for 2 hours at 4°C. Images were captured with a Zeiss laser scanning confocal microscope. Scale bar represents 20µm.
CHAPTER III

SHORT-TERM EXERCISE INCREASES GDNF PROTEIN LEVELS IN THE SPINAL CORD OF YOUNG AND OLD RATS

Introduction

A significant loss of skeletal muscle mass and strength are commonly observed in aging individuals (Kallman et al., 1990; Frontera et al., 2000) and contribute to an increased incidence of falls and disability (Fries et al., 1994; Toullotte et al., 2003). Changes with age are observed both in skeletal muscle and motor neurons innervating skeletal muscles. Alterations in motor neurons with increased age include loss of somatic motor neurons (Jacob, 1998) and loss of inputs to motor nerve cell bodies (Kullberg et al., 1998). In the aging rat there is a decrease in muscle innervation, loss of myelinated nerve fibers and changes in expression of neuropeptides and growth factors, similar to what is observed following axon lesion (Johnson et al., 1999). One possible contributing factor for the loss of motor neurons with age could be diminished neurotrophic factor signaling (Bergman et al., 1999).

Glial cell line-derived neurotrophic factor (GDNF) was first discovered in glial cells (Lin et al., 1993), and its expression has been found in a variety of tissues both in the central and peripheral nervous systems (Henderson et al., 1994; Suter-Crazzolara and Unsicker, 1994; Springer et al., 1995; Suzuki et al., 1998). To date, GDNF is the most potent survival factor identified for motor neurons (Henderson et al., 1994), where heterozygous GDNF knockout mice lack 22% of their lumbar motor neurons (Moore et
al., 1996), and GDNF receptor alpha-1 (GFRα1) knockout mice lack 24% of their lumbar motor neurons (Cacalano et al., 1998). One possible source of GDNF for somatic motor neurons is skeletal muscle, where GDNF is transported in a retrograde fashion (Yan et al., 1995; Trupp et al., 1997; Wang et al., 2002).

Increased expression of GDNF in developing skeletal muscle leads to increased axonal branching and increased motor unit size (Nguyen et al., 1998; Zwick et al., 2001), while treatment with exogenous GDNF causes continuous synaptic remodeling at the neuromuscular junction (Keller-Peck et al., 2001) and prevents motor neuron degeneration following axotomy (Oppenheim et al., 1995). GDNF increases choline acetyltransferase (ChAT) activity of embryonic motor neurons (Zurn et al., 1994), rescues somatic motor neurons from natural occurring cell death (Oppenheim et al., 2000) and from axotomy-induced cell death (Oppenheim et al., 1995), and protects motor neurons from chronic degeneration (Corse et al., 1999). Neurotrophic factors, such as brain derived neurotrophic factor (BDNF), insulin-like growth factor 1, and vascular endothelial growth factor (Wu et al., 2008; Trejo et al., 2001; Fabel et al., 2003) have been suggested to play a role in exercise-mediated neuroprotective effects; however, it is not known if GDNF plays a similar role. While independent studies have found similar beneficial effects following exercise to those observed with exogenous treatment with GDNF, no one has been able to link the two together. One of the goals of our studies was to determine if the beneficial effects of exercise for the motor nervous system may, in part, be driven by changes in GDNF levels. Here, we report that short-term exercise
increases GDNF protein content in the lumbar spinal cord of young (6-month-old) and old (24-month-old) rats, corresponding to morphological changes of motor neuron cell bodies.

The findings from this chapter have been accepted for publication in the journal Neuroscience at the time of submitting this dissertation.

**Experimental procedures**

**Subjects**

All 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 Sprague-Dawley rats (Charles River, Kalamazoo, MI) were given access to food and water *ad libitum* and were maintained on a 12h light/dark cycle. Rats were euthanized via CO₂ asphyxiation followed by thoracotomy.

**Training protocol**

We tested the effects of 2 weeks of exercise on GDNF protein content in the lumbar spinal cord of young (6-month-old) and old (24-month-old) animals. Two weeks of exercise was chosen as we have previously shown that this duration alters GDNF protein content in rat skeletal muscle (McCullough et al., 2011). The 6-month-old rats
were randomly divided into four groups. One group was kept as sedentary controls (n=12). The remaining groups underwent different exercise protocols (swimming, voluntary running and involuntary running). The voluntary running group (n=6) had continuous access to individually housed running wheels, where activity was recorded with an activity wheel monitoring system (Lafayette Instruments, Lafayette, IN). The involuntary running group (n=5) were placed in individual forced running wheels (Lafayette Instruments). These animals underwent 5 bouts of 24 min of running plus 10 min of rest, at a pace of 10m/min (McCullough et al., 2011). Two hours of involuntary exercise was chosen to match the distance run by the voluntary running group. The swimming group (n=6) had 3 rats/barrel placed in water (35°C) and these animals swam for a total of 2 hours, with bouts of rest, to match the animals of the running groups. The 24-month-old rats were randomly divided into two groups, a voluntary running group (n=6), as this was the least stressful of our exercise protocols, and an age-matched sedentary control group (n=5). Aged animals reached a peak running speed of only 2m/min.

**Tissue processing**

In order to minimize the number of animals used for each of our studies, we selected different regions of the spinal cord from each animal to quantify and visualize GDNF protein. The lumbar spinal cord region of L1 – L3 was chosen for quantification of GDNF protein content, as these motor neurons innervate the quadriceps, gluteus, adductor muscles, flexor muscles and extensor muscles, including the extensor hallucis
longus and extensor digitorum longus, and the soleus (Nicolopoulous-Stournaras and Iles, 1983), and as others have published GDNF protein content data from this region (Tokumine et al., 2003). The lumbar spinal cord region of L4 - L5 was chosen to examine localization of GDNF protein, as these motor neurons innervate the muscles of the hamstrings, adductor muscles, flexor muscles, extensor muscles including the extensor hallucis longus and extensor digitorum longus, gastrocnemius and the soleus (Nicolopoulous-Stournaras and Iles, 1983). Others have used immunohistochemical techniques to localize GDNF in this lumbar spinal cord region (Tokumine et al., 2003). To determine GDNF protein content, lumbar spinal cord sections (L1–L3) were removed and frozen on dry ice and samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin, and 0.05% Tween-20) was added and was homogenized on ice. Samples were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C.

**GDNF protein quantification**

GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (McCullough et al., 2011). Briefly, 96-well plates were incubated overnight at room temperature in a humidified chamber with a monoclonal antibody raised against GDNF (R&D Systems, Minneapolis, MN). The following day, plates were rinsed with wash buffer and blocked with phosphate buffered
saline containing 1% bovine serum albumin and 5% sucrose for 1 hour at room temperature. Plates were rinsed with wash buffer and the GDNF standard (R&D Systems) or tissue supernatants were added to the wells. For each assay, a standard curve was calculated from the known GDNF standard concentration, ranging from 2 – 1000pg/ml. Following a 2 hour incubation at room temperature, the plates washed then incubated with biotinylated anti-GDNF secondary antibody (R&D Systems) for 2 hours at room temperature. The plates were then washed and coated with β-galactosidase conjugated to streptavidin (Molecular Probes, Eugene, OR) for 20 minutes at room temperature. The plates had a final wash and chlorophenol red-β-D-galactopyranoside (CPRG) substrate was added (in phosphate buffered saline + bovine serum albumin) and incubated until the color had developed.

**Western blot**

Total protein content of the spinal cord samples were measured by a Pierce® BCA protein assay (Thermo Scientific, Rockford, IL) according to manufacturers specifications. Tissue samples were prepared for Western blot analysis of GDNF protein as previously described (Vianney and Spitsbergen, 2011). Briefly, protein extracts (20µg), a protein ladder (New England BioLabs, Ipswich, MA) and a loading control of α-Tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA) were prepared with Laemmli 2X loading buffer and loaded into a 15% polyacrylamide gel. The gel was submerged and was run in separating buffer at different voltages followed by transfer to a polyvinylidene difluoride (PVDF; Invitrogen) membrane in tris-glycine buffer. The
PVDF membrane was blocked with I-Block (Applied Biosystems, Foster City, CA) followed by overnight incubation with a primary antibody against GDNF (Santa Cruz Biotechnologies). The following day, the membrane was washed in buffer followed by incubation with a HRP-conjugated secondary antibody (ECL; GE Healthcare) in I-Blocking buffer. The ECL detection kit was used to detect the proteins and was visualized on BioMax XAR film (Kodak). ImageJ software was used to measure the relative density of GDNF bands and values were expressed as ratios of controls.

**Immunohistochemistry**

Lumbar spinal cord sections (L4–L5) were fixed in 4% paraformaldehyde overnight at 4°C and then washed in fresh phosphate buffered saline. Tissues were embedded in O.C.T. compound mounting medium, cut into 40μm transverse sections on a cryotome, and thaw mounted onto Histobond® slides (VWR International, Bridgeport, NJ). Slides were incubated overnight at 4°C with primary antibodies (1:200) of rabbit anti-GDNF (Santa Cruz Biotechnology, Santa Cruz, CA), (1:200) mouse anti-ChAT (Millipore, Temecula, CA), and (1:50) goat-anti GM130 (Santa Cruz Biotechnology) in phosphate buffered saline containing 1% bovine serum albumin and 0.1% triton X-100. Slides were then incubated with secondary antibodies (1:500) of donkey anti-mouse conjugated to Alexafluor 568, donkey anti-rabbit conjugated to Alexafluor 488, and donkey anti-goat conjugated to Alexafluor 568, for 2 hours at room temperature. Negative control slides had primary antibodies omitted. Slides were viewed with a Zeiss
LSM 510 laser scanning confocal microscope and images were examined with the Zeiss LSM 5 Image Examiner program.

Measurement of motor neuron cell body size

Since somatic motor neurons stain positively for ChAT immunoreactivity (Wetts and Vaughn, 1996), ChAT-positive cells were measured in Lamina IX of the spinal cord from all animals. Twenty randomly selected motor neurons from the L4-L5 spinal cord levels were counted from each animal to determine cell body area. The cells that had a mid-section through the nucleus were examined. Cell body areas were determined with the Zeiss LSM 5 Image Examiner program.

Statistical analysis

All data values are reported as mean ± the standard error of the mean (SEM). GDNF protein values are expressed as pg GDNF/mg of wet tissue weight. Data were analyzed using a one-way ANOVA and Tukey’s post-hoc comparison to test for differences between groups, where p values ≤ 0.05 were considered as statistically significant.
Results

Short-term exercise increases GDNF protein in the lumbar spinal cord of 6-month-old rats

Six-month-old animals underwent voluntary running, involuntary running, or swimming for 2 weeks. Animal weights were lower in the pooled exercised animals (388.8g ±16.7g) compared to sedentary controls (401.9g ± 24.8g). The maximum running speed of the voluntary running group was 28m/min, whereas the involuntary running group was maintained at 10m/min. While the intensities were quite different between the two running groups, the average distance run per day was similar (Table 1). Two weeks of all modes of exercise significantly increased GDNF protein content in the lumbar spinal cord as compared to sedentary controls (8.6 ± 1.5pg GDNF/mg tissue weight). Involuntary running resulted in the greatest change in GDNF protein content in the lumbar spinal cord (56.3 ± 26.4pg GDNF/mg tissue weight), followed by swimming (25.1 ± 9.5pg GDNF/mg tissue weight) and voluntary running (15.7 ± 1.9pg GDNF/mg tissue weight) (Figure 11a); however, there were no significant differences between the exercise groups. The involuntary running group had a 6.5-fold increase of GDNF protein content as compared to controls, followed by a 2.9-fold increase from the swimming group and a 1.8-fold increase from the voluntary running group (Table 1).

Using Western blot analysis we found that GDNF had a molecular weight of 30kDa (Figure 11B), which is close to the 34kDa previously reported in human fetal spinal cord (Koo and Choi, 2001). Densitometry analysis of GDNF bands showed a 3.5-
fold increase in GDNF expression for the involuntary runners, followed by a 1.7-fold increase for the swimmers and a 1.5-fold increase for the voluntary runners, which follows the same trend as our ELISA results (Table 1).

**Table 1.** Exercise parameters and GDNF protein expression following 2 weeks of exercise.

<table>
<thead>
<tr>
<th>Age of animals (Months)</th>
<th>Duration and type of exercise</th>
<th>Fold change of GDNF protein content (via ELISA) from controls</th>
<th>Fold change of GDNF protein content (via Western blot) from controls</th>
<th>Maximum Intensity of exercise (m/min)</th>
<th>Distance Run/day (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2 Weeks Voluntary Running</td>
<td>2 ↑</td>
<td>1.5 ↑</td>
<td>28</td>
<td>1434.9 ± 77.4</td>
</tr>
<tr>
<td>6</td>
<td>2 Weeks Involuntary Running</td>
<td>6.5 ↑</td>
<td>3.5 ↑</td>
<td>10</td>
<td>1200</td>
</tr>
<tr>
<td>6</td>
<td>2 Weeks Swimming</td>
<td>3 ↑</td>
<td>1.7 ↑</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>24</td>
<td>2 weeks Voluntary Running</td>
<td>2 ↑</td>
<td>2 ↑</td>
<td>2</td>
<td>132.3 ± 86.6</td>
</tr>
</tbody>
</table>

**Table 1.** Low-intensity, forced running (10m/min) elicited the greatest increase in GDNF protein content in spinal cord compared to other modalities of exercise. Changes in
GDNF protein content in spinal cord, as detected by Western blot, show similar trends as those measured via ELISA. Old rats (24-month-old) ran a shorter distance and at a lower intensity than young (6-month-old) rats.

**GDNF protein expression is increased in the lumbar spinal cord following 2 weeks of exercise in 6-month-old rats**

![Graph showing GDNF protein content](image)

**Figure 11.** GDNF protein content was increased in the spinal cord after 2 weeks of exercise in 6-month-old rats. The lumbar spinal cord (L1-L3) was removed from control and exercised 6-month-old animals. (A) Tissues were processed for GDNF protein content using an ELISA. A significant increase in GDNF protein content was detected in the spinal cord of animals that had undergone 2 weeks of voluntary running, involuntary
running and swimming as compared to sedentary control animals. Values are displayed as mean ± SEM. Asterisk (*) indicates significance (p≤0.05) from controls. (B) Tissues were processed for Western blot to determine GDNF protein content (top) and a loading control of α-tubulin (bottom). An increase in GDNF protein content was detected in the spinal cord of animals that had undergone 2 weeks of involuntary running, followed by swimming and then voluntary running as compared to controls.

Positive immunoreactivity for GDNF was found in ChAT-positive cells, which are presumed to be motor neurons (Wetts and Vaughn, 1996), in the lumbar spinal cord from control and exercised animals (Figure 12). We observed more vesicle-like structures containing GDNF surrounding motor neurons in exercised rats (arrows in Figure 2B-D) compared to those from sedentary controls (Figure 12A).

Analysis of motor neuron cell body areas revealed a significant increase following voluntary running (841.5 ± 49.6μm²), involuntary running (749.8 ±29.5μm²), and swimming (879.9 ± 46.2μm²), compared to that in controls (454.8 ± 25.6μm²). No significant differences were observed between exercise groups. Histogram analysis of motor neuron cell body areas displayed more occurrences of the large-sized motor neurons (>1500 μm²) belonging to the fast motor units (Deforges et al., 2009) among the voluntary and swimming groups than the involuntary running group and controls (Figure 13).
**Figure 12.** Exercise increased motor neuron size and vesicle-like structures of GDNF in 6-month-old rat spinal cord. Representative lumbar spinal cord sections from a 6-month-old sedentary control animal (A), voluntary exercised animal (B), swimming exercised...
animal (C) and an involuntary exercised animal (D). Spinal cord sections were immunolabeled with primary antibodies against ChAT (red) and GDNF (green). ChAT immunoreactivity is co-localized with GDNF immunoreactivity in the lumbar spinal cord. Exercised animals appeared to have more vesicle-like structures containing GDNF that surround the motor neurons (indicated by arrows), as compared to controls. The scale bar represents 20μm.

Histogram analysis of 6-month-old ChAT-positive motor neuron cell body area

![Histogram graph showing the distribution of cell body area for control and swimming animals.](image)

<table>
<thead>
<tr>
<th>Control Cell Body Area (μm²)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>2</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>700</td>
<td>6</td>
</tr>
<tr>
<td>900</td>
<td>8</td>
</tr>
<tr>
<td>1100</td>
<td>10</td>
</tr>
<tr>
<td>1300</td>
<td>12</td>
</tr>
<tr>
<td>1500</td>
<td>14</td>
</tr>
<tr>
<td>1700</td>
<td>16</td>
</tr>
<tr>
<td>1900</td>
<td>18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Swimming Cell Body Area (μm²)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>2</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>700</td>
<td>6</td>
</tr>
<tr>
<td>900</td>
<td>8</td>
</tr>
<tr>
<td>1100</td>
<td>10</td>
</tr>
<tr>
<td>1300</td>
<td>12</td>
</tr>
<tr>
<td>1500</td>
<td>14</td>
</tr>
<tr>
<td>1700</td>
<td>16</td>
</tr>
<tr>
<td>1900</td>
<td>18</td>
</tr>
</tbody>
</table>
Figure 13. Histogram analysis of ChAT-positive motor neuron cell body area of 6-month-old rats. Following 2 weeks of exercise, the voluntary running group and swimming group displayed a higher frequency of cells >1500μm² as compared to the involuntary running group and controls.

**Short-term exercise increases GDNF protein in the spinal cord of 24-month-old rats**

To determine if short-term exercise alters GDNF protein content in the spinal cord of old animals, 24-month-old rats underwent voluntary running for 2 weeks. These animals ran at a peak speed of only 2m/min (Table 1), which is considered to be low-intensity running. Average animal weights were significantly lower in the exercised
animals (355.0g ± 34.9g) as compared to age-matched sedentary controls (415.4g ± 56.0g). The average distance run per day by these old animals was around 1km less than the 6-month-old runners (Table 1). Two weeks of voluntary running significantly increased GDNF protein content 2-fold in the lumbar spinal cord of the 24-month-old rats (87.4 ± 4.6pg GDNF/mg Tissues) as compared to age-matched sedentary controls (54.4 ± 9.3pg GDNF/mg Tissue) (Figure 14a). GDNF protein levels in the spinal cord were significantly higher among the 24-month-old controls than the 6-month-old controls.

Western blot analysis also confirmed that 2 weeks of exercise increased GDNF protein expression 2-fold in the lumbar spinal cord of old rats as compared to age-matched sedentary controls (Figure 14b). Interestingly, the molecular weight of GDNF appeared to be around 41kDa in old rats compared to 30kDa in young adult rats.

Immunoreactivity for GDNF followed a similar pattern to that observed in 6-month-old rats, where we found more vesicle-like structures containing GDNF outside of ChAT-positive motor neurons in exercised animals compared to sedentary controls (Figure 15A-B). Antibody staining for colocalization of GDNF to vesicle-like structures was confirmed with the GM130 antibody (Figure 15C).
GDNF protein expression is increased in the lumbar spinal cord following 2 weeks of exercise in 24-month-old rats

Figure 14. GDNF protein content was increased in the spinal cord after 2 weeks of exercise in 24-month-old rats. The lumbar spinal cord (L1-L3) was removed from control and exercised 24-month-old animals. (A) Tissues were processed for determination of GDNF protein content using an ELISA. A significant increase in GDNF protein content was detected in the lumbar spinal cord of animals that had undergone 2 weeks of voluntary running as compared to age-matched sedentary controls. Values are displayed as mean ± SEM. Asterisk (*) indicates significance (p≤0.05) from controls. (B) Tissues
were processed for Western blot to determine GDNF protein content (top) and a loading control of α-tubulin (bottom). An increase in GDNF protein content was detected in the spinal cord of animals that had undergone 2 weeks of voluntary running as compared to controls.

**GDNF protein localization within ChAT-positive motor neurons in the lumbar spinal cord in 24-month-old rats**

<table>
<thead>
<tr>
<th>A. Control</th>
<th>Mouse anti-ChAT</th>
<th>Rabbit anti-GDNF</th>
<th>Overlay</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. Voluntary Runner</td>
<td>Rabbit anti-GDNF</td>
<td>Goat anti-GM130</td>
<td>Overlay</td>
</tr>
</tbody>
</table>

C. Vesicle-like staining
Figure 15. GDNF immunoreactivity and motor neuron cell body size increased with exercise in 24-month-old rats. Representative lumbar spinal cord sections from a 24-month-old control animal (A) and a 2 week voluntary exercised animal (B). Spinal cord sections were immunolabeled with primary antibodies against ChAT (red) and GDNF (green). Exercised animals appeared to have more vesicle-like structures containing GDNF that surrounded the motor neurons (arrows in B) as compared to controls (arrows in A). GDNF immunoreactivity was also colocalized to the GM130 antibody for vesicle-like structures (C). The scale bar represents 20μm.

ChAT-positive motor neuron cell body area in 24-month-old animals was significantly greater following 2 weeks of exercise (618.8 ± 31.5 μm$^2$) compared to that in age-matched sedentary controls (387.1 ± 18.0 μm$^2$). The ChAT-positive motor neuron cell body area from the 24-month-old sedentary controls were significantly smaller than the 6-month-old sedentary controls (p=0.02). Furthermore, the ChAT-positive motor neuron cell body area from the 24-month-old voluntary runners were significantly smaller than the 6-month-old voluntary runners (p=0.003). Again, histogram analysis of ChAT-positive motor neuron cell body areas displayed a higher frequency of the large-sized motor neurons (>1500μm$^2$) following 2 weeks of voluntary running as compared to controls (Figure 16).
Figure 16. Exercise increased ChAT-positive motor neuron cell body area of 24-month-old rats. Histogram analysis of ChAT-positive motor neuron cell body area. Following 2 weeks of exercise, voluntary runners displayed a higher frequency of cells >1500μm² as compared to 24-month-old sedentary controls.
Discussion

While other investigators have found that exercise increases neurotrophin levels in the spinal cord (Gomez-Pinilla et al., 2001, 2002; Dupont-Versteegden et al., 2004), few studies have examined the effects of exercise on spinal cord GDNF levels. The present study was designed to determine if short-term exercise (2 weeks) would alter GDNF protein content in the spinal cord of young and old animals. In young rats, involuntary running resulted in the greatest fold-change in GDNF protein content in the spinal cord, followed by swimming and voluntary running, however these levels were not statistically different. Interestingly, both young and old voluntary runners displayed the same fold-change in GDNF protein content as compared to their age-matched controls. In the exercise groups, ChAT-positive motor neuron cell body area doubled in size compared to that from age-matched sedentary controls.

Motor neuron size increases at the same time as GDNF levels following short term exercise

In animal models of aging, there is selective atrophy of large-sized motor neurons and a decrease in the total number of motor neurons that innervate hindlimb muscles (Hirofuji et al., 2000; Hashizume and Kanda, 1990). Our results confirm that motor neuron cell body size decreases with advancing age in sedentary animals. Mature motor neurons obtain trophic support from various types of cells, including Schwann cells, skeletal muscle cells and other neurons (Nishi, 1994; Oppenheim, 1996), and may resist death by increasing production of neurotrophic factors in these tissues. While there may
not be a direct correlation to the increase in neurotrophic factor content with the decrease in motor neuron size observed with aging, our observations of increased neurotrophic levels with advancing age may suggest a steady increase is in response to motor neuron loss with senescence. Acute effects of exercise were also found to increase production of neurotrophic factors. Recent studies have demonstrated links between beneficial effects of exercise, changes in neurotrophic factor levels and neuronal plasticity. Our observations of increased neurotrophic factor expression in the lumbar spinal cord following short-term exercise coupled with the increase in motor neuron size suggest acute bouts of exercise as a possible mechanism to protect motor neurons from undergoing atrophy with senescence.

**Low-intensity exercise is a potent stimulus for enhancing neurotrophic factor levels**

While some studies report that voluntary exercise increases mRNA and protein levels for neurotrophic factors in the spinal cord (Ferraiuolo et al., 2009; Macias et al., 2002; Skup et al., 2002; Ying et al., 2003), others show decreasing levels of neurotrophic factors in the spinal cord following exercise (Siamilis et al., 2009; Engesser-Casar et al., 2007). One possible contributing factor to these discrepancies may be due to variations in intensity and duration of exercise. BDNF and neurotrophin-3 mRNA and protein levels in the lumbar spinal cord are known to increase following short-term exercise (Gomez-Pinilla et al., 2001; Molteni et al., 2002; Neeper et al., 1996). Moderate-intensity exercise (13m/min) increases BDNF, but high intensity exercise decreases BDNF levels in the
brain (Aguiar et al., 2007), suggesting that low to moderate levels of exercise may be a more potent stimulus for increasing neurotrophic factor levels. Our results lend support to this idea, where we find that our moderate-intensity involuntary running protocol yielded the greatest change in GDNF protein content of all exercise regimens examined. Together, these results may suggest that short-term, moderate-intensity exercise programs may be a better stimulus for enhancing neurotrophic factor content in the spinal cord.

**Punctate immunoreactivity for GDNF is altered with exercise and age**

A punctate staining pattern for GDNF has been found in neuronal cell bodies, dendrites and axons (Kawamoto et al., 2000). In cultured neuroendocrine cells, the staining pattern for GDNF appears to be localized to vesicle-like structures (Lonka-Nevalaita et al., 2010). Moreover, in axons of dorsal root ganglion neurons GDNF is present in dense-core vesicles (Ohta et al., 2001) and in rat primary cortical and hippocampal neurons, the immunostaining pattern of GDNF appears in vesicle-like structures at the tips of neurites, where the authors suggest transportation of GDNF to the cell periphery (Lonka-Nevalaita et al., 2010). Within the spinal cord, we observed vesicle-like staining for GDNF that was confirmed with the GM130 antibody, where our results suggest that exercise increases the incidence of GDNF positive immunoreactivity. These observations are in accordance with our ELISA values, where GDNF protein content is increased following exercise in both young and old animals as well as increased with advancing age in control animals.
Molecular weight of GDNF changes with age

The reported molecular weight of GDNF varies in the literature depending on the cells/tissues examined. Lin et al. (1994) describe GDNF from cultured dopaminergic cells as having a molecular weight of 33-45kDa in non-reduced gels and 15-21kDa from reduced gels. GDNF protein size was reported to be 24kDa in cultured primary fibroblasts (Blesch and Tuszynkski, 2001), 30kDa in cultured neuroblastoma cells (Larsen et al., 2006) and 34kDa in 18-week-old human fetal spinal cord (Koo and Choi, 2001). We found the molecular weight of GDNF in the rat lumbar spinal cord to be 30kDa in young animals and 41kDa in old animals. Similarly, several NGF isoforms have been reported in various whole tissues of both humans and animals (reviewed by Al-Shawi et al., 2007). One explanation for the variations in molecular weights may be due to modifications of the prodomain regions of the protein. Pro-neurotrophins, which are the precursor forms of neurotrophins, are synthesized and then cleaved by furin and other proteases to produce mature neurotrophins (Lee et al., 2001). Mature NGF and BDNF induce neuronal survival, differentiation and synaptic modulation (Huang and Reichardt, 2001). Results of other studies suggest that the precursor of NGF may be either neurotoxic (Ibanez, 2002) or significantly less neurotrophic than the mature form of NGF (Fahnestock et al., 2004). Pro-neurotrophins, such as pro-BDNF and pro-NGF, induce cell death by activating an apoptotic cascade via binding to cell death complexes involving sortilin and p75 receptors (Lee et al., 2001; Nykjaer et al., 2004). Moreover, pro-NGF has been found to be upregulated with aging and disease. Pro-NGF is increased
in the superior cervical ganglia of old rats (Bierl and Isaacson, 2007), the parietal cortex of patients with Alzheimer's disease (Peng et al., 2004) and in spinal cord oligodendrocytes from a murine model of spinal cord injury (Beattie et al., 2002). Our observation of increasing molecular weight for GDNF with advancing age may suggest a similar phenomenon is occurring in spinal cord of rat, possibly demonstrating increased expression of a higher molecular weight pro-form of GDNF in the older animals. It has been shown that pro-NGF is secreted by reactive astrocytes, and may affect motor neuron survival (Domeniconi et al., 2007). The spinal cord samples processed in the current study would contain motor neurons, interneurons and glial cells, which could account for the pro-GDNF, if it is associated with glial cells. While it is still unknown if the pro form of GDNF activates similar apoptotic pathways as NGF, it is known that post-translational modifications of GDNF are due to prohormone convertase that cleaves five consensus sites giving rise to four different peptide forms of processed GDNF (Oh-hashi et al., 2009; Immonen et al., 2008). Future studies are warranted to determine if proGDNF is less neuroprotective or neurotoxic and how aging affects its expression.

**GDNF transport following exercise**

GDNF is produced by motor neurons, oligodendrocytes and Schwann cells in the spinal cord (Henderson et al., 1994; Rind and von Bartheld, 2002; Russell et al., 2000; Yamamoto et al., 1996) as well as by skeletal muscles (Yamamoto et al., 1996). Both anterograde and retrograde transport between neurons and target tissues have been demonstrated for GDNF (Rind and von Bartheld, 2002; Russell et al., 2000). While our
results did not determine which cells are producing the GDNF protein observed in the spinal cord, we have previously shown that GDNF protein is increased in skeletal muscle following short term exercise (McCullough et al., 2011; Wehrwein et al., 2002), which could be transported back to the spinal cord, resulting in the elevated levels observed in the current study. It has been shown that skeletal muscle derived GDNF has more potent effects for the neuromuscular system than that supplied by anterograde transport (Li et al., 2007), thus elevated retrograde transport of GDNF from the muscle following exercise may represent an important stimulus for enhanced plasticity.

**Exercise stressors**

There is evidence that stress is unlikely to be the critical factor underlying the differential effects of voluntary and forced running (Leasure and Jones, 2008). Forced running acutely elevates corticosterone levels, the rodent stress hormone, more than voluntary running (Ploughman et al., 2005, 2007); however, these levels return to baseline within a few hours after exercise (Stranahan et al., 2006; Ploughman et al., 2007) and after several weeks of exercise these levels are no longer elevated (Fediuc et al., 2006). Stress is also known to activate microglia (Nair and Bonneau, 2006; Sugama et al., 2007); however, neither forced nor voluntary running enhances microglial activity in the brain (Leasure and Jones, 2008). These observations may suggest that the exercise-induced changes in neurotrophic factor expression observed in the current study are not likely to be dependent on a stress response.
Conclusion

In conclusion, the results demonstrate that short-term exercise increases GDNF protein content, GDNF immuno-labeling and motor neuron size in the spinal cord of young and old animals. These results are consistent with our hypothesis that the neural protection/neural plasticity caused by exercise may be driven, in part, by enhanced GDNF production. In addition, there may be a relationship between the intensity of exercise and the amount of GDNF protein produced, where a low-intensity exercise protocol yields the greatest increase in GDNF protein content. We believe that exercise has the advantage of enhancing neurotrophic factor levels by physiological means using intrinsic mechanisms in the spinal cord rather than attempting to increase neurotrophic factor levels via exogenous administration where all the physiological implications are not well understood.
CHAPTER IV

CHRONIC EXERCISE DOES NOT ALTER GDNF PROTEIN CONTENT IN ADULT RAT SPINAL CORD

Introduction

Neurotrophic factors are extracellular signaling proteins that are important for motor neuron survival during developmental programmed cell death and in response to injury and neurodegeneration (Henderson, 1996; Gould and Oppenheim, 2006). One of the most potent neurotrophic factors for peripheral motor neurons is GDNF (Henderson et al., 1994). GDNF is critical for proper motor neuron development and alterations in GDNF levels can have effects on early motor neuron survival (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995; Nguyen et al., 1998; Keller-Peck et al., 2001; Zwick et al., 2001; Nagano and Suzuki, 2003). GDNF is found in a wide variety of cells both in the central nervous system and in the peripheral nervous system, and may have more target neurons than any other neurotrophic factor (Bohn, 2004). The spinal cord and skeletal muscles also expresses the GDNF receptors, GDNF family co-receptor α1 (GFRα-1) and receptor tyrosine kinase (RET) (Henderson et al., 1994; Widenfalk et al., 1997; Hase et al., 1999). Increased expression of GDNF in developing skeletal muscle leads to increased axonal branching and increased motor unit size (Nguyen et al., 1998; Zwick et al., 2001), while treatment with exogenous GDNF causes continuous synaptic remodeling at the neuromuscular junction (Keller-Peck et al., 2001). GDNF is involved in the maintenance of cell body size and the cholinergic phenotype of motor neurons
(Ulfhake et al., 2000) and it has been hypothesized that GDNF may regulate the physiological function of developing and adult motor neurons (Wang et al., 2001; Martin-Caraballo and Dryer, 2002).

GDNF is crucial in the maintenance of mature adult motor neurons. Results of studies examining loss of neurons following nerve injury or in neurodegenerative diseases have revealed the trophic importance of GDNF to mature motor neurons. Exogenous GDNF prevents neuronal loss and maintains motor function in a mouse model of Huntington’s disease (Ebert et al., 2010). GDNF mRNA is upregulated in injured nerves, skeletal muscle, and Schwann cells after transection (Naveilhan et al., 1997; Frostick et al., 1998; Lie and Weis, 1998). More importantly, following transection, GDNF mRNA remains elevated for 5 or 6 weeks; in contrast with members of the neurotrophin family whose upregulation exists for only 2 weeks (Frostick et al., 1998; Michalski et al., 2008). This elevation of GDNF mRNA may suggest an immediate and longer-term trophic need by the damaged nerve. Results from these studies suggest that adult motor neurons depend primarily on GDNF as their trophic factor for survival and that GDNF is critical for the maintenance of the neuromuscular system.

Decreased size and loss of spinal motor neurons innervating skeletal muscle are observed in aging humans and rodents (Kawamura et al., 1977; Ishihara et al., 1987; Hashizume et al., 1988; Jacob, 1998). These negative motor neuron alterations contribute to the process of sarcopenia, which is the loss of skeletal muscle mass and strength (Rosenberg, 1997; Welle, 2002; Doherty, 2003). Mature motor neurons obtain trophic
support from various types of cells, including Schwann cells, skeletal muscle fibers and other neurons (Nishi, 1994; Oppenheim, 1996). Since increased amounts of physical activity have been shown to prevent the loss of motor neurons in aging individuals (Kanda and Hashizume, 1998), exercise may also be a way to increase production of neurotrophic factors.

There is an increasing body of evidence that suggests physical activity mediates neurotrophic factor expression and thus promote neuroprotective effects (Trejo et al., 2001; Fabel et al., 2003; Wu et al., 2008). Exercise increases levels of neurotrophic factors in the central nervous system (Neeper et al., 1995; Vaynman et al., 2001) and in the peripheral nervous system (Wehrwein et al., 2002; McCullough et al., 2011). An activity-dependent relationship of neurotrophic factors has been demonstrated in skeletal muscle where neurotrophin-4 expression is altered in proportion to the intensity of exercise within skeletal muscle (Funakoshi et al., 1995). The resulting change in neurotrophic factors has positive effects for neuronal plasticity and regeneration. Both long-term and short-term exercise are found to increase neurotrophic factor protein and mRNA levels at the level of the spinal cord in adult rats (Macias et al., 2002; Skup et al., 2002; Ying et al., 2003). We have previously found that GDNF protein content is increased in the slow-twitch soleus muscle but is decreased in the fast-twitch extensor digitorum longus muscle following short-term exercise (McCullough et al., 2011), and increased in young and old rat spinal cord following short-term exercise (submitted for
publication). The purpose of this study was to determine if GDNF protein content is altered in the lumbar spinal cord following long-term (chronic) exercise.

Materials and methods

Experimental design

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. Adult male Sprague-Dawley rats (Charles River, Kalamazoo, MI) aged 12 months at the beginning of the studies were randomly assigned to a sedentary control group (n=10) and an exercise group. Animals from the exercise group had undergone a total duration of voluntary running for either 4 weeks (n=5) or 6 months (n=5). We thought that voluntary running would have the advantage over involuntary running because it allows the animals to choose their own desired duration and intensity of running, unfortunately even with this thought, there was a casualty among the 6 month exercise group. The exercise groups had continuous access to individually housed running wheels where running activity, such as speed, distance, and duration, was recorded with the activity wheel monitoring system (Lafayette Instruments, Lafayette, IN). All animals were given access to food and water ad libitum and were maintained on a 12h light/dark cycle. At the end of the experimental period, all rats were euthanized via
CO₂ asphyxiation followed by thoracotomy and the lumbar spinal cord was removed. All tissues were stored at -80°C until processed.

**Tissue processing**

The lumbar spinal cord (L1 – L3) was removed and frozen on dry ice for protein quantification. The L1 – L3 spinal cord section was chosen because these motor neurons innervate to the muscles of the quadriceps, gluteus, soleus, adductor muscles, flexor muscles, and extensor muscles including the extensor hallucis longus and extensor digitorum longus (Nicolopoulous-Stournaras and Iles, 1983). To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, and 0.05% Tween-20) was added in a 20:1 (buffer volume : tissue weight) dilution and the mixture was homogenized on ice. Samples were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C.

**GDNF protein quantification**

GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (McCullough et al., 2011). Briefly, 96-well plates were incubated overnight at room temperature in a humidified chamber with a monoclonal antibody raised against GDNF (R&D Systems, Minneapolis, MN). The following day, plates were rinsed with wash buffer and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose for 1 hour at room temperature. Plates
were rinsed with wash buffer and the GDNF standard (R&D Systems) or tissue supernatants were added to the wells. For each assay, a standard curve was calculated from the known GDNF standard concentration, ranging from 1000pg/ml to 2pg/ml of GDNF protein. Following a 2 hour incubation at room temperature, the plates were rinsed with wash buffer followed by the incubation of a biotinylated anti-GDNF secondary antibody (R&D Systems) for 2 hours at room temperature. The plates were then washed and coated with β-galactosidase conjugated to streptavidin (Molecular Probes, Eugene, OR) for 20 min at room temperature. The plates had a final wash and chlorophenol red-β-D-galactopyranoside (CPRG) substrate was added (in PBS + BSA) and plates were read at an absorbance of 575nm.

**Western blot**

Total protein content was measured by a Pierce® BCA protein assay (Thermo Scientific, Rockford, IL) according to manufacturer specifications. Homogenized lumbar spinal cord (L1 – L3) supernatant was used to determine total protein concentration using a bicinchoninic (BCA) protein assay with BSA as the protein standard, ranging from 1000µg/ml to 25µg/ml. All samples and standards were read at an absorbance of 562nm. Tissue samples were then prepared for Western blot analysis of GDNF protein content as previously described by Vianney and Spitsbergen (2011). Briefly, 20µg of the tissue samples were prepared for loading into gels with Laemmli 2X Loading buffer. A protein ladder (New England BioLabs, Ipswich, MD), a positive control of a GDNF standard, and a negative loading control were added. The samples were boiled for 5 min then
loaded into a 15% polyacrylamide gel. The gel was submerged in migration buffer and run at 100V for 30 min and then 200V for 1 hour at room temp. The gel was transferred to a polyvinylidene difluoride membrane (PVDF; Invitrogen, Carlsbad, CA). The gel and membrane were submerged in Tris-glycine transfer buffer and transferred at 12V. After transferring, the PVDF membrane was blocked non-specifically with Tropix® I-Block (Applied Biosystems, Foster City, CA) for 1 hour at 4°C under agitation, followed by incubation with a primary antibody against GDNF (Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C under agitation. The membrane was washed in buffer, and then was incubated with a HRP-conjugated secondary antibody (ECL; GE Healthcare) for 1 hour at room temperature under agitation. The ECL detection kit was used to visualize the proteins and developed on x-ray films (Kodak). ImageJ software was used to measure the relative density of GDNF bands and values were expressed as ratios of controls.

**Immunohistochemistry**

Lumbar spinal cord sections (L4–L5) were fixed in 4% paraformaldehyde overnight at 4°C and then washed in fresh phosphate buffered saline. Tissues were embedded in O.C.T. compound mounting medium, cut into 40µm transverse sections on a cryotome, and thaw mounted onto Histobond® slides (VWR International, Bridgeport, NJ). Slides were incubated overnight at 4°C with primary antibodies (1:200) of rabbit anti-GDNF (Santa Cruz Biotechnology, Santa Cruz, CA) and (1:200) mouse anti-ChAT (Millipore, Temecula, CA) in phosphate buffered saline containing 1% bovine serum
albumin and 0.1% triton X-100. Slides were then incubated with secondary antibodies (1:500) of donkey anti-mouse conjugated to Alexafluor 568 and donkey anti-rabbit conjugated to Alexafluor 488 for 2 hours at room temperature. Negative control slides had primary antibodies omitted. Slides were viewed with a Zeiss LSM 510 laser scanning confocal microscope and images were examined with the Zeiss LSM 5 Image Examiner program.

**Measurement of motor neuron cell body size**

Since somatic motor neurons stain positively for ChAT immunoreactivity (Wetts and Vaughn, 1996), ChAT-positive motor neuron cell body areas were measured in Lamina IX of the L4-L5 region of the spinal cord (Jacob, 1998) from all animals. Twenty representative motor neuron cell bodies were selected from each animal. Motor neuron cell body areas were calculated with the Zeiss LSM 5 Image Examiner program and exercised animals were compared to age-matched sedentary controls.

**Statistical analysis**

All data values are reported as mean ± the standard error of the mean (SEM). GDNF protein values are expressed as picogram per milligram of wet tissue weight. Data were analyzed using one-way ANOVA and Tukey’s post-hoc comparison for differences among the independent groups, where p values ≤ 0.05 were considered as statistically significant.
Results

Four weeks of voluntary running

Previous results from our laboratory found that GDNF protein content was increased in skeletal muscle following 4 weeks of treadmill running (Wehrwein et al., 2002). Thus we tested 4 weeks of wheel running to determine if GDNF protein content in the spinal cord would also increase in adult animals. Animal weights from exercised animals (545.9 ± 21.0g) were not different from their 12-month-old age-matched sedentary controls (544.7 ± 29.4g). The exercised animals reached a maximum speed of 15m/min, which was lower than the 6-month-old voluntary runners, ran a total distance of 14.6km ± 3.7km and averaged 0.5km ± 0.04km per day. A positive correlation (p =0.03) was observed between maximum speed and total distance run among individual animals.

Four weeks of voluntary running did not alter GDNF protein content in the lumbar spinal cord of the 12-month-old animals (13.7 ± 2.9pg GDNF/mg Tissue weight) as compared to their age-matched sedentary controls (11.4 ± 2.7pg GDNF/mg Tissue weight) (Figure 17A). Western blot analysis of GDNF showed a similar trend as the ELISA, where the relative density of the exercised animals had a 1.2-fold increase as compared to their respective age-matched controls. The molecular weight of GDNF was observed to be at ~30kDa in both the exercised and control animals. We visualized a more prominent double band of GDNF among the exercise animals as compared to the controls (Figure 17B).
4 weeks of exercise does not alter GDNF protein content in the adult spinal cord

Figure 17. Four weeks of voluntary running has no effect on GDNF protein content in the lumbar spinal cord of 12-month-old rats. The lumbar spinal cord (L1-L3) was removed from control and exercised animals. (A) Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM. (B) Both control and exercised animals express GDNF protein at a molecular weight of 30kDa in the lumbar spinal cord. Exercised animals display a double band of GDNF as compared to sedentary controls.
A negative correlation (p=0.04) was observed between GDNF protein content and total distance run by each animal (Figure 18). This demonstrated that the animals that ran the greatest distances had the lowest amount of GDNF protein content in their lumbar spinal cord. No relationship was observed between GDNF protein content and maximum running speed among individual animals.

ChAT-positive motor neurons in the lumbar spinal cord were observed to have positive immunoreactivity for GDNF in both the control and exercised 12-month-old animals (Figure 19), however differences were visualized between the two groups. The control animals had GDNF immunoreactivity within ChAT-positive cells as well as within the entire lumbar spinal cord section. Vesicle-like structures of GDNF were not co-localized to the motor neurons cells. Among the exercised animals, GDNF immunoreactivity was co-localized to the ChAT-positive motor neurons. Unlike the images following short-term exercise in young and old rats, we observed very few vesicle-like structures of GDNF within the motor neurons. ChAT-positive motor neuron cell body area was significantly greater following 4 weeks of voluntary exercise (967.9 ± 46.2μm²) as compared to age-matched sedentary controls (764.4 ± 26.5μm²). Histogram analysis of ChAT-positive motor neuron cell body areas displayed a higher frequency of the large-sized motor neurons (>1500μm²) following 4 weeks of voluntary running as compared to controls (Figure 20).
Negative correlation between GDNF protein content in the spinal cord versus total distance run by each animal

Figure 18. The 12-month-old animals that ran the farthest distance had lower values of GDNF protein content in their lumbar spinal cord. Individual animals had their total distance run in 4 weeks compared to their GDNF protein value found in the spinal cord. Regression analysis found a significant correlation (p=0.04) between these two variables.
GDNF immunoreactivity is localized to motor neurons in 12-month-old control and exercised animals

Figure 19. Representative lumbar spinal cord sections of a sedentary 12-month-old control animal (A) and a voluntary exercise animal (B). Spinal cord sections were immunolabeled with primary antibodies against ChAT (red) and GDNF (green). The motor neurons from the exercised animals have a larger cell body area as compared to 12-month-old age-matched, sedentary control motor neurons. The scale bar represents 20μm.
Figure 20. Exercise increased ChAT-positive motor neuron cell body area of 12-month-old rats. Histogram analysis of ChAT-positive motor neuron cell body area. Following 4 weeks of exercise, voluntary runners displayed a higher frequency of cells >1500μm² as compared to 12-month-old sedentary controls.
Six months of voluntary running

Six months of exercise had no significant effect on animal weight (519.6 ± 14.3g) as compared to the 18-month-old age-matched sedentary controls (544.7 ± 36.4g). The exercised animals reached a maximum speed of only 6m/min, ran a total distance of 59.9km ± 13.6km and averaged 0.3km ± 0.01km per day.

Six months of voluntary running did not alter GDNF protein content in the lumbar spinal cord (27.1 ± 9.3pg GDNF/mg tissue weight) compared to the sedentary controls (32.0 ± 10.7pg GDNF/mg tissue weight) (Figure 21A). When comparing the 18-month-old controls to the 12-month-old controls there was a trend toward an increase in GDNF protein content (p = 0.07) with advancing age. Unlike the 12-month-old animals there was no correlation observed between GDNF protein content in the spinal cord and max speed or between GDNF protein content and total distance run among the individual 18-month-old rats following 6 months of voluntary running. Western blot analysis of GDNF revealed a similar trend as the ELISA, where the relative densities for the 18-month-old exercised animals had a 0.9-fold decrease as compared to their sedentary controls. Interestingly, both the exercised and sedentary animals revealed a molecular weight of GDNF at ~40kDa in the lumbar spinal cord (Figure 21B), compared to the 30kDa molecular weight found with the 12-month-old animals.
6 months of voluntary running does not alter GDNF protein content in the rat spinal cord

**Figure 21.** Long-term of exercise of 6 months has no effect on GDNF protein content in the lumbar spinal cord of 18-month-old rats. The lumbar spinal cord (L1-L3) was removed from control and exercised animals. (A) Tissues were processed for GDNF protein content using an ELISA. Voluntary exercise did not alter GDNF protein content in the rat spinal cord as compared to controls. Values are displayed as mean ± SEM. (B) Tissues were processed for GDNF protein content using an ELISA. The molecular weight of GDNF is ~40kDa in the lumbar spinal cord from 18-month-old rats.
We were able to visualize GDNF within the ChAT-positive motor neurons of the lumbar spinal cord of both the control and exercised 18-month-old animals (Figure 22). Vesicle-like structures of GDNF were not co-localized to the motor neurons cells. Among the exercised animals, GDNF immunoreactivity was co-localized to the ChAT-positive motor neurons. Unlike the images following short-term exercise in young and old rats, we observed very few vesicle-like structures of GDNF surrounding the motor neurons. ChAT-positive motor neuron cell body area was significantly greater following 6 months of voluntary exercise (610.8 ± 18.0μm²) as compared to age-matched sedentary controls (354.4 ± 18.0μm²), where the histogram displays the shift in size (Figure 23).

GDNF in the spinal cord of 18-month-old control and exercised rats
**Figure 22.** Representative lumbar spinal cord sections of a sedentary 18-month-old control animal (A) and a voluntary exercise animal (B). Spinal cord sections were immunolabeled with primary antibodies against ChAT (red) and GDNF (green). Few vesicle-like structures of GDNF were observed in the spinal cord of the control and exercised animals (arrows). The motor neurons from the exercised animals have a larger cell body area as compared to 18-month-old age-matched, sedentary control motor neurons. The scale bar represents 20μm.

**Histogram analysis of the 18-month-old motor neurons**

![Histogram](image)
Figure 23. Exercise increased ChAT-positive motor neuron cell body area of 18-month-old rats. Histogram analysis of ChAT-positive motor neuron cell body area. Following 6 months of exercise, voluntary runners displayed a higher frequency of large cells as compared to 18-month-old sedentary controls.

Discussion

The purpose of this study was to determine if GDNF protein levels are altered in the lumbar spinal cord with advancing age and following chronic exercise. Long-term voluntary running of 4 weeks and 6 months does not alter GDNF protein content in the lumbar spinal cord as it does with short-term exercise. Interestingly, the molecular weight of GDNF in the spinal cord increases with advancing age. These results suggest that while the amount of GDNF protein does not change with long-term exercise, the size of the protein expressed is altered in the spinal cord with senescence. Both durations of chronic exercise did increase ChAT-positive motor neuron cell body area; however,
GDNF appears to be more localized within the motor neurons and does not surround the motor neurons.

**Aging increase molecular weight of GDNF in the spinal cord**

In accordance to our previous findings, the molecular weight of GDNF in the lumbar spinal cord increased with advancing age. We have shown that the lowest molecular weight of GDNF is mostly expressed in the youngest animals examined (6 months) followed by a sequential increase in size expressed with advancing age. Prodomain regions of neurotrophins, such as proBDNF and proNGF, have been found to induce cell death (Lee et al., 2001; Nykjaer et al., 2004), and are expressed in aging and diseased conditions (Beattie et al., 2002; Peng et al., 2004; Bierl and Isaacson, 2007). While it is still unknown if the GDNF family of neurotrophic factors are involved in this similar cell death process, others have shown that the proregion of the transforming growth factor β affects the dimerization, folding and activity of mature proteins (Gray and Mason, 1990). Since GDNF is a member of this superfamily, the altered size of GDNF in the spinal cord may be an indication of altered activity of GDNF, such as inducing neurotoxic effects or less potent neurotrophic effects.

**Chronic exercise does not increase neurotrophic factor protein levels**

Chronic physical activity has been suggested to have both a neuroprotective and neurogenerative influence (Rhodes et al., 2003; Smith and Zigmond, 2003) and can increase neurotrophic factors in the neuromuscular system (Wehrwein et al., 2002; Adlard and Cotman, 2004). However not all neurotrophic factors may respond the same
to increased levels of physical activity in the neuromuscular system. For instance, brain-
derived neurotrophic factor (BDNF) was the only neurotrophic factor to have an increased expression following long-term exercise, whereas expression of nerve growth factor (NGF) and fibroblast growth factor-2 were increased following short-term exercise in the hippocampus (Molteni et al., 2002). More evidence is accruing that short, but not long durations, of exercise increases neurotrophic factor mRNA levels, but not protein levels in hindlimb skeletal muscles (Gomez-Pinilla et al., 2001; Ying et al., 2003; Cuppinf et al., 2007), suggesting that neurotrophic factors may be differentially regulated in the neuromuscular system depending on the level of physical activity. Protein levels for neurotrophic factors in the lumbar spinal cord, as measured both by ELISA and immunohistochemistry, are increased following short-term, moderate-intensity running protocols (Gomez-Pinilla et al., 2001; Ying et al., 2003). Our data lends support to this notion as we have found that long durations of exercise do not affect GDNF protein levels, as measured by ELISA and Western blot, in the spinal cord. Recently, we have found that short-term exercise of 2 weeks increases GDNF protein content in hindlimb skeletal muscle (McCullough et al., 2011) and in the lumbar spinal cord (McCullough et al., in press). Coupled together, these results may suggest that short-term exercise may be a more potent stimulus to modulate GDNF protein expression in the neuromuscular system than long-term exercise in adult animals.

Decreased expression of neurotrophins (Ming et al., 1999; Bergman et al., 2000), their receptors (Bergman et al., 1996, 1999), and other growth factors (Renganathan et
al., 1997; Owino et al., 2001) have all been observed in the neuromuscular system of aged rats. Neurotrophic factors are known to bind to membrane-associated receptors where they undergo internalization, and then are actively transported along the axon. Both anterograde and retrograde transport of GDNF from motor neurons has been reported (Tomac et al., 1995; Leitner et al., 1999; Russell et al., 2000; Rind and von Bartheld, 2002). While there is some debate whether axonal transport of the neurotrophic factor/receptor complex is absolutely necessary for survival (MacInnis and Campenot, 2002), transport of neurotrophic factors to the motor neuron cell body is required to elicit a full cellular response (Ye et al., 2003). Evidence has shown that disruption of retrograde axonal transport decreases trophic factors from maintaining the nerve terminal (Griffin and Watson, 1988; LaMonte et al., 2002). While our results did not determine which cells are producing the GDNF protein observed in the spinal cord, we have previously shown that GDNF protein is increased in skeletal muscle following exercise (Wehrwein et al., 2002; McCullough et al., 2011), which could be transported back to the spinal cord. This may be limited to acute responses in activity, such as with short-term exercises, as we did not observe increases in GDNF protein content in the spinal cord following long-term exercise, nor did we see as many vesicle-like structures of GDNF with immunohistochemistry. Our observations of increased GDNF protein content with short-term but not long-term exercise suggests that the demand for this neurotrophic factor may be limited to its production or be needed in different cells. Future studies need to determine what cells are producing GDNF and where the protein is being transported in response to acute and chronic exercise.
**Hypertrophy of motor neurons with chronic exercise**

It is well known that exercise increases motor neuron cell body area and we found that following chronic exercise, our representative sample of motor neurons examined demonstrated an increased area in both groups of animals, with co-localization of GDNF immunoreactivity within the motor neurons. Volume of motor neuron cell body areas have been reported to show an increase (Nakano et al., 1997) as well as a decrease (Beaumont and Gardiner, 2003) depending on the intensity of the exercise training. The running intensities of both groups of exercised animals in this study was of moderate- to low-intensity. Moderate- intensity treadmill running (13m/min) has been demonstrated to activate the small motor neurons belonging to the slow motor units (Deforges et al., 2009), and are associated with low hindlimb movement amplitude and frequency that activates subpopulations of small lumbar spinal cord motor neurons (Grondard et al., 2008). Chronic exercise has been demonstrated to increase average diameter of motor neurons that innervate the slow-twitch soleus (Nakano et al., 1997). However, we did not observed changes in GDNF protein content that correlate to changes in size of motor neurons measured as we had previously observed following short-term exercise. There is a possibility that neurotrophic factors do not exclusively increase the survival of cells but rather induce hypertrophy of cells or cell nuclei (Li et al., 1995; von Bartheld et al., 1995), which may lead to false identification of γ-motor neurons as α-motor neurons (Simon et al., 1996). It would be important to determine if differences in durations of
exercise affect the survivability or motor neurons or hypertrophy of motor neurons to induce protein synthesis in the cell bodies.

**Conclusion**

In conclusion, we have found that chronic voluntary running does not elicit an increase of GDNF protein content nor a punctate pattern of immunolabeling of GDNF in the lumbar spinal cord. Aging increases the molecular weight of GDNF that is expressed as well as an increase in GDNF content in the spinal cord. Understanding how neurotrophic factors are regulated by physical activity are crucial for developing therapeutic strategies targeted at individuals suffering decreased levels of GDNF, such as with nervous system injury or disease. Based on the results of alterations of GDNF protein within the spinal cord of aging animals due to differences in exercise protocols, we decided to examine changes in GDNF protein in the spinal cord of wild type mice and transgenic models of advanced neuromuscular degeneration to determine a further role for GDNF in the motor nervous system following exercise.
CHAPTER V

IN Voluntary Exercise Increases GDNF Protein Content In the Lumbar Spinal Cord Of Wild Type Mice

Introduction

Changes in motor neuron structure and function caused by GDNF resemble the changes observed with altered physical activity. Increased expression of GDNF in developing skeletal muscle leads to increased axonal branching and increased motor unit size (Nguyen et al., 1998; Zwick et al., 2001), while treatment with exogenous GDNF causes continuous synaptic remodeling at the neuromuscular junction (Keller-Peck et al., 2001) and prevents motor neuron degeneration following axotomy (Oppenheim et al., 1995). Choline acetyltransferase (ChAT) activity of embryonic motor neurons is increased by GDNF (Zurn et al., 1994). GDNF rescues somatic motor neurons from natural occurring cell death (Oppenheim et al., 2000) and from axotomy-induced cell death (Oppenheim et al., 1995), and protects motor neurons from chronic degeneration (Corse et al., 1999).

One suggested role for neurotrophic factors includes contributing to the beneficial neuroprotective effects of exercise-induced changes in the motor nervous system (Gomez-Pinilla et al., 2001, 2002; Trejo et al., 2001; Wu et al., 2008). Activity-dependent relationships between levels of exercise and neurotrophins has been demonstrated in skeletal muscle (Funakoshi et al., 1995). We have previously shown that
both 2 weeks and 4 weeks of exercise can increase GDNF protein content in rat hind limb skeletal muscles (Wehrwein et al., 2002; McCullough et al., 2011). Neurotrophin levels in the spinal cord have been shown to be increased following exercise (Gomez-Pinilla et al., 2001, 2002; Dupont-Versteegden et al., 2004; Hutchinson et al., 2004), however it is not known if GDNF plays a similar role. Our previous results suggest that GDNF protein content is increased in the rat spinal cord following short-term exercise but not following long-term exercise. We have found that GDNF protein content is increased in the spinal cord of young and old rats following 2 weeks of exercise, yet chronic exercise does not alter GDNF protein content in the rat lumbar spinal cord. Based on those results, the goal of this study was to determine if GDNF protein content increases following 2 weeks of low-intensity exercise in the mouse spinal cord. We tested the effects of short-term exercise on GDNF protein levels in the wild type mouse prior to performing studies in transgenic mouse models of amyotrophic lateral sclerosis to determine if alterations in GDNF would follow a similar trend in the mouse spinal cord as in the rat.

Materials and methods

Training protocol

Male C57BL/6 mice (Charles River, Kalamazoo, MI) aged 3 months were randomly assigned to a age-matched, sedentary control group (n=6) and an involuntary running group (n=6). The exercised animals were placed in individual forced running wheels (Lafayette Instruments, Lafayette, IN). These animals were exercised at 8 m/min
for a total of 30min/day, including a brief warm-up and cool-down period. The exercised mice were acclimated to the running wheels for one week prior to the beginning of the study. All animals were given access to food and water *ad libitum* and were maintained on a 12h light/dark cycle. 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. At the end of the experimental period, all mice were euthanized via CO₂ asphyxiation followed by thoracotomy and the lumbar spinal cord was removed. All tissues were stored at -80°C until processed.

**Tissue processing**

The lumbar spinal cord sections of L1 – L3 were removed and frozen on dry ice for protein quantification. This region was chosen because these motor neurons innervate to the muscles of the quadriceps, gluteus, adductor muscles, flexor muscles, extensor muscles including the extensor hallucis longus and extensor digitorum longus, and the soleus (Nicolopoulous-Stournaras and Iles, 1983). To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, and 0.05% Tween-20) was added in a 20:1 dilution factor and the mixture was homogenized on ice. Samples were centrifuged for 30 min at 4°C and supernatant was collected and stored at -80°C until ready for processing.
GDNF protein quantification

GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (McCullough et al., 2011). Briefly, 96-well plates were incubated overnight at room temperature in a humidified chamber with a monoclonal antibody raised against GDNF (R&D Systems, Minneapolis, MN). The following day, plates were rinsed with wash buffer and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose for 1 hour at room temperature. Plates were rinsed with wash buffer and the GDNF standard (R&D Systems) or tissue supernatants were added to the wells. For each assay, a standard curve was calculated from the known GDNF standard concentration, ranging from 1000pg/ml to 2pg/ml of GDNF protein. Following a 2 hour incubation at room temperature, the plates were rinsed with wash buffer followed by the incubation of a biotinylated anti-GDNF secondary antibody (R&D Systems) for 2 hours at room temperature. The plates were then rinsed with wash buffer and coated with β-galactosidase conjugated to streptavidin (Molecular Probes, Eugene, OR) for 20 minute incubation at room temperature. The plates had a final wash and chlorophenol red-β-D-galactopyranoside (CPRG) substrate was added (in PBS + BSA) and the plates incubated until the color had developed. Plates were read at an absorbance of 575nm.

Western blot

Homogenized lumbar spinal cord (L1 – L3) supernatant was used to determine total protein concentration using a Pierce® bicinchoninic (BCA) protein assay (Thermo
Scientific, Rockford, IL) with BSA as the protein standard, ranging from 1000µg/ml to 25µg/ml, according to manufacturer specifications. All samples and standards were read at an absorbance of 562nm. Tissue samples were then prepared for Western blot analysis of GDNF protein content as previously described by Vianney and Spitsbergen (2011). Tissue samples (20µg) were prepared for loading into gels with Laemmli 2X Loading buffer. A protein ladder (New England BioLabs, Ipswich, MD) and a negative loading control of NGF were added. The samples were boiled for 5 min then loaded into a 15% polyacrylamide gel. The gel was submerged in migration buffer and run at 100V for 30 min and then 200V for 1 hour at room temp. The gel was transferred to a polyvinylidene difluoride membrane (PVDF; Invitrogen, Carlsbad, CA). The gel and membrane were submerged in Tris-glycine transfer buffer and transferred at 12V. After transferring, the PVDF membrane was blocked non-specifically with Tropix® I-Block (Applied Biosystems, Foster City, CA) for 1 hour at 4°C under agitation, followed by incubation with a primary antibody against GDNF (Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C under agitation. The membrane was washed in buffer, and then was incubated with a HRP-conjugated secondary antibody (ECL; GE Healthcare) for 1 hour at room temperature under agitation. The ECL detection kit was used to visualize the proteins and developed on x-ray films (Kodak).

**Immunohistochemistry**

To determine localization of GDNF protein within the lumbar spinal cord, the sections of L4 - L5 were used because these motor neurons innervate the muscles of the
hamstrings, adductor muscles, flexor muscles, extensor muscles including the extensor hallucis longus and extensor digitorum longus, gastrocnemius and the soleus (Nicolopoulous-Stournaras and Iles, 1983). Spinal cord sections were fixed in 4% paraformaldehyde overnight at 4°C and cryoprotected in 30% sucrose overnight. Tissues were embedded in optimal cutting temperature (O.C.T. compound) mounting medium and cut into 40µm transverse sections on a cryotome that were thaw mounted onto Histobond® slides (VWR International, Bridgeport, NJ). Slides were incubated overnight (4°C) with primary antibodies that were diluted 1:200 in PBS containing 1% BSA and 0.1% triton X-100. Sections were exposed to rabbit anti-GDNF (Santa Cruz Biotechnology) and mouse anti-choline acetyltransferase (ChAT; Millipore). Slides were washed in PBS before the addition of secondary antibodies of donkey anti-mouse conjugated to Alexafluor 568, donkey anti-rabbit conjugated to Alexafluor 488 that were diluted 1:500. Slides were incubated with secondary antibodies for 2 hours at room temperature, followed by a final wash. Slides were viewed using a Zeiss laser scanning confocal microscope at a magnification of 63x.

Motor neuron cell body area analysis

Motor neuron cell body areas were measured in Lamina IX of the spinal cord (L4-L5; Jacob, 1998) from all animals. Twenty representative motor neuron cell bodies were selected from each animal from each group to determine motor neuron cell body size. The motor neuron cell body areas were calculated with the Zeiss LSM 5 Image
Examiner program. Motor neurons from exercised animals were compared to those from age-matched sedentary control animals.

**Statistical analysis**

All data values are reported as mean ± the standard error of the mean (SEM). GDNF protein values are expressed as picogram per milligram of wet tissue weight. Data were analyzed using one-way ANOVA and Tukey's post-hoc comparison test for differences among the independent groups, where p values \( \leq 0.05 \) were considered as statistically significant.

**Results**

**2 weeks of low-intensity forced running increases GDNF protein content in the mouse spinal cord**

No difference in animal weights was observed between the exercise animals (25.0g ± 0.3g) and the sedentary controls (24.8g ± 0.3g). Two weeks of involuntary running significantly increased GDNF protein content in the lumbar spinal cord of the mice (7.2 ± 2.1 pg GDNF/mg Tissue weight) as compared to their age-matched sedentary controls (0.4± 0.2 pg GDNF/mg Tissue weight), which is a 16-fold increase (Figure 24).

Western blot analysis further confirmed that 2 weeks of exercise at a low-intensity forced running protocol resulted in a higher expression of GDNF protein in the mouse
lumbar spinal cord as compared to age-matched sedentary controls (Figure 25). We observed a dimeric protein of GDNF, with molecular weights of ~25kDa and ~28kDa, for both the exercised and control mice. Densitometry analysis of the GDNF bands demonstrated a 1.3-fold increase of the 25kDa molecular weight among the exercised animals compared to controls and a 1.5-fold increase of the 28kDa molecular weight. The negative loading control of NGF did not reveal any bands, suggesting specificity of the GDNF antibody labeling within the mouse spinal cord.

**GDNF protein content is increased in the mouse spinal cord following 2 weeks of involuntary running**
Figure 24. Glial cell line-derived neurotrophic factor (GDNF) protein content is increased in the mouse spinal cord following 2 weeks of exercise. The lumbar spinal cord (L1-L3) was removed from 3-month-old C57BL/6 control (n = 6) and exercised mice (n = 6). Tissues were processed for GDNF protein content using an ELISA. Low-intensity running of 8 m/min increased GDNF protein content in the lumbar spinal cord as compared to age-matched sedentary controls. Values are displayed as mean ± SEM.

![Western blot of GDNF in mouse spinal cord]

Figure 25. Western blot analysis of GDNF in the mouse lumbar spinal cord revealed a dimeric protein with two molecular weights at 25kDa and 28kDa. The lumbar spinal cord (L1-L3) was removed from 3-month-old C57BL/6 mice that had undergone 2 weeks of involuntary running (8m/min) and age-matched sedentary controls. Tissues homogenates were exposed to antibodies for GDNF. An increase in GDNF expression was detected in the spinal cord of animals that had undergone 2 weeks of involuntary running as compared to controls. No band for GDNF was visualized from the negative loading control of NGF.
Positive immunoreactivity for GDNF was found in ChAT-positive cells, which are presumed to be motor neurons (Wetts and Vaughn, 1996), in the lumbar spinal cord from both control and exercised mice (Figure 26). We noticed a more punctate pattern of ChAT surrounding the cell bodies of the exercised mice as compared to the sedentary controls. We observed few vesicle-like structures of GDNF surrounding the motor neurons in the exercised mice and even less of these structures in the control animals. Instead, GDNF appears to have immunoreactivity dispersed throughout the cell bodies.

Analysis of the ChAT-positive motor neuron cell bodies revealed that 2 weeks of involuntary running significantly increased motor neuron cell body area (408.9 ± 15.2 μm²) as compared to their age-matched sedentary controls (272.5 ± 15.1 μm²). This trend of almost a doubling in ChAT-positive motor neuron cell body area in the mouse is consistent with our observations of the changes following exercise in the rat lumbar spinal cord.
Exercise increases GDNF immunolabeling in ChAT-positive cells

Figure 26. Exercise increased motor neuron size and GDNF immunolabeling in 3-month-old mouse spinal cord. Representative lumbar spinal cord sections from a 3-month-old sedentary control animal (A), and a voluntary exercised animal (B). Spinal cord sections were immunolabeled with primary antibodies against ChAT (red) and GDNF (green). ChAT immunoreactivity is co-localized with GDNF immunoreactivity in the lumbar spinal cord. Exercise increased vesicle-like structures of ChAT and GDNF (as indicated by the arrows). The scale bar represents 20μm.
Discussion

Similar to our findings with the rat spinal cord, 2 weeks of exercise significantly increased GDNF protein content in the mouse spinal cord of young animals. However, the values of GDNF protein content in the spinal cord of the mice were less than what we observed in the rat spinal cord. Among the rats, the lowest value of GDNF content was found to be at 8.6 ± 1.5 pg GDNF/mg tissue weight for the 6-month-old control animals (Chapter 3). Others have reported that in CF1 wild type mice aged 5 months have spinal cord GDNF protein levels at 26 pg/mg of total protein (Zhao et al., 2004), and GDNF protein content has been reported to have a range of 0.5-2pg GDNF/mg total protein in the striatum of C57BL/6 mice (Boger et al., 2006). Together, our results of GDNF protein levels in the C57BL/6 mouse lumbar spinal cord appear to fall within a realistic range.

Studies are finding that beneficial effects of exercise for neurogenesis and improved neurological function of diseased and ischemic individuals are more potent following low- and moderate- intensities of exercise (Aguiar et al., 2007; Hayes et al., 2008). Forced running can acutely elevate corticosterone levels, which is a typical sign of chronic stress, more than voluntary running (Ploughman et al., 2005, 2007). However, corticosterone levels return to baseline within a few hours after exercise (Stranahan et al., 2006; Ploughman et al., 2007) and after several weeks of exercise these levels are no longer elevated (Fediuc et al., 2006). Increased levels of corticosterone have been found to decrease body weight (Brown et al., 2007). The acute effects of decreasing body
weight may have a beneficial effect to induce an increase in neurotrophic factor levels following short-term exercise. Similarly, caloric restriction has been found to increase both GDNF and BDNF protein levels in the brain of a primate model of Parkinson's disease (Maswood et al., 2004). However, long-term exercise may lead to a prolonged influx of corticosterone levels that can have more of a negative effect by down regulating neurotrophic factors. This has been demonstrated in the rat hippocampus, where increasing corticosterone levels decreased the availability of BDNF mRNA and protein levels (Schaaf et al., 1998).

**Expression of GDNF protein increases with 2 weeks of exercise**

The molecular weight of GDNF has been reported to range anywhere from 15kDa to 45kDa depending on the tissues and cells examined (Lin et al., 1993, 1994; Blesch and Tuszynski, 2001; Koo and Choi, 2001; Larsen et al., 2006). In the rat spinal cord we have observed the molecular weight of GDNF to range from 30kDa to 41kDa, depending on the age of the animal examined. Others have reported a molecular weight of GDNF in the adult rat spinal cord to be 34kDa (Zhou et al., 2008). One possibility that we cannot rule out for the difference in molecular weight of GDNF observed from the current study could be due to the different type of animal that we examined. The difference in molecular weight of GDNF that was detected in the current study could be due to the age of these animals, which were only 3-months-old, as compared to our other studies where the youngest rats that we examined were 6-months-old. Neurotrophic factors are synthesized as larger precursor forms that undergo intracellular enzymatic cleavage to
yield their mature, biologically active ligands. Prodomain regions of the precursor forms of NGF and BDNF have been found to initiate cellular death (Nykjaer et al., 2004; Teng et al., 2005). The higher molecular weight of GDNF that is expressed among the older animals could be proGDNF. An increased expression of higher molecular weight of neurotrophic factors has been observed with BDNF. Homogenates of the human cortex from Alzheimer’s patients have been shown to express the high molecular weights of proNGF and proBDNF (Seidah et al., 1996; Mowla et al., 2001; Peng et al., 2004; Pedraza et al., 2005). These precursor forms of secreted neurotrophins are glycosylated but can vary in the degree or composition of N-linked glycosylation or N-terminal cleavage (Kolbeck et al., 1994; Mowla et al., 2001; Teng et al., 2005), where they can activate different cellular pathways. Our results may suggest that with advancing age, the modifications of the GDNF protein may also be altered and may be one contributing factor to damage or apoptosis of neurons.

**Motor neuron size increases at the same time as GDNF levels following short-term exercise**

Following 2 weeks of involuntary running, GDNF immunoreactivity in the mouse spinal cord was not similar to that in the rat spinal cord. GDNF immunoreactivity in the rat spinal cord showed a distinctive punctate staining pattern for GDNF that increased with age and exercise, however GDNF immunoreactivity in the mouse spinal cord showed very little punctate staining following exercise. The volume of motor neuron cell bodies have been reported to show an increase (Nakano et al., 1997) as well as a decrease
(Beaumont and Gardiner, 2003) depending on the intensity of the exercise training. There is increasing evidence is finding that the nervous system responds to increased levels of physical activity due to changes in motor neuron properties, including alterations in cellular morphology and protein synthesis. An increased cell size of fast motor neurons, but a decrease in the slow motor neurons was observed following intense chronic exercise (Beaumont and Gardiner, 2003). Dendritic arbors have been found to increase in size per dendrite following as little as 5 days of running (Gazula et al., 2004). Our results suggest that low-intensity running can alter the morphology of ChAT-positive motor neurons following 2 weeks; however, we do not know which type(s) of motor neurons are being altered. Future studies are warranted to determine if this hypertrophy of motor neurons is specific to either α- or γ-motor neurons or both. Exercise training has been found to increase protein synthesis within motor neurons (Edstrom, 1957; Gerchman et al., 1975) and are capable of transporting larger amounts of proteins in both retrograde and orthograde directions (Dahlstrom et al., 1978; Jasmin et al., 1988; Kang et al., 1995;). Our results of increased amounts of GDNF suggest that neurotrophic factors may also increase in motor neurons in response to exercise. If GDNF protein is increased in the motor neurons following exercise, it would be important to determine if the increased supply of protein to and from the periphery are important for the motor units to adapt as a whole.
Conclusion

In conclusion, the results from this study demonstrated that short-term and low-intensity exercise increases GDNF protein content, GDNF immunolabeling and motor neuron size in the mouse spinal cord. These results are similar to those of what we have previously found in the rat spinal cord. Based on our findings, we proceeded to test alterations in GDNF protein following exercise in an advanced transgenic mouse model of advanced aging and to test neutralizing antibodies against GDNF to determine a causative role for GDNF in motor nervous system plasticity following exercise.
CHAPTER VI

GDNF PROTEIN IS ALTERED IN A TRANSGENIC MOUSE MODEL OF RAPID MOTOR NERVOUS SYSTEM DEGENERATION

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurological disorder that causes degeneration of motor neurons, primarily affecting the motor neurons of the lower spinal cord, brainstem, and motor cortex, leading to paralysis and death due to respiratory failure within 1-5 years (Brown, 1995; Traynor et al., 2000). The skeletal muscles that are innervated by these motor neurons undergo denervation with grouped fiber atrophy leading to muscle weakness. The α-motor neurons are the most vulnerable subtype of motor neurons as their number is decreased significantly with disease progression; however, γ-motor neurons remain relatively unchanged (Mohajeri et al., 1998; Fischer et al., 2004). The majority of ALS cases are sporadic with unknown etiology; however, nearly 10% of cases are inherited in an autosomal dominant manner, termed familial ALS (FALS). Of the FALS cases, about 20% of ALS patients have a point mutation caused by missense mutations in Cu/Zn superoxide dismutase in the antioxidant enzyme SOD1 gene, which encodes for the cytosolic copper- and zinc-dependent SOD (Rosen et al., 1993; Deng et al., 1993; Pasinelli and Brown 2006). SOD1 normally converts superoxide ion, a by-product of mitochondrial metabolism, into water and hydrogen peroxide. This becomes disrupted with ALS. Oxidative stress damages spinal cord proteins and motor neurons are particularly vulnerable to oxidative stress (Rosen et al., 1993; Rowland and
Shneider, 2001). Several hypotheses for the SOD1 mutant mediated neuronal loss include excitotoxicity, oxidative damage, impaired energy metabolism, inflammation, and insufficient growth factor signaling (Leitner et al., 2009).

Neurotrophic factors have been proposed as targets for ALS treatment and therapy. Transgenic mice deficient in ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) have been reported to develop earlier onset of ALS as compared to transgenic wild type ALS mice (Giess et al., 2000, 2002) and individuals with low levels of circulating vascular endothelial growth factor (VEGF) have an increased risk of developing ALS (Lambrechts et al., 2003). Studies have shown that there are decreased amounts of GDNF in skeletal muscles from individuals with ALS (Yamamoto et al., 1996), where exogenous treatment with GDNF can slow the progression of the disease in a mouse model (Mohajeri et al., 1999; Lu et al., 2003) and intramuscular injections of GDNF have been shown to prevent motor neuron loss in ALS mice (Wang et al., 2002). A single intramuscular injection of an adeno-associated virus vector of GDNF was found to be highly expressed for at least 10 months in skeletal muscle and in motor neurons due to retrograde transport (Lu et al., 2003). Furthermore, target-derived, but not centrally-derived, GDNF has been shown to delay the onset of disease, increase lifespan and improve locomotor function of ALS mouse models (Li et al., 2007).

Exercise has also been demonstrated to slow the disease progression in humans with ALS (Drory et al., 2001) and in mouse models of ALS (Kirkinezos et al., 2003). Running-based exercises of transgenic animals with ALS show a significant increase in
lifespan and motor neuron protection from death compared to sedentary ALS animals (Kirkinezos et al., 2003; Veldink et al., 2003; Liebetanz et al., 2004; Kaspar et al., 2005). Others have implicated that physical exercise may be an environmental risk factor associated with ALS, where individuals that are exposed to high levels of physical exercise and with a genetic susceptibility may be at an increased risk to develop the disease (Harwood et al., 2009). In animal models of ALS, a negative effect of high intensity exercise has been demonstrated (Mahoney et al., 2004). A critical factor among ALS exercise studies is the time frame in which the onset of the disease occurs. Studies have found that exercising presymptomatic ALS mice (9 weeks of age) run greater distances than the nontransgenic control animals. The transgenic ALS mice reduce their running distance 2-3 weeks prior to the onset of visible motor impairment and decrease their running speed 1 week after the decline in distance (Bruestle et al., 2009). There is still debate as to whether exercise-induced neuroprotection of motor neurons occurs in the spinal cord of ALS mouse models (Veldink et al., 2003; Kaspar et al., 2005), and whether protection depends on the modality of exercise, such as wheel running versus treadmill running, intensity, and the duration of exercise.

One alternate method for preventing apoptosis of neurons would be to activate anti-apoptotic pathways by regular exercise (Mattson et al., 2004). Since we have shown that exercise can increase GDNF protein levels in skeletal muscle of healthy individuals (Wehrwein et al., 2002; McCullough et al., 2011) and in the spinal cord of healthy individuals, regular exercise may be one way to naturally prevent motor neuron cell death.
of individuals with compromised motor neurons (Henderson et al., 1994; Oppenheim et al., 1995; Ulfhake and Bergman, 2000). Thus far, no studies have linked exercise to elevated GDNF levels and to the delay of disease progression in animal models of neurodegeneration. Studying changes in motor neurons with age is a slow process and any countermeasures used to prevent changes may need to be run for a year or more to be effective. By using an animal model of ALS we may observe similar changes to those observed with aging in a shorter time period, which may allow for countermeasures to be tested in a short time frame. The transgenic mouse model of ALS displays degradation of motor neurons and the skeletal muscles they innervate. Using this animal model of ALS would allow us the ability to focus on the degenerative changes that occur during a much shorter time period as the animals only survive on average for 5 months (Gurney et al., 1994). Our hypothesis is that low-intensity walk-training will increase GDNF levels in the spinal cord while slowing degeneration of motor neurons, whereas blocking circulating levels of GDNF with antibodies will block the beneficial effects of exercise in a mouse model of ALS.

Materials and methods

Subjects

All 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.
Transgenic mice that express the human mutant of SOD1 have been shown to develop age-dependent clinical and pathological features that closely mirror those found in human ALS subjects (Gurney et al., 1994; Rothstein, 2003). Male transgenic B6SJL-Tg(SOD1*G93A)1Gur/J mouse models of ALS (The Jackson Laboratory, Bar Harbor, ME), which expresses a mutant form of human SOD-1 with a gly93ala substitution (Gurney et al., 1994), were used for all studies. These mice begin to show signs of hindlimb skeletal muscle weakness by 3 – 4 months of age but variations from this timeframe may occur (Gurney et al., 1994; Leitner et al., 2009). Animals demonstrate massive death of motor neurons in the ventral horn of the spinal cord and loss of myelinated axons in ventral motor roots that lead to muscle atrophy and eventually paralysis (Leitner et al., 2009). Disease onset was determined when the first sign of motor impairment was visible. This included the inability of a mouse to completely extend its hind limbs and the manifestation of a slight tremor in one or both hind limbs while the mouse was lifted by its tail (Li et al., 2007, 2005; Mohajeri et al., 1998, 1999). Mice were given access to food and water ad libitum and extra bedding was added to the cages to ensure access when the animals displayed muscle weakness.

**Low-intensity training protocol**

The transgenic ALS mice, aged 10 weeks, were randomly assigned to a sedentary age-matched control group (SED; n=3) or an involuntary running group (EX; n=6), where exercise began when the first animal started to exhibit signs of disease onset (day 77). This exercise start point was chosen as disease onset was evident and motor neuron degeneration had occurred (De Bono et al., 2006). The EX mice were placed in
individual forced running wheels (Lafayette Instruments, Lafayette, IN), and exercised at a speed of 8 m/min for a total of 30min/day which included an additional brief warm-up and cool-down period. The EX mice were acclimated to the running wheels for one week prior to the beginning of the study. It has previously been shown that running speeds of 10m/min or lower are considered to be a low-intensity exercise protocol for mice (Bey and Hamilton, 2003). Animal weights and neurological score of disease progression were monitored daily. All mice were euthanized via CO₂ asphyxiation followed by thoracotomy at 115 days of age (Deforges et al., 2009) and the lumbar spinal cord was removed. All tissues were stored at -80°C until processed.

**Blocking of circulating GDNF**

Three of the EX mice were randomly selected to receive twice daily subcutaneous injections of human anti-GDNF (R&D Systems) for the entire duration of the study (ANTI-GDNF). These animals received injections of 25ng anti-GDNF/gram of animal weight every 12 hours, which was similar to the antibody treatment regimen developed by Ferrer-Alcon et al. (2008).

**Tissue processing**

The lumbar spinal cord section of L1 – L3 was removed and frozen on dry ice for protein quantification. To determine GDNF protein content, samples were subsequently dipped in liquid nitrogen and smashed into a fine powder. Sample processing buffer (0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, and 0.05% Tween-20)
was added and the mixture was homogenized on ice. Samples were centrifuged for 30 min at 14 000g in 4ºC and supernatant was collected and stored at -80ºC.

**GDNF protein quantification**

GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (McCullough et al., 2011). Ninety-six well plates were incubated overnight at room temperature in a humidified chamber with a monoclonal antibody raised against GDNF (R&D Systems, Minneapolis, MN). The following day, plates were rinsed with wash buffer and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose for 1 hour at room temperature. Plates were rinsed with wash buffer and the GDNF standard (R&D Systems) or tissue supernatants were added to the wells. For each assay, a standard curve was calculated from the known GDNF standard concentration, ranging from 1000pg/ml to 2pg/ml of GDNF protein. Following a 2 hour incubation at room temperature, the plates were rinsed with wash buffer followed by the incubation of a biotinylated anti-GDNF secondary antibody (R&D Systems) for 2 hours at room temperature. The plates were then rinsed with wash buffer and coated with β-galactosidase conjugated to streptavidin (Molecular Probes, Eugene, OR) for 20 minute incubation at room temperature. The plates had a final wash and chlorophenol red-β-D-galactopyranoside (CPRG) substrate was added (in PBS + BSA) and the plates incubated until the color had developed. Plates were read at an absorbance of 575nm.
Western blot

Total protein content of the spinal cord samples were measured by a Pierce® BCA protein assay (Thermo Scientific, Rockford, IL) according to manufacturers specifications. Tissue samples were prepared for Western blot analysis of GDNF protein as previously described (Vianney and Spitsbergen, 2011). Briefly, protein extracts (20µg), a protein ladder (New England BioLabs, Ipswich, MA) and a negative loading control of nerve growth factor (NGF; R&D Systems) were prepared with Laemmli 2X loading buffer and loaded into a 15% polyacrylamide gel. The gel was submerged and was run in separating buffer at different voltages followed by transfer to a polyvinylidene difluoride (PVDF; Invitrogen) membrane in tris-glycine buffer. The PVDF membrane was blocked with I-Block (Applied Biosystems, Foster City, CA) followed by overnight incubation with a primary polyclonal antibody against GDNF (1:200, rabbit anti-GDNF, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The following day, the membrane was washed in buffer followed by incubation with a donkey peroxidase-conjugated secondary antibody directed against rabbit immunoglobulins (1:500; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Bound antibody complexes were developed using the ECL plus kit and exposed to X-ray films (Kodak). After stripping, membranes were incubated with a mouse anti-GFRα-1 monoclonal antibody (1:200, Santa Cruz Biotechnologies), mouse anti-alpha tubulin (1:200; Developmental Studies Hybridoma Bank) and activated caspase-3 was detected with a rabbit antibody specific for cleaved forms of caspase-3 (1:200, Cell Signaling Technologies, USA). Antibodies were
revealed with a sheep peroxidase-conjugated secondary antibody directed against mouse immunoglobulins or a donkey peroxidase-conjugated secondary antibody directed against rabbit immunoglobulins (1:500, GE Healthcare). Bound antibody complexes were developed using the ECL plus kit and exposed to X-ray films. ImageJ software was used to measure the relative density of GDNF bands and values were expressed as ratios of controls.

**Immunohistochemistry and histological examination**

The L4-L5 lumbar spinal cord was immediately removed, fixed in 4% paraformaldehyde overnight at 4°C and cryoprotected overnight in 30% sucrose made in PBS (Li et al., 2007). Tissues were embedded in optimal cutting temperature (O.C.T. compound) mounting medium and cut into 40µm transverse sections on a cryotome that were thaw mounted onto Histobond® slides (VWR International, Bridgeport, NJ). Slides were incubated overnight (4°C) with primary antibodies that were diluted 1:200 in PBS containing 1% BSA and 0.1% triton X-100. Sections were exposed to rabbit anti-GDNF (Santa Cruz Biotechnology), mouse anti-choline acetyltransferase for motor neuron identification (Millipore), rabbit anti-activated caspase-3 (Cell Signaling Biotechnologies), or mouse anti-GFRα1 (Santa Cruz Biotechnologies). Slides were washed in PBS before the addition of secondary antibodies of donkey anti-mouse conjugated to AlexaFluor 568 or 647 and donkey anti-rabbit conjugated to AlexaFluor 488 that were diluted 1:500 in PBS containing 1% BSA and 0.1% triton X-100. Slides were incubated with secondary antibodies for 2 hours at room temperature. Since others
have demonstrated autofluorescence within the spinal cord of diseased mouse models (Messer and Plummer, 1993), the slides were incubated with the Autofluorescence Eliminator Reagent (Millipore, Temecula, CA) as specified by the manufacture's protocol, followed by mounting in 3:7 glycerol:0.5M bicarbonate buffer mounting media. Slides were viewed using a Zeiss laser scanning confocal microscope at a magnification of 63x.

ChAT-positive motor neuron cell body areas were measured in Lamina IX of the spinal cord (Jacob, 1998) from all animals. Twenty representative motor neuron cell bodies were selected from each animal. To obtain a representation of motor neuron cell death, three random sections within the lumbar spinal cord of each animal was evaluated by counting ChAT-positive cells in the ventral horn of the L4-L5 segment of the spinal cord (Chavany et al., 1998). A 100µm x 100µm box was placed in the ventral horn of the grey matter and ChAT-positive cells were counted. Images were measured and examined with the Zeiss LSM 5 Image Examiner program.

**Statistical analysis**

All data values are reported as mean ± the standard error of the mean (SEM). GDNF protein values are expressed as picogram per milligram of wet tissue weight. Data were analyzed using one-way ANOVA and Tukey's post-hoc comparison that tested for differences among the independent groups, where p values ≤ 0.05 were considered as statistically significant.
Results

Behavioral analysis

Initially, the running protocol was set to be 8m/min for the duration of the study. Unfortunately, some of the animals began exhibiting symptoms of disease onset, such as curling of hind limbs and mild tremors, so the running protocol was adjusted to a decreased speed to prevent additional injury to the animals. The final protocol for 5 of the 6 exercised animals had started at 8m/min for the first 3 weeks and then the speed was decreased to 5m/min for the last week of the program. However, one of the ANTI-GDNF mice had the speed reduced to 4m/min during the second week of the study and then down to 2.5m/min for the remainder of the study.

Symptoms of hind limb weakness began to appear first among the ANTI-GDNF mice around 92 days of age, followed by the SED mice at 97 days of age, and then by the EX mice at 101 days of age. No difference in animal weights were observed between the three groups of mice (Figure 27). The SED mice had a peak body weight at 94 days of age, the EX animals had a peak body weight at 105 days of age, and the ANTI-GDNF mice had a peak body weight at 99 days of age; however, these were not statistically different.
Figure 27. Body weights of the ALS mice. Neither exercise or exercise in combination with treatment with ANTI-GDNF had an effect on animal weight. Weights were recorded each morning at the same time prior to the exercise training. There was an apparent trend of the ANTI-GDNF animals to have a larger weight than the EX mice. Values reported as mean ± SEM.

GDNF protein content

Neither exercise (22.0 ± 1.5 pg GDNF/mg tissue weight) or exercise in combination with treatment with anti-GDNF (23.8 ± 8.2 pg GDNF/mg tissue weight) had
a significant effect on GDNF protein content in the lumbar spinal cord compared to SED mice (49.1 ± 11.2pg GDNF/mg tissue weight) (Figure 28).

**Western blot analysis**

Western blot analysis of GDNF revealed three bands at 17, 25 and 46kDa within the lumbar spinal cord of all the ALS mice (Figure 29). Densitometry analysis found only a small decrease of the 46kDa band of GDNF of the EX mice (0.3-fold decrease) and the ANTI-GDNF mice (0.4-fold decrease) as compared to SED mice (Table 2). There was also a slight decrease of the 25kDa band of GDNF of the EX mice (0.3-fold decrease) compared to SED mice. Western blot analysis of the primary GDNF receptor, GFRα-1, showed an increased expression in both the EX mice (25.6-fold increase) and the ANTI-GDNF mice (47.4-fold increase) compared to SED mice. The expression of caspase-3, a marker of apoptosis, increased at the 17kDa molecular weight of both the EX mice (2.6-fold increase) and the ANTI-GDNF mice (4.5-fold increase) compared to SED mice.
GDNF protein content in lumbar spinal cord of ALS mice

Figure 28. Glial cell line-derived neurotrophic factor (GDNF) protein content is not altered in the lumbar spinal cord of ALS mice following low intensity running or following low intensity running and anti-GDNF treatment. The lumbar spinal cord (L1-L3) was removed from 115-day-old B6SJL-Tg(SOD1*G93A)1Gur/J transgenic SED mice (n = 3), EX mice (n = 3), and ANTI-GDNF mice (n = 3). Tissues were processed for GDNF protein content using an ELISA. Values are displayed as mean ± SEM.
Western blot analysis of the lumbar spinal cord of ALS mice

**Figure 29.** Western blot analysis of GDNF, GFRα-1, Caspase-3, and Tubulin in the lumbar spinal cord of ALS mice. The lumbar spinal cord (L1-L3) was removed from 115-day-old B6SJL-Tg(SOD1*G93A)1Gur/J transgenic sedentary control ALS mice (SED; n = 3), exercised ALS mice (EX; n = 3), and anti-GDNF treated ALS mice (ANTI-GDNF; n = 3).
Densitometry analysis of Western blot of ALS mice

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (kDa)</th>
<th>Fold-change from ALS controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDNF</td>
<td>46</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1.2</td>
</tr>
<tr>
<td>GFRα-1</td>
<td>53</td>
<td>25.6</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>19</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>2.6</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>55</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (kDa)</th>
<th>EX</th>
<th>ANTI-GDNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDNF</td>
<td>46</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>GFRα-1</td>
<td>53</td>
<td>25.6</td>
<td>47.4</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>19</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>2.6</td>
<td>4.5</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>55</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 2. Fold-change of GDNF, GFRα-1, Caspase-3, and α-tubulin in the lumbar spinal cord of ALS mice. The molecular weights are displayed as fold-change from the SED mice as measured by densitometry analysis with the ImageJ software program.

Immunohistochemistry and analysis

Omission of primary antibodies of mouse anti-ChAT, rabbit anti-GDNF and mouse anti-GFRα-1 did not result in immunoreactivity within the spinal cord in ALS mice (Figure 30). Similar to what others have shown (Ryu et al., 2011), we observed dispersed immunoreactivity of ChAT among all of the lumbar spinal cord sections in
ALS mice. Positive immunoreactivity for GDNF was observed within the ChAT-positive cells, although immunoreactivity for both ChAT and GDNF were easier to visualize with the EX mice followed by the ANTI-GDNF mice as compared to the SED mice (arrows in Figure 31A,B). Vesicle-like structures of ChAT that surrounding the motor neurons were visualized among all the transgenic animals (Figure 31A). Few vesicle-like structures of GDNF were visualized from the ALS mice that were not localized to the ChAT-positive motor neurons (Figure 31B). Both caspase-3 and GFRα-1 were evident in all animals and somewhat localized to ChAT-positive motor neurons (Figure 31C, D). To determine if anti-GDNF used for treatment was still found in tissues, tissues were stained with donkey anti-mouse 568, and the ANTI-GDNF mice displayed positive immunoreactivity with the addition of the secondary fluorescent antibodies compared to the SED and EX mice (Figure 32).

Analysis of the ChAT-positive motor neuron cell body area revealed a significant decrease in the EX mice (228.4 ± 16.5μm²) and the ANTI-GDNF mice (212.1 ± 9.9μm²) compared to the SED mice (258.2 ± 19.1μm²). We previously examined the motor neuron cell body size of age-matched wild-type C57BL/6 control mice (Chapter 5) and a significant decrease (p≤0.05) in ChAT-positive motor neuron cell body size was observed in all ALS animals compared to the C57BL/6 control mice (272.5 ± 15.1μm²). To determine if our exercise protocol had elicited neuroprotection of ChAT-positive motor neurons, cells were counted from sections of the lumbar spinal cord of all animals. The C57BL/6 mice had significantly more averaged ChAT-positive motor neurons than the
ALS animals (8.7 ± 0.3 ChAT-positive cells per section). A significant increase was observed from the EX mice (6.7 ± 0.7 ChAT-positive cells per section) compared to the ANTI-GDNF mice (4.0 ± 0.6 ChAT-positive cells per section). No difference in cell number was observed in the SED mice (5.3 ± 0.3 ChAT-positive cells per section) compared to the EX and ANTI-GDNF mice (Figure 33).

**Immunohistochemistry with no primary antibodies in the ALS mouse spinal cord**

![Image of immunohistochemistry](image)

**Figure 30.** A representative image of a negative control with the omission of primary antibodies. The lumbar spinal cord (L4-L5) was removed from an ALS control mouse.Slides were drained incubated with the secondary antibodies (1:500) of donkey anti-

131
mouse 568, donkey anti-mouse 647 and donkey anti-rabbit 488. Images were captured with a Zeiss laser scanning confocal microscope. Scale bar represents 20μm.

**Immunohistochemistry in the lumbar spinal cord of ALS mice**

A.

B.

C.

D.
**Figure 31.** Localization of ChAT, GDNF, Caspase-3 and GFRα-1 in the lumbar spinal cord of ALS mice. The lumbar spinal cord (L4-L5) was removed from SED, EX and ANTI-GDNF mice. Sections were exposed to primary antibodies against (A) ChAT (red), (B) GDNF (green), (C) activated caspase-3 (green), and (D) GFRα-1 (blue). Images were captured with a Zeiss laser scanning confocal microscope. Scale bar represents 20µm.

**Detection of neutralizing antibodies in ALS spinal cords**

![Image A](image1.png)
![Image B](image2.png)
![Image C](image3.png)
Figure 32. Animals injected with neutralizing antibodies display positive staining for these antibodies. Representative images from (A) SED, (B) EX, and (C) an ANTI-GDNF mice. Slides were incubated with the secondary antibodies (1:500) of donkey anti-mouse 568. Images were captured with a Zeiss laser scanning confocal microscope. Scale bar represents 20μm.

Cell counts of ChAT-positive motor neurons
Figure 33. Cell counts of ChAT-positive motor neurons in the mouse lumbar spinal cord. Sections were exposed to primary antibodies against ChAT. Images were captured with a Zeiss laser scanning confocal microscope. A 100µm x 100µm box was randomly placed within Lamina IX in the ventral horn of the grey matter in three random sections. Cells were counted from (A) control C57BL/6 mice, (B) SED mice, (C) EX mice, and (D) ANTI-GDNF mice. Scale bar represents 20µm.

Discussion

The study was designed to determine if low-intensity walk-training would alter GDNF protein expression in the lumbar spinal cord of a transgenic mouse model of rapid neuromuscular degeneration and if anti-GDNF treatments would block the beneficial effects of exercise on motor neurons. Low-intensity exercise initiated after the onset of the disease in ALS mice does not alter GDNF protein content in the lumbar spinal cord. Three molecular weights of GDNF were observed in the spinal cord of transgenic ALS mice. Western blot analysis revealed an increased expression of GFRα-1 in the lumbar spinal cord following exercise. We observed fragmented ChAT-positive motor neurons among all the ALS mice, where exercise decreased the cell body area of motor neurons, but did rescue the number of motor neurons within the lumbar spinal cord.
Long-term exercise does not alter GDNF protein content in the spinal cord of ALS mice

Physical exercise has consistently emerged as a key factor in maintaining nervous system health and function, yet the role it plays in altering neurotrophic factor expression remains unclear. While exercise has been demonstrated to be beneficial for the motor nervous system of individuals with ALS (Drory et al., 2001; Kirkinezos et al., 2003), other studies report the complete opposite (Mahoney et al., 2004; Harwood et al., 2009). Low-intensity exercise does not limit the apoptotic process in the spinal cord of ALS animals whereas exercise-activated motor neurons can be selectively protected against cell death in a mouse model of ALS (Deforges et al., 2009). We have previously demonstrated that exercise can increase GDNF protein content within skeletal muscle and the lumbar spinal cord of healthy animals, however to our knowledge, the current study is the first report on alterations in GDNF protein in animal models of ALS following exercise. GDNF protein content was found to increase following exercise in wild-type mice but not ALS mice. A high degree of variability of protein content was observed among the ALS mice which may have resulted from the low sample size of each group (n=3) and the disease onset for each individual animal may have also affected the amount of protein that was expressed. A possible explanation for the differences observed between the two strains of animals may be due to the longer durations of exercise for the ALS animals. Since we ended the ALS study when the animals displayed severe hindlimb weakness, this resulted in an extended duration of exercise. Our previous findings in rat demonstrated that longer term exercise does not alter GDNF protein
content in the spinal cord. Interestingly, our results demonstrated that GDNF protein content in the spinal cord of age-matched wild-type C57BL/6 control mice were much lower as compared to that of age-matched ALS mice. Another explanation for the differences in GDNF protein content observed among the ALS individuals is that GDNF protein content is found in the cerebral spinal fluid of ALS patients, but not in healthy control individuals (Grundstrom et al., 2000). This might suggest that GDNF is increased in the spinal cord of ALS individuals in part due to increased demands for GDNF in the motor neurons.

**Increased molecular weights of GDNF occur at the same time as motor neuron disruption**

Both proBDNF and proNGF trigger cell death via the p75NTR and sortilin receptors (Lee et al., 2001; Nykjer et al., 2004; Teng et al., 2005) and the amount of proNGF increases during spinal cord injury and neurodegenerative disease (Fahnestock et al., 2001; Harrington et al., 2004). Although it is currently not known if proGDNF triggers apoptotic pathways, there may be a possibility that increased levels of proGDNF are found in individuals with ALS. We observed three molecular weights of GDNF in the ALS mice, of which the two largest sizes of GDNF were not expressed in the age-matched wild-type C57BL/6 mice. Increasing molecular weights of GDNF were also found with advancing age in rat spinal cord. Similar to what others have shown, we observed a loss of ChAT-positive motor neuron staining and more vesicle-like fragments outlining the motor neurons in transgenic ALS mice (Ryu et al., 2011). Transgenic
animal models of motor neuron disease have been found to have an increase of autofluorescence in lumbar spinal cord sections that are presumed to be lysed motor neurons (Messer and Plummer, 1993). Levels of activated caspase-3, a marker of neuronal apoptosis, are known to be increased in the spinal cord of ALS patients as compared to control patients (Martin, 1999). Our observations of increased expression of activate caspase-3 along with positive immunostaining in the motor neurons with the ANTI-GDNF mice may suggest accelerated apoptosis of motor neuron cell death when circulating levels of GDNF are blocked.

GDNF plays a vital role in the maintenance and survival of the motor nervous system. Studies that have examined disease, aging and injury have demonstrated alterations in signal transduction patterns of GDNF and its receptors. GDNF mRNA levels are decreased with advancing age in the mouse striatum (Blum and Weickert, 1995) but increased in skeletal muscle following denervation (Ulfhake et al., 2000). GDNF protein levels decrease in skeletal muscle with advancing age (Nagano and Suzuki, 2003; McCullough et al., 2011). In a mouse model of ALS, only target-derived GDNF but not centrally-derived GDNF has been shown to be neuroprotective (Li et al., 2007). The receptors for GDNF, GFRα-1 and RET, are both expressed in motor neurons in normal spinal cord (Widenfalk et al., 1997) and in ALS spinal cord (Mitsuma et al., 1999). An upregulation of both receptors is found in the spinal cord of old animals and GFRα-1 is upregulated following nerve lesion (Frotick et al., 1998). Our results demonstrated that GFRα-1 expression was increased with the systemic addition of
neutralizing antibodies for GDNF and following exercise. In spinal cord injury models, GDNF and GFRα-1 are upregulated in glial cells and in motor neurons (Burazin and Gundlach, 1998; Mitsuma et al., 1999; Widenfalk et al., 2001), however RET mRNA levels are decreased in motor neurons of ALS patients (Mitsuma et al., 1999). An increased expression of the primary GDNF receptor (GFRα-1) may suggest the cells are anticipating more GDNF to be produced however disruptions in transport, such as with disease or injury, prevent this process from occurring efficiently.

**Anti-GDNF treatment abolishes the neuroprotective effects of exercise**

Several studies have demonstrated prevention of beneficial neural adaptations following exercise with the addition of antibodies against growth and trophic factors. Blocking the p75 low-affinity neurotrophin receptor with neutralizing antibodies prevents the survival effects of neurotrophic factors for motor neurons (Ricart et al., 2006). Daily injections of a specific BDNF inhibitor in the hippocampus abolishes the beneficial effects of energy regulators following exercise as well as decreases other neurotrophic factors (Gomez-Pinilla et al., 2008). Furthermore, daily injections with antibodies against IGF-1 block the beneficial effects of low-intensity exercise on life-span in a mouse model of progressive motor neuropathy (Ferrer-Alcon et al., 2008). Since we have previously found that GDNF protein levels can vary from 0.2pg/mg of tissue weight to 150pg/mg of tissue weight in rodents, we chose a concentration of a neutralizing GDNF antibody that was physiologically relevant to the concentrations normally found within these animals. Only a handful of studies have examined the effects of anti-GDNF
for neuronal death while none have examined these effects with exercise. Neutralizing antibodies against GDNF injected directly into the extraocular muscle of chick embryos increased the number of apoptotic cells in the oculomotor nuclei (Chen et al., 2003). Systemic injections of neutralizing antibodies against GDNF decreased hindlimb function of rats that had transected spinal cords as compared to sham controls (Zhou et al., 2008). Administration of neutralizing antibodies against human GDNF inhibit avian ciliary neuron target innervation (Hashino et al., 2001) and lumbar spinal cord expression of large conductance calcium-activated potassium channels (Martin-Caraballo and Dryer, 2002). Our results demonstrate that the ALS mice that received neutralizing antibodies against GDNF had a decrease in motor neuron number and a trend toward a decrease in motor neuron size, suggesting that GDNF may be important for protecting motor neurons.

**Enhancing GDNF signaling in compromised motor neurons**

A prominent feature of ALS is the generation and migration of new cells, especially microglia, that surround damaged regions (Barbeito et al., 2004). One role that has been hypothesized for microglia in neurodegenerative diseases includes altering secretion of trophic and/or toxic molecules (Ryu et al., 2011). In animal models of ALS, microglia release reactive oxygen and nitrogen species that can damage motor neurons. Oxidative stress induces expression of RET in microglia, where it may divert GDNF from motor neurons, eliciting neuronal damage (Ryu et al., 2011). The GDNF receptor RET is derived from a newly synthesized component in motor neuron cell bodies and a
retrogradely transported component from nerve terminals (Jing et al., 1996). In RET-bearing motor neurons, GDNF appears to act in a target-derived mode from muscle, a paracrine mode from glial cells, and an autocrine mode within neurons (Yamamoto et al., 1996). Both non-phosphorylated and phosphorylated forms of RET are altered in motor neurons of the lumbar spinal cord of ALS transgenic mice (Ryu et al., 2011), suggesting abnormal GDNF signaling. Recently, the use of macrophages to deliver GDNF has shown promising results in neuroprotection for Parkinson’s disease, as these cells are able to cross the blood brain barrier and then differentiate into microglia (Biju et al., 2010). If oxidative stress does alter retrograde transport of GDNF in individuals with compromised motor neurons, such as with disease, injury and aging, then short-term exercise may be a potential way to increase the supply of GDNF for motor neurons. Future studies would need to determine mechanisms to intrinsically increase GDNF levels as therapies for compromised motor neurons.

ALS patients are affected by abnormal membrane electrical properties, such as axonal hyperexcitability (Kanai et al., 2006) and membrane hyperpolarization (Vucic et al., 2007). A disregulation or malfunctioning of ion channels, such as K\(^+\) channels, in motor neurons may be reversed by exercise. Interestingly, GDNF secretion is increased by high potassium depolarization (Oh-hashi et al., 2009). While this study did not demonstrate that exercise alters GDNF protein expression, we did find differences in neuronal plasticity and survival following exercise that were abolished when treated with neutralizing antibodies of GDNF. The results from this study will provide insight to
intrinsic mechanisms that can alter neurotrophic factors and cellular function and survival of motor neurons.

**Conclusion**

In conclusion, these results are not consistent with our hypothesis. We demonstrate that low-intensity exercise initiated after the onset of the disease does not alter GDNF protein content in the spinal cord in animal models of ALS. Addition of circulating levels of anti-GDNF did abolish the increase in motor neuron number following low-intensity exercise along with the trend toward a decrease in motor neuron cell body size. These observations may suggest a relationship of exercise-induced neural protection that is dependent on GDNF. Future studies will need to examine the effects of blocking circulating levels of GDNF in healthy animals that have undergone short-term exercise.
CHAPTER VII

DISCUSSION AND CONCLUSIONS

The results from these studies have established short-term exercise (2 weeks) as a mechanism to increase GDNF protein content in the spinal cord of young and old animals (Chapter 3, 5). Low-intensity forced exercise was found to elicit the greatest fold-change of GDNF protein expression among the young animals as compared to swimming and voluntary running at a higher intensity (Chapter 3). Chronic exercise (4 weeks to 6 months) did not significantly alter GDNF protein expression in the spinal cord of adult and diseased animals (Chapter 4, 6). Even at a low-intensity forced exercise regimen, chronic exercise did not alter GDNF protein expression in the spinal cord (Chapter 6). The molecular weight of GDNF was found to increase in size with advancing age and with disease (Chapter 3, 4, 5, 6). All exercise prescriptions increased motor neuron cell body area with all the ages examined of the healthy animals (Chapter 3, 4, 5). Interestingly, the motor neuron size decreased following low-intensity chronic exercise of the ALS mice, yet exercise did increase the number of motor neurons that survived with the disease progression (Chapter 6). The observations from the following studies suggest that low-intensity exercise, such as walking, even at short durations, are beneficial for the motor nervous system health as individuals continue to age.

All of our studies that quantified GDNF protein content in the lumbar spinal cord tissue homogenates were prepared from the entire lumbar spinal cord region. This
processing technique included all cell types of the spinal cord, including somatic and autonomic motor neurons, glial cells, oligodendrocytes, cerebral spinal fluid, blood cells, sensory neurons, spinal meninges, interneurons, astroglia, microglia, satellite cells, Schwann cells, and stem cells. Thus our reports on changes in GDNF protein content and expression are not strictly limited to just one cell type found within the lumbar spinal cord. Future studies need to be performed to determine what cell types are producing GDNF, such as with *in situ* hybridization, and how production of GDNF is altered with exercise and aging at the spinal cord. Since GDNF is a neurotrophic factor that can also be retrogradely transported from target tissues, such as skeletal muscle (Yan et al., 1995), we cannot be sure if the increase in GDNF following exercise is strictly from the spinal cord or from the target tissues. If there are differences in GDNF expression in the motor neurons, it would be interesting to determine if GDNF is differentially expressed in the α- or γ-motor neurons and this would provide more information as to which motor neuron subtypes are undergoing hypertrophy or atrophy with exercise and aging.

**Control levels of GDNF from rodents undergoing senescence**

One of the interesting results from our studies of analyzing GDNF protein content in the lumbar spinal cord of Sprague-Dawley rats was the pattern of protein levels that occurred with advancing age. In our preliminary studies, levels of GDNF protein were analyzed from the lumbar spinal cord of control Sprague-Dawley rats at 1-, 2-, and 3-months of age, where we found that GDNF protein content steadily decreased with advancing age. In skeletal muscle, our lab and others have previously demonstrated that
GDNF protein content decreases during this timeframe as the neuromuscular apparatus becomes established (McCullough et al., 2011; Nagano and Suzuki, 2003). We have demonstrated that Sprague-Dawley rats aged 6-, 12-, 18- and 24-months steadily increase their GDNF protein content in the lumbar spinal cord with advancing age. Similarly, we found that GDNF protein content in the spinal cord of 3-month-old wild type mice were much lower when compared to the age-matched transgenic ALS mice, a mouse model of advanced motor nervous system degeneration. This increased amount of GDNF protein content that was observed within the spinal cord with the aging individuals may be a mechanism to protect the negative effects of the neuromuscular system that are associated with aging, including loss of motor neurons (Jacob, 1998) and loss of inputs to motor nerve cell bodies (Kullberg et al., 1998). Since mature motor neurons obtain trophic support from various types of cells, including Schwann cells, skeletal muscle cells and other neurons (Nishi, 1994; Oppenheim, 1996), they may resist death by increasing production of neurotrophic factors in these tissues.

Interestingly, our results revealed an increase in the molecular weight of GDNF that was expressed with advancing age among the rats. This was also true for the ALS mice as compared to the age-matched wild-type mice. The reported molecular weight of GDNF varies in the literature from 15-34kDa depending on the cells/tissues examined (Lin et al., 1994; Blesch and Tuszynkski, 2001; Larsen et al., 2006; Koo and Choi, 2001). We found the molecular weight of GDNF in the rodent lumbar spinal cord to range from 25- 46kDa. Similarly, several NGF isoforms have been reported in various whole tissues.
of both humans and animals (reviewed by Al-Shawi et al., 2007). One possible explanation for the variations in molecular weights may be due to modifications of the prodomain regions of the protein. Pro-neurotrophins, which are the precursor forms of neurotrophins, are synthesized and then cleaved by furin and other proteases to produce mature neurotrophins (Lee et al., 2001). Mature NGF and BDNF induce neuronal survival, differentiation and synaptic modulation (Huang and Reichardt, 2001). Results of other studies suggest that the precursor of NGF may be either neurotoxic (Ibanez, 2002) or significantly less neurotrophic than the mature form of NGF (Fahnestock et al., 2004). Pro-neurotrophins, such as pro-BDNF and pro-NGF, induce cell death by activating an apoptotic cascade by binding to cell death complexes involving sortilin and p75 receptors (Lee et al., 2001; Nykjaer et al., 2004). Moreover, pro-NGF has been found to be upregulated with aging and disease, such as in Alzheimer's disease and following spinal cord injury (Bierl and Isaacson, 2007; Peng et al., 2004; Beattie et al., 2002), and may affect motor neuron survival (Domeniconi et al., 2007).

While it is still unknown if the pro form of GDNF activates similar apoptotic pathways as other neurotrophins, it is known that post-translational modifications of GDNF are due to prohormone convertase that cleaves five consensus sites giving rise to four different peptide forms of processed GDNF (Oh-hashi et al., 2009; Immonen et al., 2008). Partial deletion of the prodomain region of GDNF reduces the molecular size of intracellular GDNF and decreases its secretion, suggesting a role for the prodomain region in trafficking and sorting of the GDNF protein (Oh-hashi et al., 2009).
Interestingly, short duration of stimulation was found to have different effects for the secretion of the truncated and full-length GDNF molecules, where short duration of stimulation increased secretion of truncated GDNF but long duration of stimulation increased secretion of full-length GDNF (Lonka-Nevalaita et al., 2010; Geng et al., 2011), suggesting differences in presynaptic sorting of neurotrophic factors to secretory pathways due to differences in activity-dependent biological functions (Geng et al., 2011). Our observation of a punctate staining pattern for GDNF following short-term exercise within neuronal cell bodies might also suggest an alteration in the secretion pattern of GDNF. Interestingly, this punctate pattern of GDNF immunoreactivity was not observed with long-term exercise. Future studies are warranted to determine if proGDNF is less neuroprotective or neurotoxic and how aging affects its expression. Along these lines, determining which pathways are activated and/or inhibited with increasing amounts of proGDNF would be an important mechanism to provide insight for neuronal death. It would be important to known what factors are responsible for intracellular transport of GDNF and how this process is altered with aging and if it can be slowed or reversed with different exercise prescriptions.

**GDNF protein content is not modulated by all forms of exercise**

Our results have demonstrated that GDNF protein content does not increase in the lumbar spinal cord with all the exercise protocols tested. Similar reports have found that voluntary exercise can increase mRNA and protein levels for neurotrophic factors in the spinal cord (Ferraiuolo et al., 2009; Macias et al., 2002; Skup et al., 2002; Ying et al.,
as well as decrease levels of neurotrophic factors in the spinal cord following exercise (Siamilis et al., 2009; Engesser-Casar et al., 2007). One possible contributing factor to these discrepancies may be due to variations in intensity and duration of exercise. Short-term and moderate intensity exercise protocols have been found to increase mRNA and protein levels of neurotrophic factors in the lumbar spinal cord (Gomez-Pinilla et al., 2001; Molteni et al., 2002; Neeper et al., 1996; Aguiar et al., 2007). However, high intensity exercise decreases levels of neurotrophic factor in the brain (Aguiar et al., 2007). We have found that short-term exercise increases GDNF protein content in the spinal cord of young adult and old rats (Chapter 3) and in young mice (Chapter 5), yet long-term exercise does not alter GDNF protein content in the spinal cord of middle-aged adult rats (Chapter 4) or in the ALS mice (Chapter 6). It is interesting that the forced wheel-running protocol at a low intensity elicited the greatest fold-change of GDNF protein content in young rats as compared to all the other exercise regimens examined. Even though the animals that had undergone long-term exercise and had run at a moderate- to low-intensity running protocol, there was no effect on GDNF protein content in the spinal cord. Our results lend support to what others have previously shown with other neurotrophic factors. Specifically, we found that moderate-intensity involuntary running yields the greatest change in GDNF protein content of all exercise regimens examined. These results may suggest that a short-term exercise program may be a better stimulus for enhancing neurotrophic factor content in the spinal cord. It may be possible that acute exercise activates prosurvival pathways, such as PI3-K and MAPK pathways, to initiate rapid changes within the nervous system. Another
possibility could be that acute exercise decreases amounts of stress, which are known to
decrease amounts of neurotrophic factor expression (Ang et al., 2003). However, 
continuous chronic exercise may not be able to elicit the same neuroprotective role of
decreasing levels of stress. Future studies will need to determine what prosurvival and 
stress pathways are activated following short-term and long-term exercise in age-matched 
animals. If there are differences in expression of prosurvival pathways with the different 
exercise regimens, determining what transcriptional regulators promoting neuronal 
survival, such as cyclic AMP responsive-element binding protein (CREB), to help 
facilitate GDNF levels are warranted.

Conclusion

As we revisit our three hypotheses of the current dissertation, we have answered the following questions: Exercise does modulate GDNF protein in the spinal cord. However, differences in duration and intensity vary GDNF differently. Our observations have demonstrated that short-term exercise may be a more potent stimulus to increase GDNF protein than long-term exercise. Furthermore, forced low-intensity exercise may also be a more potent mechanism to increase GDNF protein than voluntary and moderate-intensity exercise. We did not find that the forced low-intensity exercise program for the duration chosen significantly alters GDNF protein in transgenic ALS mice. Treatment with antibodies against GDNF demonstrated there was a decrease in motor neuron size and number, thus blocking the beneficial effects of exercise in ALS mice. We believe that enhancing neurotrophic factors may be a contributing factor to the
neural protection/neural plasticity caused by exercise. Exercise has the advantage of enhancing neurotrophic factor levels by physiological means using intrinsic mechanisms in the spinal cord rather than attempting to increase neurotrophic factor levels via exogenous administration where all the physiological implications are not well understood. Finding an exercise regimen that elicits the best response to increase neurotrophic factor production and increase neuronal function and survivability is critical as we continue to age and undergo degeneration of our nervous system.
REFERENCES


Rat Genome Sequencing Project Consortium (2004).


Appendix

Institutional Animal Care and Use Committee (IACUC) Forms
WESTERN MICHIGAN UNIVERSITY
Institutional Animal Care and Use Committee
ANNUAL REVIEW OF VERTEBRATE ANIMAL USE
I.A.C.U.C.

PROJECT OR COURSE TITLE: Activity Dependent Regulation Of Neurotrophic Factor Expression In Skeletal Muscle.
IACUC Protocol Number: 09-0260
Date of Review Request: 12/22/10
Date of Last Approval: 02/01/10
Purpose of project (select one): ☒Teaching ☐Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: John Spitsbergen Title: Professor
Department: BGS Electronic Mail Address: john.spitsbergen@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Title: Select one
Department: Electronic Mail Address:

1. The research, as approved by the IACUC, is completed:
☐Yes (Continue with items 4-5 below.) ☒No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐Yes ☒No
3. Have there been any new findings or publications relative to this research? ☐Yes ☐No

Describe the sources used to determine the availability of new findings or publications:
☐No search conducted (Please provide a justification on an attached sheet.)
☐Animal Welfare Information Center (AWIC)
☒Search of literature databases (select all applicable)
☐AGRICOLA ☐Current Research Information Service (CRIS)
☐Biological Abstracts ☒Medline
☐Other (please specify):
Date of search: 12/20/10 Years covered by the search: 2000-2010
Key words:
☐Additional search strategy narrative: Key words: GDNF, exercise training, nervous system plasticity,

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research? ☐Yes ☒No

Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 12 Cumulative number of animals used to date: 118

Principal Investigator/Faculty Advisor Signature Date 12/22/10

Co-Principal or Student Investigator Signature Date

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

IACUC Chair Signature Date 4/1/2011

Revised 10/01 WMU IACUC
All other copies obsolete
PROJECT OR COURSE TITLE: Activity Dependent Regulation Of Neurotrophic Factor Expression In Skeletal Muscle And Spinal Cord.

IACUC Protocol Number: 19-12-02
Date of Review Request: 11/14/11 Date of Last Approval: 12/19/10
Purpose of project (select one): ☑Teaching ☑Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: John Spitsbergen Title: Select one
Department: 3005 Electronic Mail Address: john.spitsbergen@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Title: Select one
Department: Electronic Mail Address:

1. The research, as approved by the IACUC, is completed:
   ☑Yes (Continue with items 4-5 below.) ☒No (Continue with items 2-5 below.)

   If the answer to any of the following questions (items 2-4) is “Yes,” please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☑Yes ☒No

3. Have there been any new findings or publications relative to this research? ☑Yes ☐No

   Describe the sources used to determine the availability of new findings or publications:
   ☑No search conducted (Please provide a justification on an attached sheet.)
   ☐Search of literature databases (select all applicable)
     ☐AGRICOLA ☐Current Research Information Service (CRIS)
     ☐Biological Abstracts ☑MEDLINE
     ☐Other (please specify):

   Date of search: 11/14/11 Years covered by the search: 2010-11

   Key words:
   ☐Additional search strategy narrative: 

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research? ☑Yes ☒No

   Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 12 
   Cumulative number of animals used to date: 21

Principal Investigator/Faculty Advisor Signature: 11/14/11

Co-Principal or Student Investigator Signature: Date

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature:

Revised 10/01 WMU IACUC
All other copies obsolete.
Date: January 13, 2012

To: John Spitsbergen, Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol Number 12-01-03

Thank you for submitting your protocol with the required revisions. Your revised protocol entitled “Activity Dependent Regulation of Neurotrophic Factor Expression in Skeletal Muscle” 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: January 13, 2013