The Effects of Aging and Activity on Glial Cell Line-Derived Neurotrophic Factor Expression in Skeletal Muscle

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THE EFFECTS OF AGING AND ACTIVITY ON GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR EXPRESSION IN SKELETAL MUSCLE

by

Nathan Peplinski

A Dissertation
Submitted to the
Faculty of The Graduate College
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Glial cell line-derived neurotrophic factor (GDNF) is an extracellular signaling protein that is produced by skeletal muscle and is important for the motor neurons that control muscle movement. GDNF has been shown to keep neurons alive under conditions that they would otherwise not persist. In skeletal muscle, GDNF has been shown to be one of the most potent neurotrophic factors that influence motor neuron survival. While the role of GDNF has been well studied during early development, not much is known about what happens to GDNF expression in the adult and with advanced aging. Previous results from our lab have demonstrated that GDNF protein content in the adult can be significantly altered with exercise. It was the purpose of these studies to determine the effects of aging and activity on GDNF protein expression in rat skeletal muscle.

We found that GDNF expression was not significantly altered throughout the majority of adulthood until it significantly increased at 19 and 23 months of age. This time frame overlaps with onset of sarcopenia (age-related muscle loss and weakness). We also found that changes in GDNF, both in early and in late-life
stages, appeared to be specific to skeletal muscle type. These results suggest that changes in GDNF and/or its ability to signal to motor neurons may be involved in age-related changes that occur in skeletal muscle.

Additionally we investigated how GDNF expression is altered with activity. When we stimulated skeletal muscle to contract we observed significant changes in GDNF protein content in skeletal muscle. Again we found that these responses were specific to skeletal muscle type. Further investigation determined that the receptors for the neurotransmitter, acetylcholine, appeared to be involved in the regulation of GDNF protein expression with activity. These results provide a better understanding of how GDNF protein expression is regulated in skeletal muscle.
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Nathan Peplinski
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CHAPTER I

INTRODUCTION

Neurotrophic Factors

Neurotrophic factors are a class of proteins that support the development, maintenance and survival of neurons. They are produced by various cell types which can either be the targets of the neurons themselves or supportive, ancillary cells. They are released and diffuse across extracellular regions to act as extracellular signaling proteins. Neurotrophic factors have many roles within an organism.

The word “neurotrophic” literally means nerve-nourishing. These classes of molecules demonstrate the ability to increase the survival of neurons under various conditions that they may not otherwise persist (Lin et al. 1993; Oppenheim et al. 1995; Arce et al. 1998). Early observations of this physiological phenomenon brought about an exciting new class of proteins to investigate with unknown possibilities.

Neurotrophic factors are part of a larger family of growth factors present throughout the body. Growth factors are important for improving cell proliferation and growth. They guide development of tissues and act to help maintain homeostasis. Two main families of growth factor proteins have been identified as neurotrophic. These include the Neurotrophin family and the Glial cell line-derived neurotrophic factor (GDNF) Family.

1
The Neurotrophin family of neurotrophic factors include; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). These ligands share a binding affinity for a family of related tyrosine kinase receptors (Trk) that promote neuron survival as well as an affinity toward a separate, pro-apoptotic p75NR receptor. All of the neurotrophin family ligands demonstrate the ability to bind to the p75NR receptor (Chao 1994), while the affinity for the Trk family of receptors is ligand/receptor specific; NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 binds to TrkC (Barbacid 1994; Bothwell 1995; Kaplan and Miller 2000). While both the Neurotrophin family and the GDNF families of neurotrophic factors promote the survival of neurons they are two distinct classes of molecules.

The GDNF family of neurotrophic factors consists of GDNF, neurturin (NTRN), artemin (ARTN), and persephin (PSPN). Like the Neurotrophin family, they too activate a tyrosine kinase receptor called c-Ret, however signaling through c-Ret is mediated by a specific co-receptor for each molecule. These co-receptors are glycosyl phosphatidylinositol (GPI)-linked receptors that preferentially bind to the GDNF receptors in the following manner; GDNF binds to GFRα1, NTRN binds to GFRα2, ARTN binds to GFRα3, and PSPN binds to GFRα4 (Airaksinen et al. 1999). However a varying degree of ligand/receptor crosstalk within the GDNF family does exist.

While the aforementioned ligand/receptor pairing is the preferred interaction \textit{in vivo}, some ligands demonstrate weak affinities towards other receptors in the family. Both NTRN and ARTN can associate with GFRα1. GDNF may have the
ability to signal through GFRα2 and GFRα3 however PSPN has been shown to only signal through GFRα4 (Lindahl et al. 2001; Airaksinen and Saarma 2002). Furthermore GDNF has been shown to signal through GFRα1 independent of c-Ret (Poteryaev et al. 1999; Trupp et al. 1999) while NTRN is unable to signal in the absence of c-Ret (Pezeshki et al. 2001). While signaling in the GDNF family of neurotrophic factors may be complicated this overlap in cellular signaling may be a method of conservation.

The GDNF family of neurotrophic factors has been shown to be important for normal physiological development. Mice that lack both gene copies for either GDNF, GFRα1, or c-Ret all die shortly after birth due to multiple neurological failures during development including improper renal formation (Sanchez et al. 1996; Airaksinen and Saarma 2002). Neurturin or GFRα2 knock-out mice grow poorly after weaning and demonstrate a loss of multiple types of neuronal populations within an organism (Heathcote and Sargent 1987; Enomoto et al. 2000; Nanobashvili et al. 2000). This is in contrast to both the ARTN or GFRα3 and the GFRα4 knock-out animals that demonstrate only minor phenotypic changes and the selective loss of only a few neuronal subtypes solely in the peripheral nervous system (Nishino et al. 1999; Hiltunen et al. 2001).

Of the GDNF family of neurotrophic factors only GDNF and NTRN were discovered for their functional activity while both ARTN and PSPN were later discovered by similarities in their gene sequence to the other two family members (Balo et al. 2000). While both GDNF and NTRN molecules are important for normal neurological development and function, the consequences of GDNF knockout
in relative comparison to NTRN knockout has a distinctly more severe phenotypic effect. Due to GDNF’s important biological activity and its role in both the normal neuronal development and function it is an excellent candidate for functional studies.

**GDNF**

Glial cell line-derived neurotrophic factor (GDNF) was first discovered in 1993 and found to enhance the survival of midbrain dopaminergic neurons (Lin et al. 1993). It was further characterized as belonging to the TGF-β superfamily of growth factors. It is an extracellular signaling protein that forms a homodimer to become biologically active. Each mature GDNF protein monomer is comprised of 134 amino acids and is approximately 15 kDa before it becomes glycosylated. The full mRNA sequence is 633 base pairs (bp) of nucleotides which encodes for 211 amino acids. It is believed that alternative spicing of the mRNA is responsible for producing a second mRNA of 555-558 bps (Springer et al. 1995; Trupp et al. 1995; Suzuki et al. 1998).

This shorter version of GDNF cDNA is a result of a 78 bp deletion in the prepro region of the coding sequence (Trupp et al. 1995). The resultant 26 amino acid deletion also results in a change of Gly25 into an Ala. However no differences in mobility were observed between the mature GDNF proteins formed from either of the cDNA sequences (Trupp et al. 1995). It is still unclear if the alternative forms of GDNF have distinctly separate functions.

Springer et al. (1995) speculates that this may be a method of auto-regulation, as proteins produced from the smaller splice variant of acidic fibroblast
growth factor act as an antagonist to the larger form of this growth factor (Yu et al. 1992). In the Neurotrophin family both NGF and BDNF have biologically active pro- forms of their respective neurotrophic factors. For NGF it is the pro-NGF form that binds with high affinity to the pro-apoptotic p75 receptor (Hempstead 2006). This is in direct contrast to the pro-survival pathways activated by NGF signaling through the TrkA receptor. For BDNF it is even more complex as 7 different splice variants of mRNA are present, from which 4 different pro-BDNF proteins are derived. Pro-BDNF 1-3 isoforms can all be processed to become the mature BDNF with similar characteristics and are ~14 kDa monomers, while pro-BDNF 4 has a sequence deletion and its protein is expressed as a 10 kDa monomer (Diamond et al. 2005).

The exact sequences of the events involved in GDNF protein processing are not well characterized. It is known that the mature GDNF protein monomer is smaller than the amino acid sequence after translation suggesting a protease cleavage of the full protein. The enzymes involved and the exact locations of these events are not known at this time. The resulting mature GDNF protein monomer becomes glycosylated and is disulfide bonded to another monomer to become a homodimer (Lin et al. 1993). This mature homodimer is released by target tissues and is considered the biologically active form which can initiate signaling with the GFRα1/c-Ret complex.

Once GDNF binds to GFRα1/c-Ret complex the internal tyrosine residues on c-Ret become phosphorylated. Activation of c-Ret initiates several signaling cascades that regulate cell survival, proliferation, neurite outgrowth, differentiation,
and synaptic plasticity (Sariola and Saarma 2003). c-Ret also forms from a splice variant and the receptor proteins can be displayed in either a long or a short isoform. While most of the physiological activity associated with c-Ret knockout studies can be rescued by solely expressing the short form (de Graaff et al. 2001), the short-form only has three tyrosine residues that become phosphorylated while the long-form has an additional tyrosine (Tyr1096) that is phosphorylated. These two isoforms of c-Ret activate different signaling complexes (Tsui-Pierchala et al. 2002) and each complex is dependent upon the association of c-Ret to lipid rafts in the cell membrane. GDNF has also been shown to initiate cell signaling independent of c-Ret.

Recently it has been demonstrated that GDNF can signal in a c-Ret independent manner by binding to GFRα1 and associating with a yet unknown transmembrane protein. Signaling in the absence of c-Ret initiates Src-family kinase phosphorylation and activation of ERK/MAP kinase, PLC-γ, Fos, and the transcription factor CREB protein (Trupp et al. 1999). GDNF signaling requires heparin sulfate glycosaminoglycans (Barnett et al. 2002) as well as GFRα1. It is suggested that this unknown transmembrane protein may use heparin sulfate proteoglycans to associate with GFRα1 and initiate this c-Ret independent signaling cascade (Sariola and Saarma 2003).

By studying the signaling cascades activated by GDNF we are provided with specific molecular insight as to how GDNF affects the neuron and how it generates its neurotrophic activity. However it still remains that in order for GDNF to initiate these cellular pathways it must first be produced by a source and released before it can have an impact on the neuron. There are many sources of GDNF throughout the
body (Suter-Cazzolara and Unsicker 1994; Trupp et al. 1995; Golden et al. 1999). These tissues may work individually or collectively to provide trophic support to nearby neurons. One such source is skeletal muscle.

**Skeletal Muscle**

The nerves that communicate with skeletal muscle cells rely on GDNF and other neurotrophic factors for survival. Skeletal muscle is a diversely innervated tissue. It receives nerve input from a specific group of neurons called motor neurons that originate from the spinal cord. These neurons are responsible for stimulating the skeletal muscle to contract and provide locomotion. Skeletal muscle is also innervated by sensory neurons that communicate with the CNS about muscle fiber length to help coordinate locomotion and proprioception. Both the motor neurons and sensory neurons are trophically supported by GDNF. However, the association with sensory neurons decreases shortly after development and its effects on sensory neurons are minimal when compared to other neurotrophic factors (Buj-Bello et al. 1995; Trupp et al. 1995).

Skeletal muscle fibers themselves are a diverse population of adaptable cells with various phenotypes. There are two main categories of skeletal muscle fibers; intrafusal and extrafusal muscle fibers. Each skeletal muscle fiber type has a specific role within the tissue. Intrafusal muscle fibers, often called muscle spindle fibers, have contractile regions on the distal ends of the cell and a non-contractile region in the center. In this non-contractile region these muscle fibers have adapted sensory apparatus that detect the tension and position relationship of the muscle. Sensory
neurons can then relay sensory information about muscle position in both a reflex arc and to the motor cortex of the brain for advanced coordination of muscle movement.

Along with the sensory innervation of intrafusal muscle fibers they are also contacted by motor neurons. These neurons are responsible for controlling the contractile portions of the intrafusal muscle fibers to “reset” the muscle fiber so that tension remains constant as skeletal muscle position changes with coordinated movement. It has been recently demonstrated that GDNF is highly expressed in these intrafusal muscle fibers and that its role in these fibers may be to trophically support the motor neuron innervation of these fibers (Whitehead et al. 2005).

Extrafusal muscle fibers are the muscle fibers responsible for generating the force associated with muscle contraction. These make up the majority of the composition of the skeletal muscle and can further be divided into two basic phenotypes. These two extrafusal skeletal muscle fiber phenotypes are classified by their metabolic function associated with myosin ATPase activity. These skeletal muscle fibers can be classified as either slow-(type I or slow oxidative) or fast-twitch muscle fibers.

Of the fast-twitch muscle fibers there exist three different subtypes. These subtypes include; type IIA (fast-oxidative, fast fatigue-resistant), type IID/X (fast intermediate fatigability), and IIB (fast glycolytic, fast fatigable)(Schiaffino et al. 1989; Staron et al. 1999). Some skeletal muscles do contain muscle fibers that appear as combinations of these four phenotypes however their presence is usually minimal and may or may not be present in various skeletal muscles. Skeletal muscle fiber type may be related to GDNF protein expression. Nagano et. al. (2003)
determined the pattern of GDNF expression of both mRNA and protein content of developing rat skeletal muscles differed when comparing the soleus (a primarily slow-twitch skeletal muscle) to the gastrocnemius (a primarily fast-twitch skeletal muscle). Fast- and slow-twitch muscle fibers are contacted by motor neurons that also display fast and slow signaling dynamics (Edstrom and Kugelberg 1968; Burke et al. 1973). It may be inferred then that if GDNF is selectively expressed in a particular skeletal muscle fiber subtype, then it may be important for supporting a selective subtype of motor neuron as well.

GDNF in Skeletal Muscle

The role of GDNF in skeletal muscle has been primarily associated with the support and maintenance of motor neurons. It has been demonstrated that GDNF is one of the most potent neurotrophic factors for motor neurons (Henderson et al. 1994; Oppenheim et al. 1995; Trupp et al. 1995; Yan et al. 1995; Nguyen et al. 1998). GDNF has been shown to rescue motor neurons from cell death due to both naturally occurring apoptosis in development (Henderson et al. 1994; Oppenheim et al. 1995; Yan et al. 1995; Nguyen et al. 1998; Oppenheim et al. 2000; Zwick et al. 2001) as well as recovery from motor neuron injury (Lie and Weis 1998; Chen et al. 2001; Boyd and Gordon 2003). GDNF has also been shown to decrease the motor neuron damage associated with degenerative neuropathies (Sagot et al. 1996; Suzuki et al. 1998; Corse et al. 1999; Yamamoto et al. 1999; Wang et al. 2002). While its importance for motor neuron survival has been well established some have noticed that GDNF appears to only effect a subpopulation of motor neurons.
Functional studies of GDNF-deficient and GFRα1-deficient mice demonstrated that only 22-35% of spinal motor neurons were lost at birth (Moore et al. 1996; Sanchez et al. 1996; Cacalano et al. 1998; Oppenheim et al. 2000). This suggests that there may be GDNF-dependent and GDNF-independent subpopulations of motor neurons. Furthermore histochemical analyses of sciatic nerve bundles demonstrate that GFRα1 is expressed preferentially in 25% of the motor neurons (Bergman et al. 1999). And GDNF protein labeling of the axons in intramuscular nerve bundles demonstrated increased GDNF signal from the large diameter axons in human skeletal muscle (Suzuki et al. 1998). However, without a specific marker for motor neuron subtype, identification of the GDNF-dependent subtype has not yet been determined.

A recent finding in our lab demonstrated that GDNF protein expression in skeletal muscle is regulated in an activity-dependent manner (Wehrwein et al. 2002). Others have since determined that exercise after nerve injury increases the neurotrophic effects of GDNF in rat soleus (Dupont-Versteegden et al. 2004). We demonstrated that increased exercise with walk-training causes a significant increase in GDNF protein content, while skeletal muscle inactivity associated with hind limb suspension caused a significant decrease in the unloaded skeletal muscles. In contrast, the forelimb skeletal muscles in the hind limb unloaded experiments increased their GDNF protein content. An interesting comparison, however, was that GDNF protein content was significantly higher in the primarily slow-twitch skeletal muscle (soleus) compared to the primarily fast-twitch skeletal muscle (gastrocnemius). These differences in skeletal muscle expression with activity may
be associated with the fiber-type composition. Therefore, current studies were designed to address the specific responses of skeletal muscles with various fiber-type compositions in response to exercise and activity. While little is still known about the regulation of GDNF expression in skeletal muscle with exercise and activity, even less is known about GDNF expression with advanced aging.

With skeletal muscle aging there is a significant loss of skeletal muscle mass. This process, called sarcopenia, is characterized by a loss of approximately one-third of normal adult muscle mass and occurs in humans from age 20-80 (Rosenberg 1997). During the process of sarcopenia, skeletal muscles selectively lose fast-twitch muscle fibers in a process that is still unclear. Both fast-twitch muscle fibers and the fast-firing motor neurons are significantly decreased with age (Brown 1972; Brown et al. 1988; Kanda and Hashizume 1998; Roubenoff and Hughes 2000). However, slow-twitch muscle fibers appear to be less affected and often sprout axonal processes to take over the vacated NMJs left behind on the denervated fast-twitch muscle fibers (Rosenberg 1997; Roubenoff and Hughes 2000). The result is an age-dependent shift in fiber-type composition from a fast- to a slow-twitch phenotype and an overall loss of skeletal muscle mass. As expected, age-related changes associated with sarcopenia occur more in skeletal muscles that have a primarily fast-twitch muscle fiber-type composition and less in skeletal muscles that have a primarily slow-twitch muscle fiber-type composition.

One possible cause for the loss of motor neurons and the fiber-type switching that occurs with advanced aging could be due to alterations in neurotrophic factor expression and/or signaling. Since GDNF has been shown to be one of the most
potent neurotrophic factors for motor neurons, then alterations in GDNF content and/or signaling may be involved in age-related motor neuron loss. Bergman et. al. (1999) determined that both GFRα1 and c-Ret mRNA and protein are up-regulated on spinal motor neurons of aged rats. However, the effect of aging on skeletal muscle production of GDNF protein has not yet been determined. It is therefore important and necessary to determine if age-related changes in GDNF protein content occur in skeletal muscle in order to better understand the possible role of GDNF in the process of sarcopenia.
CHAPTER II

EXPERIMENTAL DESIGN

Specific Aims

The broad objective of our lab is to gain a better understanding of the bi-directional communication that occurs between neurons and the target tissues that they innervate. We are focused primarily on determining the role that neurotrophic factors have in this communication as well as investigating signals that alter their expression. We are specifically interested in the production of a type of neurotrophic factor called glial cell line-derived neurotrophic factor (GDNF). It is the aim of this research to determine where GDNF protein is expressed in skeletal muscle and to determine what can alter expression of GDNF protein in skeletal muscle. To achieve these aims we will test the following hypotheses;

1. **GDNF protein is concentrated at the area of the neuromuscular junction (NMJ).** To determine where GDNF is located in skeletal muscle:

   a. We will use antibody labeling against GDNF protein and view the NMJ region in both longitudinal and cross-sectional analyses.
2. **The process of aging alters GDNF protein expression in skeletal muscle.** To determine how aging affects GDNF protein content:

   a. We will measure GDNF protein content of various hind limb skeletal muscles in animals at early, mid, and late life stages of aging.

3. **Voluntary exercise alters GDNF protein expression in skeletal muscle.**

   To determine how voluntary exercise affects GDNF protein content:

   a. We will train animals to exercise voluntarily and measure the effect of a long bout of exercise on GDNF protein content of skeletal muscles.

   b. We will train animals to exercise voluntarily and measure the effect of an acute bout of exercise after a long bout of exercise on GDNF protein content of skeletal muscles.

4. **Skeletal muscle stretch alters GDNF protein expression.** To determine how physical stretch affects GDNF protein content:
a. We will subject skeletal muscle to repetitive stretching and measure the amount of GDNF protein expressed.

b. We will block AChRs using $\alpha$-bungarotoxin, stretch the muscle, and measure the amount of GDNF protein expressed.

5. **Acetylcholine receptor activity alters GDNF protein expression.** To determine how acetylcholine receptor activity affects GDNF protein content:

a. We will block AChRs using $\alpha$-bungarotoxin and measure the amount of GDNF protein expressed. (no ACh activity, no electrical stimulation)

b. We will block AChRs using $\alpha$-bungarotoxin, stimulate the muscle via electric field and measure the amount of GDNF protein produced. (no ACh activity, but electrical stimulation)

c. We will directly activate AChRs using carbachol and measure the amount of GDNF protein expressed.
Testing these specific hypotheses will further our understanding of neurotrophic factor regulation in skeletal muscle. The results of these experiments may elucidate mechanisms that are responsible for exercise related changes that occur in skeletal muscle as well as provide insight as to the possible role of GDNF in age-related changes in skeletal muscle.

**Preliminary Data and Troubleshooting**

The methods of all of the following experiments are explained in detail in their corresponding chapters. It is the purpose of this section to explain the trials and tribulations that led up to their current form and give insight as to how these methods came about.

**Skeletal Muscle Selection**

There appears to be an underlying theme suggested by results from ours and others’ previous studies (Wehrwein et al. 2002; Nagano and Suzuki 2003). Skeletal muscles respond to exercise with a change in GDNF protein content and it is possible that these responses may be tissue dependent. It is because of this possibility that we decided to compare the results for all of our studies using a variety of hind limb skeletal muscles. We chose both the soleus (SOL) and extensor digitorum longus (EDL) muscles because of their vast differences in muscle fiber-type composition, physiological function, neuronal input, and metabolic activity (Alnaqeeb and Goldspink 1987; Reid et al. 2003; Patterson et al. 2006). With respect to skeletal muscle fiber-type composition, these two tissues represent an almost completely
opposite expression pattern. The SOL is composed primarily of slow-twitch muscle fibers while the EDL is composed primarily of fast-twitch muscle fibers. It is because of these differences that these two tissues have been compared classically throughout skeletal muscle activity studies (Alnaqeeb and Goldspink 1987; Windisch et al. 1998; Gissel and Clausen 1999; Copray et al. 2000; Plant et al. 2001; Reid et al. 2003).

While we hoped to compare and contrast the responses of these two tissues with respect to activity and aging, an unknown technical problem did not come to light until late into our investigation. We had been mistakenly removing the flexor digitorum superficialis (FDS) skeletal muscle instead of the SOL. While results from the SOL have been well documented in the literature, the FDS has not been extensively studied. In the rat, the FDS is larger than both the SOL and EDL in size and works in opposition to the EDL. In the human, the muscle is deep and not superficial and is therefore named flexor digitorum longus (FDL). The FDL has a much longer distal tendon than the FDS in the rat and is relatively smaller in size to human EDL and SOL. These differences between rat FDS and human FDL make it increasingly difficult to draw direct comparisons between the two different species.

The relative composition of rat FDS was determined to contain 8% slow-twitch muscle fiber counts (Ariano et al. 1973) but published reports of muscle type-fiber composition from this paper have differed slightly compared to more recent values published by other researchers (Armstrong and Phelps 1984; Alnaqeeb and Goldspink 1987; Chamberlain and Lewis 1989). We have also provided visual evidence of its relative slow fiber-type composition compared to both the SOL and
the EDL (Figure 10). We concur that the FDS is similar in composition to the EDL with the majority of its muscle fibers being fast-twitch but it does appear to have slightly higher slow-twitch muscle fiber content than EDL.

It is because of this misidentification that the majority of the bath studies include results from all three muscles. It is also one of the main reasons why there are two separate aging studies; the first did not include the real SOL and only had results for the FDS and EDL. The voluntary running study was also affected. However, results were reported for the FDS and EDL and do not include data from the SOL.

In the first of the two aging studies another muscle called the vastus intermedius (VI) was used for only a few of the time points investigated (7 month, 9 month, and 19 month). This tissue was selected because the literature suggested that it may represent a hind limb skeletal muscle with a relatively mixed distribution of fast- to slow-twitch muscle fiber composition (Ariano et al. 1973). However technical difficulties in the reliable removal of this tissue cause complications. The VI inserts onto the medial portion of the rat femur in a fan-like array and removal of this insertion often resulted in a varying degree of muscle fiber damage. Furthermore, the VI had a poorly defined border with its neighboring quadriceps muscles the vastus medialis and the vastus lateralis. Removal of the VI at times also included partial removal of either of these two tissues and was inconsistent throughout. It is because of these technical difficulties that the VI was no longer used for the other time points investigated or for any of our other studies.
Another tissue selected for study was the extensor hallucis longus (EHL). It has identical function to the EDL (to extend the digits of the foot), but the EDL extends phalanges II-V while the EHL extends the hallux bone (a.k.a. the big toe). This tissue was chosen because its function is similar to that of the EDL but with its relatively much smaller size, it is more useful for histological analyses. The EHL was used in whole-mount, free-floating, preparations for longitudinal visualization of skeletal muscle histochemistry.

**Handedness**

One observation from previous studies in our lab was that even in a control group of animals, GDNF protein content of skeletal muscle appeared to be variable at times. After review of the previous methods used in the lab, whereby the right side of the animal’s paired skeletal muscles were used for GDNF protein quantification using and enzyme linked immunosorbant assay (ELISA) and the left side used for histology, we decided to examine the possibility of “handedness” in GDNF protein expression.

In a preliminary study of control animals we examined the GDNF protein content of the flexor digitorum superficialis (FDS) and extensor digitorum longus (EDL) in seven month old Sprague Dawley rats. We found that there was no significant difference between the left versus the right side of these paired hind limb skeletal muscles (*Figure 1*).
There is no significant effect of 'handedness' on GDNF protein expression in hind limb skeletal muscles of 7 month old rats.

Figure 1. There is no significant effect of ‘handedness’ on GDNF protein expression in hind limb skeletal muscles of 7 month old rats. Glial cell line-derived neurotrophic factor protein content was measured in the flexor digitorum superficialis (FDS) and extensor digitorum longus (EDL) muscles of seven month old Sprague-Dawley rats. Paired left and right hind limb muscles were removed, frozen on dry ice, and processed for determination of GDNF protein content using an enzyme-linked immunosorbant assay. There was no significant difference in the left versus the right skeletal muscle GDNF protein content for either the FDS or the EDL (n = 6).
When comparing the left versus the right side for these tissues there was no statistically significant difference in GDNF protein content. It might have been that if GDNF protein content was affected by an inherent dominate “handedness,” then our results could have been skewed by one or two of our animals being left-side dominate while the rest of the animals may have been right-side dominate, or vice versa. Without the ability to initially determine an inherent preference, we could not correlate any of these possibilities with our results. We did notice a trend in the values from each animal.

It appeared as though for each animal, while there was no distinct difference from left versus right, there was a difference in expression in one side versus the opposing side. If we ignored the left versus right classification and instead used the null hypothesis that one side has greater GDNF protein content than the other, and sorted our data so that within each animal we compared the muscle with the higher GDNF protein content to the muscle with the lower GDNF protein content then our results were significantly different for the FDS but not the EDL (Figure 2). It is because of this that we were faced with a dilemma.
There is a significant effect of 'sided-ness' on GDNF protein expression in FDS but not EDL of 7 month old rats.

Figure 2. There is a significant effect of ‘sided-ness’ on GDNF protein expression in FDS but not EDL of 7 month old rats. Glial cell line-derived neurotrophic factor protein content was measured in the flexor digitorum superficialis (FDS) and extensor digitorum longus (EDL) muscles of seven month old Sprague-Dawley rats. Paired left and right hind limb muscles were removed, frozen on dry ice, and processed for determination of GDNF protein content using and enzyme-linked immunosorbant assay. GDNF protein content of each tissue was determined for each animal and values were placed categorically into a group with the higher of the paired values belonging to the highest group, and the lower of the paired values belonging to the lowest group (n = 6). In this analysis, GDNF protein content was significantly higher in the ‘highest’ group in the FDS but not significantly higher in the EDL. These results suggest that for the FDS, within an animal, one side contains significantly more GDNF protein content than its contralateral pair. (* = p ≤ 0.05)
Without the advance knowledge of knowing which side may have started with more GDNF before experimentation, manipulations like bath experiments could have been greatly affected. In tissue bath experiments, both sides were used and compared to one another. It would have been difficult to infer physiological changes from control if one group had a significantly different basal level of GDNF protein content. It is because of this possibility that we chose to randomly sort left- and right-sided muscles into our control and experimental groups for all of the following bath experiments. This random assortment may have increased the variability of the absolute values of GDNF in the tissue, but since both the experimental and the control groups are treated in this manner, then any differences between the groups should have been due to the manipulation of the bath experiment itself and not to inherent differences in baseline levels of GDNF protein content.

_Skeletal Muscle Sectional Analysis_

One of the objectives of these studies was to determine where in the tissue GDNF protein is located. We proposed two methods to determine if GDNF protein expression is increased in the region of the neuromuscular junction. The first method was to use immunohistochemistry and the results of which are explained in the following chapter. The second method proposed was to perform a sectional analysis of skeletal muscle and determine GDNF protein content as it relates to endplate density _**(Specific Aims 1b)**_. This is based upon the assumption that most of the innervation of skeletal muscle occurs in the belly of the tissue. We decided to dissect the muscle into three equal pieces that represent the proximal, middle, and distal third
of the skeletal muscle. According to the assumption the majority of the skeletal muscle endplates should be in the middle section. If GDNF protein content is greatest near the region of the NMJ, then the middle section should have significantly more GDNF protein content than the proximal or the distal segments.

Our results showed no significant difference between any of the three sections when GDNF protein content is measured and compared (Figure 3). Upon further investigation we checked our initial assumption that the majority of the endplates do in fact occur in the middle section. We found that in the FDS, as well as in the SOL and EDL, the orientation of the muscle fibers run diagonally along the muscle body. The endplates also follow this diagonal orientation and thus, skeletal muscle endplates were found dispersed evenly throughout the majority of the length of the tissue. Sectional analyses of GDNF protein content of skeletal muscles would therefore not be a suitable method to determine if GDNF protein is preferentially expressed near the region of the NMJ.
There are no significant differences in GDNF protein content between regions of the FDS in four month old F344 rats.

Figure 3. There are no significant differences in GDNF protein content between regions of the FDS in four month old F344 rats. In order to determine if there was a regional expression of GDNF protein content in skeletal muscle, a sectional analysis of the flexor digitorum superficialis (FDS) muscle was performed. FDS were removed from four month old male Fisher 344 rats (n = 4) and divided into three equal sections representing the proximal, middle, and distal one-third of the muscle respectively. Tissue sections were frozen on dry ice and processed for quantification of GDNF protein content using an enzyme-linked immunosorbant assay. There was no significant difference between any of the three segments with regards to GDNF protein content.
CHAPTER III

HISTOLOGY

Introduction

One of the specific aims of our research is to determine where within the tissue glial cell line-derived neurotrophic factor (GDNF) protein is expressed. Researchers have attempted labeling of endogenous GDNF protein in skeletal muscle with varied success (Suzuki et al. 1998; Suzuki et al. 1998; Russell et al. 2000). Still others have decided to avoid complications associated with labeling endogenous GDNF protein and instead used genetically modified animals to express β-galactosidase-linked GDNF protein. These methods have been shown to be useful in labeling the genetically modified GDNF protein; however these manipulations can only be performed in mice and cannot be used in our system (Whitehead et al. 2005).

Suzuki et. al. (Suzuki et al. 1998) identified GDNF immunoreactivity to be increased in the area of the NMJ for transversely-sectioned human skeletal muscle biopsies. Russel et. al. (Russell et al. 2000) were unable to detect signs of increased GDNF immunoreactivity at the neuromuscular junction (NMJ) of rat skeletal muscle. This discrepancy in GDNF protein expression between these two species could be of merit, or it might have been that differing methodology led to different results in protein imaging.

We hoped to determine if GDNF protein is expressed at the NMJ in rat skeletal muscle. Additionally, we hoped to include longitudinal views of the NMJ
with the intent of improving GDNF protein imaging in skeletal muscle. Replication of these previous methods in our tissues has historically been an arduous task with inconsistent outcomes. We sought to enhance these imaging methods with the hopes of increasing the reliability of the results. The following methods are the results of many series of combined troubleshooting and tweaking that has accumulated over the years to arrive in the current form.
Materials and Methods

Tissue Preparation

Skeletal muscles were removed and placed in Zamboni’s fixative (Stefanini et al. 1967) for 15 minutes. Tissues were then washed in phosphate buffered saline solution ([PBS] 22.5 mM NaCl, 2.0 mM NaH₂PO₄, and 8.0 mM NaHPO₄) three times for 5 minutes each. Samples to be processed as whole-mount, free-floating preparations were then placed in PBS and stored at 4°C. Samples that were to be used for transverse sectioning were placed in 2-methylbutane cooled on dry ice until frozen. After freezing, tissues were placed in a sealed container and stored at -80°C.

Primary Antibodies

Stock solutions were prepared and stored according to manufacturer’s recommendations. The following primary antibodies were used at the following concentrations; rabbit anti-GDNF (SC 328, Santa Cruz Biotechnologies) at 2.0 μg/mL, goat anti-GDNF (AF-212-NA, R & D Systems) at 5.0 μg/mL, mouse anti-slow myosin (M8421, Sigma-Aldrich) at 5.0 μg/mL, goat anti-GFRα1 (AF560, R & D Systems) at 5.0 μg/mL, mouse anti-neurofilament H (MAB305, Chemicon) at 1.0 μg/mL, mouse anti-typeIIA myosin (805.503, Alexis Biochemicals), and mouse anti-intrrafusal fiber (S46-s, Developmental Studies Hybridoma Bank at the University of Iowa) as undiluted cell culture supernatant.

All of the antibodies listed were prepared in blocking solution which consisted of PBS containing 1% bovine serum albumin (1600-100, Fisher Scientific)
and 0.3% Triton X-100 (T8787, Sigma-Aldrich). Also used were α-bungarotoxin-488 (B13422, Molecular Probes), α-bungarotoxin-594 (B13423, Molecular Probes) and α-bungarotoxin-Texas Red (00015, Biotium) all at 5.0 μg/mL.

**Secondary Antibodies**

All secondary antibodies used were developed to recognize the Immunoglobulin G specific to the host organism that developed the corresponding primary antibodies. Additionally they all were developed to be directly linked to Alexa Fluor molecules for visualization. The following secondary antibodies were all purchased from Molecular Probes and were used at 4.0 μg/mL; donkey anti-mouse-647 (A31573), donkey anti-goat-568 (A11057), donkey anti-rabbit-488 (A21206), goat anti-rabbit-488 (A11008), goat anti-rabbit 594 (A110120, goat anti-mouse-488 (A21121), and goat anti-mouse-594 (A110050).

**Transverse Sections**

Skeletal muscles were removed from storage and embedded in tissue mounting medium (Tissue Tek O.C.T., Sakura). Tissues were cut into 50 μm transverse slices using a cryostat and thaw-mounted to glass slides (12-550-15, Fisher Scientific). Slides were then washed 3 x 5 minutes in PBS and a 10% secondary specific animal serum (either donkey or goat) was added to blocking serum and added to the slide for 30 minutes at room temperature. Excess blocking solution was drained from the slides and primary antibodies were added for 4 days at 4ºC. If α-bungarotoxin was used, it was applied before the primary antibodies for 30 minutes at
room temperature followed by washing with PBS 3 x 5 minutes. Following the incubation in primary antibodies, slides were washed 3 x 5 minutes each with PBS and the corresponding secondary was added for visualization. Slides were covered with a 1:1 solution of PBS:glycerol and sealed with a coverslip. All slides were viewed on a Zeiss laser scanning confocal microscope (LSM 510, Zeiss).

*Free-floating Preparations*

Skeletal muscles were removed from storage and washed 3 x 5 minutes with PBS. For the EDL and SOL, these tissues were too large to process as whole-mounts so the tissues were divided into 3 and 4 equal pieces respectively. For the extensor hallucis longus (EHL), tissues were processed as whole-mounts. All procedures for free-floating preparations mimic exactly the protocol above used for transverse sections except that sufficient volumes were added to submerge the tissues and perform each step in 1.5 mL Eppendorf tubes (T2422, Sigma-Aldrich). After the final wash, tissues were transferred to a depression slide, covered with a 1:1 solution of PBS:glycerol and sealed with a coverslip. All slides were viewed on a Zeiss laser scanning confocal microscope (LSM 510, Zeiss).
Results and Discussion

Neuromuscular Junction

According to the neurotrophic model, GDNF like other neurotrophic factors, is produced by target tissues and released for uptake by motor neurons. If GDNF protein can be detected in skeletal muscle, then the area of nerve-muscle contact may be the most probable location for visualization of GDNF protein expression. We determined that there is positive immunoreactivity in the region of the NMJ. We were able to visualize positive staining for GDNF near the region of positive α-bungarotoxin staining in transverse sections of rat EDL (Figures 4 and 5). We noticed that GDNF immunoreactivity is increased near the region of the NMJ but we were unable to determine if GDNF expression is within the skeletal muscle, on the skeletal muscle membrane, or associated with the motor nerve terminal. These results suggest that rat GDNF protein is expressed at the NMJ similar to the pattern of expression observed in human skeletal muscle (Suzuki et al. 1998; Suzuki et al. 1998; Hase et al. 1999). The negative results observed by Russel et al. (2000) might have been due to complications with their histological methods.

Suzuki et al. (1998) demonstrated positive GDNF immunoreactivity near the NMJ in human skeletal muscle after applying the primary antibody to sectioned skeletal muscle for 3 days at 4°C. Russel et al. (2000) applied primary antibody to sectioned skeletal for 2 days at 4°C and was unable to detect GDNF protein immunoreactivity at the NMJ.
GDNF is expressed in the region of the NMJ in rat EDL.

Figure 4. GDNF is expressed in the region of the NMJ in rat EDL. The extensor digitorum longus (EDL) muscle was removed from four week old male Sprague-Dawley rats. The muscle was cut into 50 μm sections and fixed using Zamboni’s fixative. Samples were treated with a blocking solution containing donkey serum for 30 minutes. Slides were drained and treated with α-bungarotoxin-488 for 30 minutes for endplate visualization (green). Samples were washed and goat anti-GDNF primary antibody was applied for 4 days. Slides were washed and donkey anti-goat-568 secondary antibody was applied for 1 hour for GDNF visualization (red). Images were captured using a Zeiss laser scanning confocal microscope. Also represented above is an overlay of transmitted light to show skeletal muscle morphology. The image above depicts increased GDNF immunoreactivity (red) in the region near the neuromuscular junction (green).
GDNF is expressed in the region of the NMJ in rat EDL.

Figure 5. GDNF is expressed in the region of the NMJ in rat EDL. The extensor digitorum longus (EDL) muscle was removed from four week old male Sprague-Dawley rats. The muscle was cut into 3 equal pieces and fixed using 4% paraformaldehyde for 15 minutes followed by ice cold methanol for 5 minutes. Samples were treated with a blocking solution containing donkey serum for 30 minutes. Tissues were drained and treated with α-bungarotoxin-488 for 30 minutes for endplate visualization (green, top left panel). Samples were washed and goat anti-GDNF primary antibody was applied for 4 days. Tissues were washed and donkey anti-goat-594 secondary antibody was applied for 1 hour for GDNF visualization (red, top right panel). Images were captured using a Zeiss laser scanning confocal microscope. The image above depicts increased GDNF immunoreactivity (red) in the region near the neuromuscular junction (green, bottom left overlay).
While the difference might have been minimal, our first attempts were unsuccessful when primary antibody was applied for 1 day at 4°C. It was not until after we changed our protocol to include incubation of the primary antibody to be 4 days in duration at 4°C were we able to successfully visualize GDNF protein at the NMJ. Furthermore, recent developments in our laboratory methods have preliminarily demonstrated that the signal of GDNF protein immunoreactivity increases and the amount of non-specific background staining decreases when we incubate the primary antibody for 7 days and increase the duration of the secondary antibody from 1 hour to 3 days at 4°C (unpublished observations). The duration of primary antibody exposure may only be partly responsible for the differences in the imaging results between our studies and that of Russel et. al. (2000).

Publication materials from the GDNF primary antibody manufacturer (R&D Systems) also suggest that the GDNF antigen may be a fixative sensitive antigen and that prolonged exposure to 4% paraformaldehyde, might completely abolish GDNF labeling. While it is unclear if this is a phenomenon specific to the antibody used, the fixation protocols used for all 3 studies were different. Suzuki et. al. (1998) used 4% paraformaldehyde for 15 minutes for fixation of skeletal muscle sections. Russel et. al. (2000) also used 4% paraformaldehyde for fixation but exposed the tissue for 30 minutes. We decided to use Zamboni’s fixative (Stefanini et al. 1967) because of its improved shelf-life and stability over that of paraformaldehyde. While it is still a paraformaldehyde-based fixative, the approximate concentration of paraformaldehyde is only 2% as opposed to 4%. We also exposed our tissues to fixative for 15 minutes as opposed to 30 minutes.
The differences in methods between our studies and the methods used by Russel et. al. (2000) may account for the discrepancy in GDNF imaging at the NMJ. These differences in methods, along with inherent differences involved in the use of two different primary antibodies for GDNF, collectively or in part might have been responsible for their lack of GDNF signal in rat skeletal muscle. We also hoped to further characterize the phenotypic expression of GDNF immunoreactivity at the NMJ by including longitudinal imaging of skeletal muscle.

Tissues were processed as free-floating preparations, as opposed to transversely sectioned skeletal muscle, and displayed similar results. We were again able to demonstrate increased GDNF immunoreactivity near the NMJ for a variety of skeletal muscles using two different GDNF primary antibodies. The EHL (Figure 6), the EDL (Figure 7), and the SOL (Figure 8) all displayed increased GDNF protein immunoreactivity near the region of the NMJ. These images were all captured using the same goat anti-GDNF antibody (AF-212-NA, R&D Systems). When these results were compared to images attained using a different GDNF primary antibody (sc-382, Santa Cruz Biotechnologies), GDNF protein was still expressed near the NMJ, but the pattern of expression differed.

This second antibody (rabbit) we tested for labeling of GDNF protein was the same antibody used by Russel et. al. (2000). We noticed a different pattern of expression using the rabbit antibody (Figure 9).
GDNF is expressed at the NMJ in rat EHL.

Figure 6. GDNF is expressed at the NMJ in rat EHL. The extensor hallucis longus (EHL) muscles were removed from four week old male Sprague-Dawley rats. The muscles were fixed using Zamboni’s fixative for 15 minutes and processed as a free-floating preparation. Tissues were treated with a blocking solution containing donkey serum for 30 minutes. Samples were drained and treated with α-bungarotoxin-594 (A, red) or α-bungarotoxin-488 (B, green) for 30 minutes for endplate visualization. Samples were washed and goat anti-GDNF primary antibody was applied for four days. Tissues were washed and donkey anti-goat-488 (A, green) or donkey anti-goat-594 (B, red) secondary antibody was applied for one hour for GDNF visualization. Images were captured using a Zeiss laser scanning confocal microscope. The images above depict increased GDNF immunoreactivity (red) in the region near the neuromuscular junction (green). Above left (A) represents endplates (red) and GDNF (green) with a wide field of view. Above right (B) shows a magnified view of endplates (green) and GDNF (red).
GDNF is expressed at the NMJ in rat EDL.

Figure 7. GDNF is expressed at the NMJ in rat EDL. The extensor digitorum longus (EDL) muscles were removed from four week old male Sprague-Dawley rats. Whole EDL were too large to process in a free-floating preparation so tissues were divided lengthwise into three equal pieces. The muscles were fixed using Zamboni’s fixative for 15 minutes. Tissues were treated with a blocking solution containing donkey serum for 30 minutes. Samples were drained and treated with α-bungarotoxin-594 (A, red) or α-bungarotoxin-488 (B, green) for 30 minutes for endplate visualization. Samples were washed and mouse anti-neurofilament H (B only) and goat anti-GDNF primary antibodies were applied for 4 days. Tissues were washed and donkey anti-goat-488 (A, green) or donkey anti-goat-594 (B, red) secondary antibody was applied for 1 hour for GDNF visualization along with donkey anti-mouse-647 (B, blue) for neurofilament visualization. Images were captured using a Zeiss laser scanning confocal microscope. The images above depict increased GDNF immunoreactivity in the region near the neuromuscular junction. Above left (A) represents endplates (red) and GDNF (green) with a wide field of view. Above right (B) shows a magnified view of endplates (green), GDNF (red), and nerve fiber (blue).
GDNF is expressed at the NMJ in rat SOL.

Figure 8. GDNF is expressed at the NMJ in rat SOL. The soleus (SOL) muscles were removed from four week old male Sprague-Dawley rats. Whole SOL muscles were too large to process in a free-floating preparation so tissues were divided lengthwise into four equal pieces. The muscle pieces were fixed using Zamboni’s fixative for 15 minutes. Tissues were treated with a blocking solution containing donkey serum for 30 minutes. Samples were drained and treated with α-bungarotoxin-488 for 30 minutes for endplate visualization (green). Samples were washed and mouse anti-neurofilament H and goat anti-GDNF primary antibodies were applied for 4 days. Tissues were washed and donkey anti-goat-568 secondary antibody was applied for 1 hour for GDNF visualization (red) along with donkey anti-mouse-647 for neurofilament visualization (blue). Images were captured using a Zeiss laser scanning confocal microscope. The images above depict increased GDNF immunoreactivity in the region near the neuromuscular junction. Above top left represents GDNF protein (red) and top right represents nAChRs (green). Above bottom left represents neurofilament (blue) and above bottom right shows an overlay of all three signals. These results suggest that GDNF protein is expressed at the NMJ and possibly in the motor nerve bundle as well.
GDNF is expressed at the NMJ in both EDL and SOL.

Figure 9. GDNF is expressed at the NMJ in both the EDL and SOL. The extensor digitorum longus (A) (EDL) and soleus (B) (SOL) muscles were removed from four week old male Sprague-Dawley rats. Whole EDL and SOL were too large to process in a free-floating preparation so tissues were divided lengthwise into three and four equal pieces respectively. The muscles were fixed using Zamboni’s fixative for 15 minutes. Tissues were treated with a blocking solution containing donkey serum for 30 minutes. Samples were drained and treated with α-bungarotoxin-488 (green) for 30 minutes for endplate visualization. Samples were washed and mouse anti-neurofilament H and rabbit anti-GDNF primary antibodies were applied for 4 days. Tissues were washed and donkey anti-rabbit-568 secondary antibody was applied for 1 hour for GDNF visualization (red) along with donkey anti-mouse-647 for neurofilament visualization (blue). Images were captured using a Zeiss laser scanning confocal microscope. The images above depict increased GDNF immunoreactivity in the region near the neuromuscular junction. Both the EDL (A) and the SOL (B) show a different pattern of expression of GDNF protein as compared to the goat anti-GDNF antibody. The GDNF immunopositive regions appear to follow the contours of the endplate region more closely and have a more definitive border than previously observed with the goat anti-GDNF antibody. Also different is the appearance of punctate labeling in regions away from the NMJ.
While the rabbit antibody appeared to be increased near the region of the NMJ, the GDNF labeling (Figure 9, red) appeared to follow the contours of the endplate region more definitively. This was in stark contrast to the GDNF labeling observed with the goat antibody (Figures 6, 7, and 8) where GDNF expression appeared hazy around the region of the NMJ. This difference in GDNF protein expression was also observed near the region of neurofilament positive staining (Figures 7 and 8 [blue] compared to Figure 9 [blue]).

We also noticed that the second (rabbit) GDNF antibody appeared to additionally label the samples in a more punctate manner away from the NMJ (Figures 8 and 9 [red]). It was not clear whether this was specific labeling of the GDNF molecule or non-specific binding of a different epitope. When primary antibody (rabbit) was omitted the punctate labeling was not present suggesting that the punctate signals were not effects of the secondary antibody.

Together these results demonstrate that GDNF protein is expressed at the region of the neuromuscular junction. We offer evidence that GDNF expression is similar in rat skeletal muscle as it appears in human skeletal muscle. This is not surprising considering that mature rat and human GDNF share a 93% overlap of amino acid sequence. We have also demonstrated that two different types of GDNF primary antibodies can label GDNF protein differently. This is also not surprising considering that GDNF protein has two isoforms of expression.

Expression of GDNF, like other neurotrophic factors, has various isoforms. It has been determined that GDNF mRNA has both an α- and a β- form and that both isoforms are expressed in skeletal muscle (Springer et al. 1995; Trupp et al. 1995).
When measured, the longer $\alpha$-isoform had approximately 633 base pairs and the shorter $\beta$-isoform had an identical sequence with a 78 base-pair deletion in the prepro region. The GDNF protein accordingly also was expressed as two isoforms, the full length protein being 211 amino acids and a mature protein isoform that is 26 amino acids shorter. It could be that the two antibodies used may have differed in their recognition epitope and since GDNF protein can be expressed in multiple isoforms, then it follows that expression patterns can vary depending upon the antibody used.

Another interesting finding from our results was the observation that not every endplate region was positive for increased GDNF protein expression. It appeared that possibly only a subset of skeletal muscle endplates might display increased GDNF protein expression. We subsequently decided to attempt to identify if there was any relationship between increased GDNF staining and skeletal muscle fiber type.

*Extratfusal Muscle Fibers*

As mentioned in chapter two, we were interested in identifying the skeletal muscle fiber-type composition of our tissues. Previous exercise and developmental studies suggested a possible relationship between skeletal muscle composition and changes in GDNF protein content. While the proportion of the specific skeletal muscle fiber-types for each of the tissues has already been previously reported (Ariano et al. 1973; Alnaqeeb and Goldspink 1987), we hoped to use skeletal muscle fiber-type immunohistochemistry to identify any possible relationship with GDNF protein expression in skeletal muscles. Our results of slow-myosin staining of the
EDL, FDS and SOL (*Figure 10*) appear to match closely the published reports of slow-twitch muscle fiber composition (2%, 8%, and 88% respectively).

One of the main objectives of skeletal muscle fiber-type staining was to determine if there was any correlation with our previous findings of increased GDNF protein expression at a subset of NMJs. We used antibodies directed against the slow myosin isoform and the type IIA (fast, intermediate) myosin isoform and looked at transverse sections of both the EHL and the EDL. We found that in the EHL the muscle fibers with GDNF positive endplate regions did not positively stain for the type IIA myosin antibody (*Figure 11*). However, these results do not conclusively rule out a possible association between this muscle fiber-type and increased GDNF immunoreactivity at the endplate since none of the endplates visualized were associated with the type IIA skeletal muscle fibers.

Our results suggest that increased GDNF protein expression at the NMJ is not solely limited to the fast-intermediate skeletal muscle fiber-type. It might be that increased GDNF protein expression at the NMJ is associated with the fast-intermediate skeletal muscle fiber-type in conjunction with other skeletal muscle fiber-types. Future studies should be directed at identifying endplate regions on these type IIA myosin immunopositive muscle fibers and determine the GDNF protein expression at these NMJs. We decided to investigate the possibility of increased GDNF protein expression at the NMJ of slow-twitch muscle fibers.
Figure 10. Slow myosin staining for three hind limb skeletal muscles. The extensor digitorum longus (EDL), flexor digitorum superficialis (FDS), and soleus (SOL) muscles were removed from four week old male Sprague-Dawley rats. Tissues were removed and cut into 50 μm sections. Samples were fixed using Zamboni’s fixative. Slides were washed and then treated with mouse anti-slow-myosin for 1 hour. Slides were washed and secondary antibody directed against the primary antibody was applied for 30 minutes for slow-twitch muscle fiber visualization (white). Images were captured using a Zeiss laser scanning confocal microscope. The image above depicts the relative composition of slow-twitch muscle fibers of each of the three tissues. These images visually support the published values of 2, 8, and 88 percent of slow-twitch muscle fibers for the EDL, FDS, and SOL respectively.
GDNF is expressed at neuromuscular junctional regions that are not associated with fast-intermediate myosin.

Figure 11. GDNF is expressed at neuromuscular junctional regions that are not associated with fast-intermediate myosin. The extensor hallucis longus (EHL) muscle was removed from four week old male Sprague-Dawley rats. The muscle was cut into 500 μm sections and fixed using Zamboni’s fixative. Samples were treated with a blocking solution containing donkey serum for 30 minutes. Slides were drained and treated with α-bungarotoxin-488 for 4 hours for endplate visualization (green). Samples were washed and goat anti-GDNF and mouse anti-typeIIA myosin primary antibodies and were applied for 7 days. Slides were washed and donkey anti-goat-568 and donkey anti-mouse-647 secondary antibodies were applied for 5 hours for GDNF visualization (red) and fast-intermediate muscle fibers (blue). Images were captured using a Zeiss laser scanning confocal microscope. The images above depict increased GDNF immunoreactivity (red) in the region near the neuromuscular junction (green). However, these GDNF positive endplate regions are not associated with fast-intermediate muscle fibers (blue). In A above, three GDNF positive endplate regions are visualized on muscle fibers that lack positive labeling of type IIA myosin antibody. In B above, again GDNF positive endplates are visualized that lack type IIA myosin labeling. However these results do not conclusively rule out a possible association between fast intermediate muscle fiber-type and increased GDNF immunoreactivity at the endplate since none of the endplates visualized were on the type IIA skeletal muscle fibers.
We found that GDNF protein expression was increased at the NMJs of slow-twitch skeletal muscle fibers (*Figure 12*). Nearby junctions that were not associated with slow-myosin antibody either did not appear to have increased GDNF protein expression associated with them, or the relative level of GDNF protein signal was greatly diminished. One caveat is that these findings are preliminary in nature and improved imaging as well as replication of this phenomenon is needed in order to be stated conclusively. Future studies should be directed at replicating and improving these preliminary findings.

While amidst our investigations into the increased GDNF signaling at the neuromuscular junction, recent work suggested that the motor neuron subset that responds most to functional down-regulation or up-regulation of GDNF expression may be the motor neurons that synapse on intrasusal muscle fibers (Whitehead et al. 2005). We therefore decided to see if we could label these skeletal muscle fiber types and determine if GDNF protein immunoreactivity is increased at their NMJs.
GDNF is expressed at the neuromuscular junctions associated with slow myosin.

Figure 12. GDNF is expressed at the neuromuscular junctions associated with slow myosin. The extensor digitorum longus (EDL) muscle was removed from four week old male Sprague-Dawley rats. The muscle was cut into 50 μm sections and fixed using Zamboni’s fixative. Samples were treated with a blocking solution containing donkey serum for 30 minutes. Slides were drained and treated with α-bungarotoxin-488 for 30 minutes for endplate visualization (green). Samples were washed and goat anti-GDNF and mouse anti-slow myosin primary antibodies were applied for 4 days. Slides were washed and donkey anti-goat-568 and donkey anti-mouse-647 secondary antibodies were applied for 1 hour for GDNF visualization (red) and slow myosin labeling (blue). Images were captured using a Zeiss laser scanning confocal microscope. The image above depicts increased GDNF immunoreactivity (red) in the region near the neuromuscular junction (green) of a slow-myosin immunopositive muscle fiber.
Intrafusal Muscle Fibers

Until this point, our investigations involved identification of increased GDNF protein expression in regards to extrafusal muscle fibers. Extrafusal muscle fibers are the skeletal muscle fibers within a muscle that are responsible for the generation of force associated with skeletal muscle contraction. These fibers make up the majority of the composition of any given skeletal muscle. There exists another muscle fiber population termed intrafusal muscle fibers. These muscle fibers are part of a specialized sensory apparatus and they do not contribute to the generation of skeletal muscle force during contraction.

The main role of the intrafusal muscle fibers is as a sensory organ to detect changes in skeletal muscle length to help regulate proprioception. These fibers are often involved in reflex pathways and are important for sensing overall changes in skeletal muscle position. They are also innervated by motor neurons. These motor neurons regulate the tension in these intrafusal muscle fibers and keep the sensory apparatus functional. As the extrafusal muscle fibers contract or relax the length of the skeletal muscle changes accordingly. In order for these intrafusal muscle fibers to be able to detect changes in tension, they also need to have the ability to contract and relax as the muscle length changes.

Early investigations into GDNF expression in these intrafusal muscle fiber-types demonstrated an increased immunoreactivity in the muscle spindle and the capsule that encloses the intrafusal muscle fibers in human skeletal muscle (Suzuki et
al. 1998). It was not further investigated until Whitehead et al. (2005) demonstrated that using gene modification of GDNF expression that the changes observed in mouse motor neuron populations with GDNF could be accounted for by changes in γ-motor neuron populations (those that synapse with intrafusal muscle fibers) and that α-motor neuron populations (those that synapse with extrafusal muscle fibers) were not significantly affected. We therefore decided to see if we could label these intrafusal skeletal muscle fibers in rat and determine if GDNF is expressed in these fibers.

We found that GDNF protein was expressed in the intrafusal fibers of rat EDL (Figure 13). When imaged using the rabbit anti-GDNF antibody, there was strong immunopositive GDNF signaling within the muscle membrane. As previously noted, this antibody appeared to label within the muscle fiber in a punctate manner. The nearby extrafusal fibers did not stain strongly for GDNF protein within the muscle fibers. However, a subset of extrafusal fibers did appear to contain GDNF within the muscle fibers. These findings are similar to those presented by Suzuki et al. (1998) in human skeletal muscle biopsies.
GDNF is expressed in intrafusal muscle fibers of rat skeletal muscle.

Figure 13. GDNF is expressed in intrafusal muscle fibers of rat skeletal muscle. The extensor digitorum longus (EDL) muscle was removed from four week old male Sprague-Dawley rats. The muscle was cut into 50 μm sections and fixed using Zamboni’s fixative. Samples were treated with a blocking solution containing donkey serum for 30 minutes. Slides were drained and treated with rabbit anti-GDNF and mouse anti-s46 primary antibodies for 5 days. Slides were washed and donkey anti-goat-488 and donkey anti-mouse-647 secondary antibodies were applied for 1 hour. Images were captured using a Zeiss laser scanning confocal microscope. For both A and B above intrafusal muscle fibers are labeled using the mouse anti-s46 antibody (blue) and the rabbit anti-GDNF antibody (green). In A GDNF protein is expressed in all of the s46-labeled intrafusal muscle fibers and in a subset of extrafusal muscle fibers. In B a series of transverse images were reconstructed and rotated to demonstrate GDNF labeling within the intrafusal muscle fibers in a longitudinal view.
Summary

We found that GDNF protein is expressed near the region of the neuromuscular junction in rat skeletal muscle. Expression at the neuromuscular junction did not appear to occur for all skeletal muscle fibers and we demonstrated that NMJs of slow-twitch muscle fibers contain increased GDNF protein labeling. We also demonstrated that GDNF protein is expressed in intrafusal muscle fibers of rat skeletal muscle. Together these results further our understanding of how GDNF protein is expressed in rat skeletal muscle.
CHAPTER IV

GDNF WITH AGING

Introduction

Many changes occur with advanced skeletal muscle aging. There is a significant loss of skeletal muscle cross-sectional area termed, “sarcopenia,” whereby the process of aging, skeletal muscle loses approximately 1/3 its normal adult weight (Rosenberg 1997). These changes are attributed to either a decrease in muscle fiber diameter, a decrease in muscle fiber number, or a combination of both. While some of the phenotypic changes in muscle fiber size can be attributed to decreased satellite cell activity (Roubenoff and Hughes 2000; Edstrom and Ulfhake 2005), others have observed a change in muscle fiber-type composition with age (Tauchi et al. 1971; Larsson et al. 1978).

One of the main changes occurring with age that contributes to the progression of sarcopenia is the loss of α-motor neurons. This leads to an overall decrease of number of motor units by 50% in people over 60 years of age (Brown 1972; Brown et al. 1988; Roubenoff and Hughes 2000). This loss in motor unit number is attributed to a selective denervation of fast twitch muscle fibers and collateral reinnervation by motor neurons from nearby slow twitch fibers. This age-related change in fiber type composition has been well studied (Tauchi et al. 1971; Larsson et al. 1978; Alnaqeeb and Goldspink 1987). One possible explanation for the
loss of motor neuron innervation with age could be due to altered neurotrophic factor signaling with age (Bergman et al. 1999).

Neurotrophic factors are extra cellular signaling proteins that are important for motor neuron survival. One of the most potent neurotrophic factors for peripheral motor neurons is glial cell line-derived neurotrophic factor (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). While the role of GDNF in skeletal muscle in early development has been extensively examined, little is known about the role of GDNF in skeletal muscles with age.

The previous studies focus primarily on changes in GDNF observed in early (pre to early post-natal) development. Studies that do involve adult organisms are generally intended to observe changes in GDNF in response to exercise, motor neuron damage, or motor neuron disease (Lie and Weis 1998; Chen et al. 2001; Wang et al. 2002; Wehrwein et al. 2002; Dupont-Versteegden et al. 2004). It is the purpose of this study to examine the GDNF protein content of skeletal muscles beyond the early post-natal time point, through adulthood, and into the later stages of skeletal muscle aging. A better understanding of how GDNF is expressed in skeletal muscle with age might provide insight as to its possible role in age-associated neurological changes that occur.
Materials and Methods

Animals

Male Sasco Sprague Dawley rats were housed with free access to food and water and maintained in accordance with the institutional animal care and use committee standards. Two separate analyses were performed; the first contained animals at 5, 7, 9, 14, and 19 months of age (n = 8,6,5,6,5 respectively). Within this group; the extensor digitorum longus (EDL) and flexor digitorum superficialis (FDS) muscles were removed for all time points and the vastus intermedius (VI) muscles were removed from the 7, 9, and 19 month groups. For the second study; EDL, FDS, and soleus (SOL) muscles were removed from animals at 1, 10, and 23 months of age (n = 5,8,5 respectively).

Tissue Processing

Tissues 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 (0.4 M NaCl, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, 0.05% Tween-20 in PBS) was added and the mixture was homogenized on ice. Samples were then centrifuged for 30 min at 4°C and supernatant collected and stored at -80°C. GDNF protein content of the supernatant was determined using enzyme-linked immunosorbant assay (ELISA).
For the first study, samples were prepared at a 1:4 dilution of tissue weight (in mg) to sample buffer volume (in mL). In the second study, samples were prepared at a 1:14 tissue weight to buffer volume dilution. All of the ELISA sample concentration values were multiplied by their respective dilution factor to determine total GDNF and then normalized to tissue weight and represented as pg of GDNF/mg tissue weight.

**Enzyme-linked Immunosorbant Assay**

Enzyme-linked immunosorbant assays were performed using capture antibody (R&D Systems, MAB212) and detection antibody (R&D Systems, BAF212) to manufacturer’s specifications (R&D Systems). The tetramethylbenzidine color reagent (Sigma, T-3405) was prepared according to manufacturer specifications. The reaction was stopped with 0.1 M phosphoric acid and absorbance measured at 450 nm.

**Data Analysis and Statistics**

Data were analyzed using the Kruskal-Wallis analyses of ranks procedure followed by the non-parametric multiple comparisons analysis published by (Simes 1986). Significance was established at $p \leq 0.05$ for all comparisons and all values reported as GDNF pg/mg tissue weight ± SEM.
Results

For the first study, GDNF values were analyzed from rats of 5, 7, 9, 14, and 19 months of age (n = 8,6,5,5,5, respectively). The oldest animals had significantly more GDNF protein content than the mid-age time points while the youngest group seemed to be only slightly elevated but not statistically significant. For the FDS, the 19 month old group (0.628 ± 0.15 pg/mg) had significantly more GDNF protein content than the 5, 7, 9, and 14 month groups (0.164 ± 0.05, 0.024 ± 0.01, 0.058 ± 0.03, and 0.073 ± 0.07 pg/mg tissue respectively) (Figure 14). For the EDL the 19 month time point (0.560 ± 0.10 pg/mg tissue) also had significantly more GDNF than the 5, 7, 9, and 14 month old groups (0.119 ± 0.06, 0.042 ± 0.02, 0.021 ± 0.01, and 0.123 ± 0.08 pg/mg tissue respectively) (Figure 15). The VI also followed this trend and the 19 month time point (3.01 ± 0.72 pg/mg tissue) had significantly more GDNF protein than both the 7 month (0.450 ± 0.23 pg/mg tissue) and the 9 month (0.447 ± 0.23 pg/mg tissue) old groups (Figure 16).
GDNF protein content of the FDS is highest at 19 months of age.

Figure 14. GDNF protein content of the FDS is highest at 19 months of age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat flexor digitorum superficialis (FDS) at various time points with age. Male Sprague-Dawley rats were euthanized at various ages and GDNF protein content of the FDS was measured using standard ELISA procedures. GDNF was highest at the latest time point of 19 months of age and significantly elevated when compared to all other ages sampled. For the 5, 7, 9, 14, and 19 month groups n = 8,6,5,5,5, respectively. (* significantly different from all other groups, p ≤ 0.05)
GDNF protein content of the EDL is highest at 19 months of age.

Figure 15. GDNF protein content of the EDL is highest at 19 months of age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat extensor digitorum longus (EDL) at various time points with age. Male Sprague-Dawley rats were euthanized at various ages and GDNF protein content of the EDL was measured using standard ELISA procedures. GDNF was highest at the latest time point of 19 months of age and significantly elevated when compared to all other ages sampled. For the 5, 7, 9, 14, and 19 month groups n = 8,6,5,5,5, respectively. (* significantly different from all other groups, p ≤ 0.05)
GDNF protein content of the VI is highest at 19 months of age.

Figure 16. GDNF protein content of the VI is highest at 19 months of age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat vastus intermedius (VI) at various time points with age. Male Sprague-Dawley rats were euthanized at various ages and GDNF protein content of the VI was measured using standard ELISA procedures. GDNF was highest at the latest time point of 19 months of age and significantly elevated when compared to both the 7 and 9 month time points. For the 7, 9, and 19 month groups n = 6,5,5, respectively. (* significantly different from all other groups, p ≤ 0.05)
In the second study, GDNF values were analyzed from animals at 1 month, 10 month, and 23 months of age (n = 5 for all groups). There were no significant differences in GDNF protein content in the EDL at any time point (Figure 17). In the FDS, GDNF protein content significantly decreased between 1 month (47.9 ± 6.5 pg/mg tissue) and 10 months (20.8 ± 4.5 pg/mg tissue) of age and significantly increased between 10 months and 23 months (35.2 ± 1.7 pg/mg tissue) of age (Figure 18). There was no significant difference in the GDNF protein content of the FDS between the 1 month and the 23 month time points. For the SOL, GDNF protein levels were significantly elevated at 1 month (96.5 ± 11.6 pg/mg tissue) compared to 10 months (41.9 ± 6.9 pg/mg tissue) of age and compared to the 23 month (65.0 ± 6.6 pg/mg tissue) old group. The 23 month old group GDNF protein content was also significantly elevated compared to the 10 month old time point (Figure 19).
Figure 17. GDNF protein content of the EDL remains constant with age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat extensor digitorum longus (EDL) at various time points with age. Male Sprague-Dawley rats were euthanized at various ages and GDNF protein content of the EDL was measured using standard ELISA procedures. GDNF protein content did not significantly differ at any of the time points sampled. For the 1, 10, and 23 month groups n = 5, 8, and 5 respectively.
Figure 18. GDNF protein content of the FDS fluctuates with age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat flexor digitorum superficialis (FDS) at various time points with age. Male Sprague-Dawley rats were euthanized at various ages and GDNF protein content of the FDS was measured using standard ELISA procedures. GDNF protein content significantly decreased from 1 month of age (47.9 ± 6.5 pg/mg tissue, n = 5) to 10 months (20.8 ± 4.5 pg/mg tissue, n = 8 [* p ≤ 0.05]). GDNF protein content significantly increased between 10 months of age and 23 months of age (35.2 ± 1.7 pg/mg, n = 5 [# = significantly different than previous group, p ≤ 0.05]).
GDNF protein content of the SOL fluctuates with age.

Figure 19. GDNF protein content of the SOL fluctuates with age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat soleus (SOL) at various time points with age. Male Sprague-Dawley rats were euthanized at various ages and GDNF protein content of the SOL was measured using standard ELISA procedures. GDNF protein content significantly decreased between 1 month (96.5 ± 11.6 pg/mg tissue) and 10 months of age (41.9 ± 6.9 pg/mg tissue). GDNF significantly increased between 10 months and 23 months of age (65.0 ± 6.6 pg/mg tissue). At 23 months of age, the SOL had significantly more GDNF than at 10 months of age, but significantly less than at 1 month of age. For the 1, 10, and 23 month groups n = 5, 8, and 5 respectively. (* = significantly different from all other groups, p ≤ 0.05)
Comparisons were also made at each time point between the different muscles. At 1 month of age (n = 5), GDNF protein content was significantly higher in the SOL (96.5 ± 11.6 pg/mg tissue) than the EDL (37.2 ± 1.7 pg/mg tissue) and the FDS (47.9 ± 6.5 pg/mg tissue) (Figure 20). At 10 months of age (n = 8), GDNF protein content in the FDS (20.8 ± 4.5 pg/mg tissue) is significantly less than in the SOL (41.9 ± 6.9 pg/mg tissue) and EDL (37.2 ± 4.7 pg/mg tissue) (Figure 21). At the 23 months of age (n = 5), GDNF protein content was again significantly higher in the SOL (65.0 ± 6.6 pg/mg tissue) than the EDL (29.3 ± 5.2 pg/mg tissue) and the FDS (35.2 ± 1.7 pg/mg tissue) (Figure 22).
The SOL contains significantly more GDNF protein than the EDL and FDS at 1 month of age.

Figure 20. The SOL contains significantly more GDNF protein than the EDL and FDS at 1 month of age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat hind limb skeletal muscle at one month of age. Male Sprague-Dawley rats (n = 5) were euthanized at one month of age and GDNF protein content of 3 different hind limb skeletal muscles were measured using standard ELISA procedures. GDNF was significantly increased in the soleus (SOL [96.5 ± 11.6 pg/mg tissue]) when compared to the extensor digitorum longus (EDL [37.2 ± 4.7 pg/mg tissue]) and the flexor digitorum superficialis (FDS [47.9 ± 6.5 pg/mg tissue]) at one month of age. (* = significantly different from all other groups, p ≤ 0.05)
The FDS contains significantly less GDNF protein than the SOL and EDL at 10 months of age.

Figure 21. The FDS contains significantly less GDNF protein than the SOL and EDL at 10 months of age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat hind limb skeletal muscle at 10 months of age. Male Sprague-Dawley rats (n = 8) were euthanized at 10 months of age and GDNF protein content of 3 different hind limb skeletal muscles were measured using standard ELISA procedures. GDNF was significantly decreased in the flexor digitorum superficialis (FDS [20.8 ± 4.5 pg/mg tissue]) when compared to the extensor digitorum longus (EDL [40.0 ± 2.9 pg/mg tissue]) and to the soleus (SOL [41.9 ± 6.9 pg/mg tissue]). (* = significantly different from all other groups, p ≤ 0.05)
The SOL contains significantly more GDNF protein than the EDL and FDS at 23 months of age.

- **Figure 22.** The SOL contains significantly more GDNF protein than the EDL and FDS at 23 months of age. Glial cell line-derived neurotrophic factor (GDNF) protein values of rat hind limb skeletal muscle at 23 months of age. Male Sprague-Dawley rats (n = 5) were euthanized at 23 months of age and GDNF protein content of 3 different hind limb skeletal muscles were measured using standard ELISA procedures. GDNF was significantly increased in the soleus (SOL [65.0 ± 6.6 pg/mg tissue]) when compared to the extensor digitorum longus (EDL [29.3 ± 5.2 pg/mg tissue]) and the flexor digitorum superficialis (FDS [35.2 ± 1.7 pg/mg tissue]) at 23 months of age. (* = significantly different from all other groups, p ≤ 0.05)
Discussion

Between Study Comparisons

When comparing the results of these two studies there is a discrepancy in the reported GDNF protein content values. The range of mean values of the first study span from 0.021 ± 0.01 to 3.01 ± 0.72 pg/mg tissue while the range of the second study is between 20.8 ± 4.5 and 96.5 ± 11.6 pg/mg tissue. When comparing relative time point values for the EDL between the two studies this becomes even more apparent. The first study found GDNF protein content in the EDL of the 9 month old group to be 0.021 ± 0.01 pg/mg tissue. This is significantly less than the GDNF value of the EDL in the 10 month old group of the second study (37.2 ± 4.7 pg/mg tissue). The reason for this discrepancy might be a result of the differences in the way the samples were processed.

The samples in the first study were processed at a 1:4 ratio of tissue weight (in mg) : volume of sample buffer (in mL). The samples of the second study were processed at a 1:14 dilution factor. These results demonstrate a significantly different expression of GDNF protein content from similarly age-matched EDL. This might have been a result of a differential extraction of GDNF from the tissue during sample processing. The data suggests that there may be an optimal dilution factor for extraction of GDNF from skeletal muscle, although future investigation is warranted to directly determine this effect. It is because of the suggested discrepancy in extraction ability, further comparisons were not made among absolute values of
GDNF protein content between the two studies. Instead, significant changes in GDNF protein content within each study were determined and results were compared with respect to the process of aging.

*Early to Midlife Comparisons*

The general trend of GDNF protein expression early in life appeared to be the same between the two studies. Early life (1 and 5 month) time points were either significantly elevated or only slightly elevated from the midlife (7, 9, 10, and 14 month) time points. This could possibly be a continuation of elevated expression from increased GDNF levels in early development. Nagano and Suzuki (2003) determined that GDNF protein content for the SOL of Wistar rats significantly increased from postnatal day 6 to postnatal day 15, was highest at 1 month of age, and significantly decreased by 3 months of age. Our results demonstrate this similar developmental trend. GDNF protein content of the SOL peaked at 1 month of age and was significantly higher than at 10 months of age (*Figure 19*). Taken together these results suggest that GDNF protein content in the SOL increases in early development, peaks at 1 month of age, and subsequently decreases into adulthood.

These early trends in GDNF protein expression of the SOL follow developmentally important time points. During the first two postnatal weeks, the neuromuscular junction (NMJ) is undergoing both pre- and post-synaptic changes in order to stabilize the neuromuscular communication. It has been demonstrated that after the first two postnatal weeks the gross structure of the NMJ remains stable and that only the overall size of the junction changes with proportion to the growth of the
muscle fibers (Balice-Gordon and Lichtman 1990). These normal changes that occur with growth and development involve a three-fold increase in size. Growth occurs until approximately 18-weeks of age and the NMJ remains relatively stable unless influenced by exercise, disuse, microgravity, or aging (for review see (Wilson and Deschenes 2005). It is interesting then, that both our results and the results of Nagano and Suzuki (2003) demonstrate that GDNF protein content of the SOL is highest shortly after the NMJ is stabilized and declines during the time of the three-fold increase in NMJ size between one and three months of age.

As for the FDS and EDL, there can be no direct comparisons made from Nagano and Suzuki (2003) since they only examined changes in GDNF protein content for the SOL and gastrocnemius (GAST). They did, however, demonstrate a difference in GDNF protein expression between the SOL and GAST in early postnatal development. They determined that while GDNF in the SOL was low initially (increased from postnatal day 6 to 1 month and then decreased from 1 to 3 months), in the GAST the pattern was almost the opposite. GDNF was elevated initially, decreased from postnatal day 6 to 15, remained unchanged through 1 month and then increased between 1 and 3 months of age. This observed difference in expression between different skeletal muscle tissue types was also present in our studies.

In the EDL, there was no significant difference when comparing any of the early life time points to any of the midlife for either of our two studies (Figures 15 and 17). This is different than the changes observed with the SOL and different still from the observations made by Nagano and Suzuki (2003) in the GAST. These
results support the suggestions that various hind limb skeletal muscles display
differential expression patterns of GDNF protein content in early life and midlife.

The results from the FDS were similar in its pattern of expression of early and
midlife GDNF protein content to that of the SOL but with a significantly smaller
magnitude. In the first study GDNF protein content was unchanged from months 5 to
14 months of age (Figure 14). The second study demonstrated a significant decrease
from 1 month to 10 months of age (Figure 18). This early to midlife decrease in
GDNF protein content appears to occur only in the SOL and FDS while the GDNF
protein content of the EDL does not significantly change throughout any of these
early time points.

One possible explanation for these inherent differences in GDNF protein
content could be based upon skeletal muscle fiber type. When comparing the relative
composition of these three tissues the SOL contains the highest percentage of slow
muscle fibers (88%), the EDL contains the least (2%), and the FDS composition is
slightly more slow than the EDL (8%) (Ariano et al. 1973; Alnaqeeb and Goldspink
1987). The SOL and FDS both had similar changes in GDNF protein content with
age, but at every time point the concentration in the SOL was significantly higher
than the FDS (Figures 20, 21, and 22). This discrepancy might also be explained by
the inherent differences in their fiber type composition.

Tissues with elevated slow muscle fiber-type composition appear to change in
GDNF protein content while tissues that have relatively low numbers of slow muscle
fibers did not show any changes in early to midlife development. However, the
results with GDNF protein content are only correlative to skeletal muscle fiber type
and may possibly be explained by other differences between these skeletal muscles. Future studies aimed to directly examine the relationship between skeletal muscle fiber type and GDNF protein expression are warranted.

It is also important to note that while the results from the vastus intermedius (VI) muscle are reported (*Figure 16*), there were technical difficulties in successful removal of this tissue. The VI was chosen as it was a good candidate to represent a muscle with an evenly mixed muscle fiber type composition; however dissection of this tissue was inconsistent throughout. Due to its direct insertion into the medial femur and unclear borders with neighboring quadriceps muscles, tissues were often damaged during removal and/or included pieces of the neighboring vastus lateralis and vastus medialis. It is because of these technical issues that the results from the VI are presented here but are not discussed in great detail.

*Late Life Comparisons*

Another aging trend in GDNF protein expression appears late in life. We analyzed changes in GDNF protein content in the late life stages (19 and 23 months) in both of our studies. One might suggest that a possible cause for the denervation of muscle fibers and subsequent fiber type change that occurs with sarcopenia could be due to a decrease in the neurotrophic protein supplied to the nerves by the target tissues. Here we demonstrate that throughout the late life stages GDNF protein content either significantly increased or had no change from the previous midlife values. None of the skeletal muscles examined displayed decreased GDNF protein
content with advanced aging. This might be explained by comparing the neurological changes that occur in skeletal muscle during this time frame.

Skeletal muscle changes in senescence include denervation of fast muscle fibers followed by partial re-innervation of fast muscle fibers by neighboring slow motor neurons. However, the re-innervation process does not occur for all of the denervated fast muscle fibers and slowly these muscle fibers are lost and overall skeletal muscle size and motor unit numbers are decreased. Others have demonstrated that denervation is a cue for increased GDNF expression (Lie and Weis 1998; Chen et al. 2001). It could be that the significant increases that we observe with our latest time point are a result of increased denervation with age. It is important to note that while GDNF protein levels do not decrease with age GDNF signaling might still be affected.

Bergman et. al. (1999) determined that the GDNF receptor alpha-1 (GFRα1) and co-receptor c-ret mRNA significantly increased in motor neurons from Sprague-Dawley rats at 30 months of age compared to 2-3 months of age. They also noted that of the motor neurons that increased GFRα1 mRNA expression, a subpopulation of motor neurons (25%) appeared to display very high levels of GFRα1 mRNA. These aged animals also exhibited a significant increase in protein expression observed via immunohistochemistry. Taken together these results suggest that even though GDNF protein content increases or remains constant with age, GDNF signaling might be affected by a change in GDNF receptor. Therefore, even without significant changes in GDNF protein content GDNF may still be a factor involved in the denervation observed with sarcopenia. Additionally, our observations are merely
correlative with the aging process and did not attempt to identify neurological signs of aging. Future studies should include identification and quantification of the denervation process with aging and the response of GDNF protein expression in respect to aging.

There was an interesting conflict of results between our two studies in the EDL with age. The first study demonstrated a significant increase in GDNF protein content at 19 months of age (Figure 15) while the second study showed no significant change from 10 months when compared to 23 months of age (Figure 17). It could be that for this tissue GDNF undergoes a series of significant changes between 10 and 23 months of age, including a significant increase at 19 months and an eventual decrease at 23 months. This would be quite different than the results observed with SOL and FDS that were both significantly higher at 19 months and remained significantly elevated at 23 months. The EDL is composed primarily of fast-twitch muscle fibers and age related changes in skeletal muscle composition have been shown to preferentially affect fast-twitch muscle fibers. Further information is needed to clarify if the differences observed in the EDL between the first and second study are in fact real differences or if they are artifact due to the different extraction methods used between the two studies.
Summary

We have examined the effects of aging on GDNF protein content of three different hind limb skeletal muscles. We have determined that the SOL and EDL contain significantly different basal levels of GDNF protein content. These inherent tissue differences may be related to differences in skeletal muscle fiber-type composition. We have also determined that GDNF protein expression changes with age and that these changes do not appear to be uniform for all muscle types. These changes overlap and might well be associated with neurological events that occur with early development and aging. These findings further characterize how GDNF protein expression is affected with aging and may provide insight as to GDNF’s possible role in neurological changes that occur in skeletal muscle with age.
CHAPTER V

GDNF WITH VOLUNTARY RUNNING

Introduction

Glial cell-line derived neurotrophic factor (GDNF) was first discovered in the brain and found to promote survival and differentiation in dopaminergic neurons (Lin et al. 1993). Further investigation determined that GDNF is critical for proper motor neuron development (Henderson et al. 1994). Over expression of GDNF in skeletal muscle in transgenic mice resulted in hyper-innervation of the neuromuscular junction (Zwick et al. 2001). Exercise after nerve injury has been shown to increase GDNF mRNA and speed the time of recovery after injury in the rat soleus (SOL) muscle (Dupont-Versteegden et al. 2004).

A recent study in our laboratory demonstrated that GDNF protein expression is regulated in an activity-dependent manner in normal, adult rat skeletal muscle (Wehrwein et al. 2002). We determined that rats exercised by walk training had an increase in GDNF protein in the SOL and the gastrocnemius (GAST) hind limb skeletal muscles. However, the use of negative reinforcement as a means for motivation in this study might have increased stress and possibly affected GDNF protein levels in these tissues.

We also noticed that while both the SOL and the GAST had significantly increased in response to forced treadmill running, the SOL had a greater increase in GDNF protein content in response to exercise than did the GAST. Additionally, the
SOL from sedentary animals contained more GDNF protein content than the GAST from sedentary animal. This suggests that there might be inherent differences in skeletal muscles that could affect GDNF protein content and that not all muscles may respond the same to exercise.

It is the purpose of this study to evaluate the effects of voluntary running on GDNF protein content in skeletal muscle without the use of negative reinforcement. We also intend to determine if changes in GDNF protein content are uniform for all skeletal muscles or if individual muscles respond in individual manners. We hypothesize that GDNF protein content will be altered by voluntary running and that the response to physical activity will vary depending upon the skeletal muscle sampled.
Materials and Methods

Male Fisher 344 rats (8 weeks old) were housed with free access to food and water and maintained in accordance with the institutional animal care and use committee standards. Four animals were housed in standard cages without access to a running wheel and composed the sedentary control group. The remaining 8 animals were allowed access to voluntary running wheels for 3 weeks. At the end of 3 weeks, the 8 animals were not granted access to running wheels for 1 week. A subset of 4 animals was then re-allowed access to the voluntary running wheels for an additional 48 hours.

All animals were euthanized via CO$_2$ asphyxiation followed by thoracotomy. The extensor digitorum longus (EDL) and flexor digitorum superficialis (FDS) muscles were removed and frozen on dry ice. Tissues were processed following standard procedures in preparation for determination of GDNF protein content using an enzyme-linked immunosorbant assay (R&D Systems, Minneapolis, MN). GDNF protein values were quantified and expressed as pg/mg of tissue weight.

Data were analyzed using the one-way analysis of variance procedure followed by the Student-Neuman-Keuls’ post hoc procedure. Significance was established at $p \leq 0.05$ for all comparisons and all values reported as GDNF pg/mg tissue weight ± SEM.
Results

After 3 weeks of voluntary running and one week of rest, GDNF protein levels in rat EDL (Figure 23) were significantly lower (18.1 ± 0.9 pg GDNF/mg tissue, n = 4) than those of sedentary control animals (24.3 ± 1.0 pg GDNF/mg tissue, n = 4). This significant decrease in GDNF protein expression is contrary to the significant increase observed previously with forced treadmill running (Wehrwein et al. 2002).

The second bout of 48 hours of voluntary running did not have any significant effect on GDNF protein levels for the EDL (18.4 ± 1.7 pg GDNF/mg tissue, n = 4) as compared to the exercised group (Figure 23). These animals did run voluntarily during this time frame and at the same daily rate as before the stoppage.

In the FDS there was no significant difference in GDNF protein content between the 3 week exercised and one week rest group (13.4 ± 0.4 pg GDNF/mg tissue, n = 4) than that of the sedentary control group (15.2 ± 1.3 pg GDNF/mg tissue, n = 4 [Figure 24]).

Similar to the EDL, the second bout of 48 hours of voluntary running did not have any significant effect on GDNF protein levels for the FDS (15.2 ± 0.6 GDNF/mg tissue, n = 4) as compared to either of the other two groups (Figure 24). These animals did run voluntarily during this time frame and at the same daily rate as before the stoppage.
Figure 23. Effects of voluntary running on GDNF protein content in rat EDL. Three weeks of voluntary running decreases GDNF protein expression in rat extensor digitorum longus (EDL) muscles. Fisher 344 rats were housed with free access to running wheels. After three weeks of running and one week of rest GDNF protein content in EDL of the exercised group (EX, n=4) was significantly less (*p ≤ 0.05) than that of age-matched sedentary controls (SED, n=4). A subset of the exercised group was then granted access again to the running wheels for 48 hours (EX+48, n=4) to determine if there were any effects of a secondary acute bout of running. The acute bout of running had no significant effect on GDNF protein levels of the EDL.
Figure 24. Effects of voluntary running on GDNF protein content in rat FDS. Three weeks of voluntary running had no significant effect on GDNF protein content in rat flexor digitorum superficialis (FDS) muscles. Fisher 344 rats were housed with free access to running wheels. After three weeks of running and one week of rest, there was no significant effect on GDNF protein content in FDS of the exercised group (EX, n=4) compared to age-matched sedentary controls (SED, n=4). A subset of the exercised group was granted access again to the running wheels for 48 hours (EX+48, n=4) to determine if there were any effect of a secondary acute bout of running. The acute bout of voluntary running had no significant effect on GDNF protein levels of the FDS.
Discussion

We have demonstrated that voluntary running is able to alter GDNF protein content in hind limb skeletal muscle. Our observations demonstrate the ability of voluntary running to enact an activity dependent change in GDNF protein content in rat skeletal muscle. Our results oppose, in direction, the previous results attained with forced treadmill running. This discrepancy in the direction of change could be due to differences in exercise protocols or the differences between muscles that were analyzed.

While the EDL had a statistically significant decrease in GDNF protein, the FDS showed no change with exercise. One explanation for these differences could be based on the different composition of these two muscles. Others have demonstrated that GDNF mRNA and protein content differs during development in various hind limb skeletal muscles (Nagano and Suzuki 2003). They suggested that observed differences may be due to inherent differences in skeletal muscle composition including skeletal muscle fiber type. These inherent differences could be a possible explanation as to why the FDS exhibited a greater response to voluntary exercise as FDS has a greater percentage of slow muscle fiber type as compared to EDL (unpublished observations). This might also account for the differences in direction of change between this study and our previous findings.

When comparing the combined results of this study and our previous study, a correlation may be drawn to fiber type composition. It has been established that the EDL and the SOL are skeletal muscle opposites in many regards. They differ vastly
in fiber-type composition, physiological function, neuronal activity patterns, and metabolic activity, and it is because of these differences that they are chosen as some of the most classically studied skeletal muscles (Alnaqeeb and Goldspink 1987; Reid et al. 2003; Patterson et al. 2006). The SOL is composed primarily of slow-twitch muscle fibers which make up 88% of the total fiber-type composition (Alnaqeeb and Goldspink 1987). The EDL conversely, is composed primarily of fast-twitch muscle fibers and only 2% of the muscle fibers are slow-twitch fibers (Alnaqeeb and Goldspink 1987). The FDS is composed primarily of fast-twitch muscle fibers and 8% of the muscle fibers are slow-twitch fibers (Ariano et al. 1973). In the EDL, GDNF protein content significantly decreased with exercise while in the SOL, GDNF protein content significantly increased (Wehrwein et al. 2002). In the FDS exercise did not elicit a significant change in GDNF protein content. This might suggest a correlation with muscle fiber type such that the primarily fast-twitch EDL had a significant decrease and the primarily slow-twitch SOL had a significant increase in GDNF protein content in response to exercise. However, these results are only observations made through correlation and may not be the only explanation for the differential responses between these three skeletal muscles. Further investigation is warranted to specifically address this possibility.

We were also interested in further characterizing the effect of exercise on GDNF protein content. It could be possible that the change in GDNF protein content observed previously with treadmill running was a transient effect and one that may have attenuated shortly after inactivity. It is because of this that we chose to exercise the animals for 3 weeks followed by 1 week of rest, as opposed to measuring GDNF
immediately after the end of the running protocol. We were able to determine that GDNF protein levels in the EDL still remained significantly different than in sedentary controls even after one week of inactivity. This suggests that the change in GDNF protein expression is not a transient effect but rather a lasting effect that was still observed a week after cessation of exercise.

We also aimed to further characterize the effects of exercise on the regulation of GDNF protein in skeletal muscle. After the one week of rest a subset of the voluntary exercise group was granted access to running wheels for an additional 48 hours to determine the acute effect of exercise on GDNF protein levels. This second bout of acute activity demonstrated that GDNF protein levels could not be significantly altered within this short time frame. This provides further support that GDNF protein expression is not regulated in a transient manner under these conditions.

One possible argument could be that the ability of skeletal muscle to alter GDNF protein content in response to exercise might have already reached a physiological plateau. It may be that any exercise beyond this point may not be physiologically capable of further altering GDNF protein content. Future studies should be intended to address not only this aspect, but to additionally characterize this effect in a time-course study with the hopes to identify when GDNF is first significantly altered by exercise, the physiological range of this response, and the longevity of the effect of exercise on GDNF protein content in rat skeletal muscle.
Summary

Together these results further our understanding of the physiology that regulates GDNF expression in skeletal muscle in vivo. We have determined that voluntary running can alter GDNF protein content and that these changes are not transient in nature. We have also determined that voluntary running affects GDNF protein content of the FDS different from EDL and suggest that individual muscles may respond differently to increased physical activity.
CHAPTER VI

BATH STUDIES

Introduction

GDNF was discovered in 1993 during a search for a neurotrophic factor for dopaminergic neurons in the central nervous system (CNS) (Lin et al. 1993). Since its discovery in CNS glial cells, GDNF mRNA has been identified in a variety of tissues throughout the body (Suter-Crazzolara and Unsicker 1994). The protein is important for dopaminergic neurons of the CNS, as well as many other neuronal populations (Buj-Bello et al. 1995). One neuronal population in particular that is affected by GDNF are motor neurons (MNs) of the peripheral nervous system that innervate skeletal muscle (Henderson et al. 1994; Oppenheim et al. 1995).

GDNF has been shown to be one of the most potent neurotrophic factors for MNs. Treatment with GDNF has rescued MNs from axotomy-induced cell death (Oppenheim et al. 1995), slowed the loss of MNs in mice exhibiting progressive motor neuropathy (Sagot et al. 1996), and protected MNs in a model of chronic motor neuron 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).

Neurotransmitters have been shown to affect neurotrophic factor expression. In vascular and bladder smooth muscle cells in culture, neurotransmitters from
sympathetic neurons altered production of nerve growth factor by smooth muscle cells (Spitsbergen et al. 1995; Clemow et al. 1999). In a previous study, Oppenheim et al. (2000) demonstrated that blockade of nicotinic acetylcholine receptors on skeletal muscle enhanced the survival of developing MNs in vivo. Others have demonstrated that denervated skeletal muscle contains elevated levels of GDNF mRNA (Lie and Weis 1998) suggesting that absence of neuronal influence leads to an elevated GDNF mRNA response in skeletal muscle.

One possible explanation is that the release of the neurotransmitter acetylcholine (ACh) from the intact nerve terminal and subsequent nicotinic acetylcholine receptor (nAChR) activation decreases GDNF protein expression in skeletal muscle. If this were the case, then extrinsic cues that lead to release of ACh and subsequent nAChR activation may decrease GDNF expression in skeletal muscle. It is the purpose of this study to determine how skeletal muscle activity may affect the short-term response of GDNF expression in skeletal muscle and establish the possible role of the nAChR in this response.
Materials and Methods

Animals

Male SASCO Sprague-Dawley (Charles River, Kalamazoo, MI) were given access to food and water ad libitum and maintained on a 12h light/dark cycle. Rats (4wk, 100g ± 22g) were euthanized via CO₂ asphyxiartion followed by thoracotomy.

Bath Studies

Bilateral soleus (SOL), flexor digitorum superficialis (FDS), and extensor digitorum longus (EDL) were removed and placed into a tissue bath containing Krebs-Ringer bicarbonate solution (in mM): 120.2 NaCl, 25.1 NaHCO₃, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.3 CaCl₂, and 5 D-glucose (Gissel and Clausen 1999). Experiments were performed at room temperature to increase the physiological stability of the tissues for the duration of the experiment (Segal and Faulkner 1985; Gissel and Clausen 1999). The bath was continuously bubbled with O₂/CO₂ (95%/5%) and maintained a working pH of 7.4. Suture (4-0 silk) was tied to tendons at each end of the tissues, mounted between a fixed glass hook and a force transducer, and placed in the bath. The FDS however had no proximal tendon and attached directly to the distal end of the femur. Due to this anatomical design, the distal end of the femur was cut and the attachment was left intact. Suture was then tied to the distal tendon and the distal head of the femur to suspend the FDS. All bath experiments were performed with contralateral tissues as controls.
**Blockade of acetylcholine receptors**

Acetylcholine (ACh) (A-2661, Sigma) was delivered to the bath at a concentration of 88.1μM. This concentration was determined to be more than adequate to obtain maximal force production for the tissues. Baths were rinsed with fresh Ringers solution between applications and incubated for 30 minutes with α-bungarotoxin (00010, Biotium) at a concentration of 0.25 mM for EDL and 0.38mM for SOL. Tissues were washed with fresh ringers for 30 minutes to remove any unbound toxin. ACh was then applied at double the original concentration and elicited no response (Harborne et al. 1978).

**Stimulation Protocol**

Muscles were removed and attached to a fixed glass hook using 4-0 silk suture in tissue baths. The bodies of the muscles were suspended between two zigzag electrodes (160152-12, ADI Instruments) and the proximal tendons were tied to a force transducer (FT03D, Grass) to determine contractile force. Tissues were field stimulated (S88, Grass) to determine optimum length. Contractile force was measured and recorded on a pen recorder (7D Polygraph, Grass). After optimum length of the tissues were determined, tissues were electrically stimulated at 0.1Hz (1.0-ms pulses) for 4 hours with supramaximal voltage (Gissel and Clausen 1999).

**Stretch Protocol**

For these experiments, resting length was measured *in situ* and tissues were placed in baths with the distal ends attached to a glass hook, while the proximal ends
were attached to a fabricated cam. The motor (Globe Motors, 409A582) was fixed above the tissue bath and the cam was offset by 3 mm from the center of the motor’s shaft. This design allowed for tissues to be stretched ±3 mm from resting length per revolution which resulted in a ±14% and 15% stretch for the SOL and EDL respectively. This is within the physiological range of stretch (10-15%) as determined for skeletal muscle (Chen and Grinnell 1997). Tissues were stretched at a rate of 2 revolutions in 0.5 second at 0.1 Hz for an overall duration of 4 hours. This protocol mimicked the contractile activity of the stimulation protocol used above.

Carbamylcholine Protocol

Muscles were removed and optimum length was determined as previously described (see stimulation protocol). After optimum length was established, carbachol was added to the bath so that the final concentration was 10μM. Tissues were exposed for four hours and contralateral control tissues were left in normal Ringer’s solution for the duration of the experiment.

Tissue Processing

At the conclusion of the bath experiments, tissues 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 (0.4 M NaCl, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, 0.05% Tween-20 in PBS) was added and the mixture was homogenized on ice. Samples were centrifuged at 13,000 x g for 30 min at 4°C and
supernatant collected and stored at -80\(^\circ\)C. GDNF protein content of the supernatant was determined using enzyme-linked immunosorbant assay (ELISA).

*Enzyme-linked immunosorbant assay.* Enzyme-linked immunosorbant assays were performed using capture antibody (R&D Systems, MAB212) and detection antibody (R&D Systems, BAF212) to manufacturer’s specifications (R&D Systems). The tetramethylbenzidine color reagent (Sigma, T-3405) was prepared according to manufacturer specifications. The reaction was stopped with 0.1 M phosphoric acid and absorbance measured at 450 nm.

**Data Analysis and Statistics**

For all bath studies, direct comparisons were made between the experimental group mean and the unmanipulated, contralateral control group mean. GDNF ELISA protein values of the experimental group were normalized to the control group values and reported and a percent change from control ± the normalized standard error of the mean. In order to determine significance a t-test was performed, except when variance was not homogeneous; in those instances Wilcoxon rank-sum comparisons were used. Significance was established at p ≤ 0.05 for all comparisons.
Results

Field Stimulation

In order to determine the effects of field stimulation on skeletal muscle GDNF content, EDL, FDS, and SOL were placed in isolated tissue baths and electrically stimulated. After 4 hours of field stimulation tissues were removed and processed for analysis of GDNF protein content. In the EDL, GDNF protein content significantly decreased (44% ±18%) as compared to unstimulated, contralateral controls. The field stimulated FDS significantly decreased compared to control (77% ±7%) while SOL muscles had a significant increase (138% ±7%) when compared to control values (Figure 25).

When tissues were pre-treated with α-bungarotoxin as previously described and subsequently underwent field stimulation, both responses, the significant decrease in the EDL and the significant increase in the SOL, were attenuated (125% ±31% and 140% ±16% respectively). In the FDS, α-bungarotoxin pre-treatment and subsequent field stimulation caused a significant decrease (51% ±15%) compared to unstimulated α-bungarotoxin treated control tissues (Figure 26). Treatment with α–bungarotoxin alone had no significant effect on GDNF expression in EDL, FDS, or SOL muscles (105% ±9%, 110% ±8%, and 74% ±16% respectively [Figure 27]).
Effects of field stimulation on GDNF protein content of hindlimb skeletal muscles.

Figure 25. Effects of field stimulation on GDNF protein content of hindlimb skeletal muscles. Four week old male Sprague-Dawley rats were euthanized and the extensor digitorum longus (EDL), flexor digitorum superficialis (FDS), and soleus (SOL) muscles were removed. Samples were placed into a tissue bath containing Ringer’s solution and the contralateral tissues were used as unmanipulated controls. Four hours of field stimulation caused a significant decrease in GDNF protein content of the EDL (44% ±19%, n = 7) as compared to unstimulated control. Field stimulation also caused a significant decrease in the FDS (77% ±7%, n = 6) In the SOL field stimulation caused a significant increase (138% ±7%, n = 6) (*p ≤0.05).
Figure 26. Effects of field stimulation on GDNF protein content of hind limb skeletal muscles after treatment with α-bungarotoxin. Four week old male Sprague-Dawley rats were euthanized and the extensor digitorum longus (EDL), flexor digitorum superficialis (FDS), and soleus (SOL) muscles were removed. Samples were placed into a tissue bath containing Ringer’s solution and the contralateral tissues were used as unmanipulated controls. Four hours of field stimulation after a 30 minute pre-treatment of the tissues with α-bungarotoxin had no significant effect on GDNF protein content of the EDL (125% ± 31%, n = 9) as compared to unstimulated, α-bungarotoxin treated controls. Field stimulation caused a significant decrease in the FDS (51% ± 15%, n = 9). In the SOL field stimulation had no significant effect on GDNF protein content compared to control (140% ±16%, n = 6) (*p ≤0.05).
Effects of α-bungarotoxin on GDNF protein content of hindlimb skeletal muscles.

Figure 27. Effects of α-bungarotoxin on GDNF protein content of hindlimb skeletal muscles. Four week old male Sprague-Dawley rats were euthanized and the extensor digitorum longus (EDL), flexor digitorum superficialis (FDS), and soleus (SOL) muscles were removed. Samples were placed into a tissue bath containing Ringer’s solution and the contralateral tissues were used as unmanipulated controls. Tissues treated with α-bungarotoxin for 30 minutes and then held at optimum length for 4 hours had no significant change in GDNF protein content in the EDL, FDS, or SOL (105% ±9%, 110% ±8%, and 74% ±16%; n = 6 for all) as compared to untreated controls that were held at optimum length.
Stretch

In order to determine the effects of passive stretching on GDNF content of skeletal muscle, EDL, FDS, and SOL muscles were removed, and placed in isolated tissue baths. Tissues were cyclically stretched every 15 seconds for 4 hours. Tissues were removed and processed to measure GDNF protein content. The EDL had a significant decrease in GDNF protein content (66% ±10%) as compared to control in response to passive stretch. There was no significant effect on FDS or SOL muscle GDNF protein content (95% ±8% and 108% ±10% respectively) after 4 hours of stretching (Figure 28).

To determine the role of the nAChRs in this response, tissues were pretreated with α-bungarotoxin to block nAChR activity. After treatment with α-bungarotoxin and subsequent washes, muscles were cyclically stretched for 4 hours and GDNF protein content was analyzed. There was no significant difference between the α-bungarotoxin treated, passively-stretched EDL or FDS (85% ±13% and 86% ±14%) and α-bungarotoxin treated, unstretched control tissues (Figure 29). For the SOL, pretreatment with α-bungarotoxin and subsequent passive stretching caused a significant increase in GDNF protein content (240% ±10%) compared to the α-bungarotoxin treated, unstretched control (Figure 29).
Figure 28. Effects of stretch on GDNF protein content of hindlimb skeletal muscles. Four week old male Sprague-Dawley rats were euthanized and the extensor digitorum longus (EDL), flexor digitorum superficialis (FDS), and soleus (SOL) muscles were removed. Samples were placed into a tissue bath containing Ringer’s solution and the contralateral tissues were used as unmanipulated controls. Four hours of cyclical, passive stretching caused a significant decrease in GDNF protein content of the EDL (66% ±10%, n = 6) as compared to unstimulated control. There was no significant effect of passive stretching on GDNF protein content of the FDS (95% ±8%, n = 6) or the SOL (108% ±10%, n = 6) as compared to the unstretched, contralateral control tissues (*p ≤0.05).
Effects of stretch with α-bungarotoxin on GDNF protein content of hindlimb skeletal muscles.

Figure 29. Effects of stretch with α-bungarotoxin on GDNF protein content of hindlimb skeletal muscles. Four week old male Sprague-Dawley rats were euthanized and the extensor digitorum longus (EDL), flexor digitorum superficialis (FDS), and soleus (SOL) muscles were removed. Samples were placed into a tissue bath containing Ringer’s solution and the contralateral tissues were used as unmanipulated controls. There was no significant effect of 4 hours of cyclical passive stretching after 30 minutes of α-bungarotoxin treatment on GDNF protein content of the EDL (80% ±9%, n = 6) or the FDS (86% ±14%, n = 5) as compared to the unstretched, α-bungarotoxin treated control tissues. Passive stretching after α-bungarotoxin treatment caused a significant increase in GDNF protein content of the SOL (240% ±10%, n = 6) as compared to unstretched, α-bungarotoxin treated controls (*p ≤0.05).
**Carbachol Treatment**

To further characterize the role of the nAChRs in response to stimuli tissues were exposed to 10μM of the nAChR agonist carbachol for 4 hours in the muscle bath. After treatment with carbachol, EDL and SOL muscle GDNF protein content was measured using an ELISA. There was a significant decrease in GDNF protein content for the EDL (49% ± 28%) as compared to control. There was no significant effect of carbachol treatment on SOL GDNF protein content (126% ± 22% [Figure 30]).
Figure 30. Effects of carbachol on GDNF protein content of hindlimb skeletal muscles. Four week old male Sprague-Dawley rats were euthanized and the extensor digitorum longus (EDL) and soleus (SOL) muscles were removed. Samples were placed into a tissue bath containing Ringer’s solution and the contralateral tissues were used as unmanipulated controls. Four hours of treatment with 10uM carbachol caused a significant decrease in GDNF protein content of the EDL (49% ±29%, n = 7) as compared to untreated control. Carbachol treatment had no significant effect on GDNF protein content in the SOL (126% ±15%, n = 6) (*p ≤0.05).
Discussion

Field Stimulation

The findings of this study demonstrate that GDNF protein content is differentially regulated in skeletal muscle. Stimulation caused a significant decrease in the EDL (44% ±19%, n = 7), while in the SOL (138% ±7%, n = 6) a significant increase in GDNF protein content was observed. These findings are interesting because the response to field stimulation seems to be both statistically significant in magnitude and physiologically opposite in direction. This suggests that both the EDL and the SOL respond to these conditions in a unique way and that inherent differences in these tissue types may be responsible for the physiologically opposing changes of GDNF protein content. One difference between these two tissue types is the composition of the muscle fibers.

The EDL is composed primarily of fast-twitch muscle fibers with published reports of 98% of the overall composition being of the fast-twitch phenotype (Alnaqeeb and Goldspink 1987). The SOL on the other hand is composed primarily of slow-twitch muscle fibers which account for approximately 89% of the overall muscle fiber composition (Alnaqeeb and Goldspink 1987). Nagano and Suzuki (2003) demonstrated a developmental difference in GDNF protein content between muscles of opposing fiber type composition (SOL [slow] and gastrocnemius [fast]) and suggested that inherent differences in muscle fiber composition might be a cause in the GDNF protein content differences observed during development. Our data also
lends support to this idea of differential GDNF protein expression between muscles of different fiber-type composition. The observation of both the field stimulated EDL and SOL to significantly change from control unstimulated tissues and change in opposing directions suggests that they respond differently to electrical activity initiated via field stimulation. Further support for a relationship to muscle fiber-type composition is exemplified in the response of the FDS to field stimulation.

In the FDS, field stimulation caused a significant decrease (77% ± 7%, n = 6) but not to the same magnitude as observed in the EDL (44% ± 19%, n = 7). Ariano et al. (1973) determined the FDS is composed primarily of fast-twitch muscle fibers, but does contain more slow-twitch muscle fibers than the EDL. They determined the slow-twitch muscle fiber content for FDS to be approximately 8%. Our laboratory observations using immunohistochemistry also support these findings (Figure 10). The results from field stimulation of the FDS demonstrate a response in between that of the SOL and EDL in regards to GDNF protein content for a muscle with a slow-twitch muscle fiber-type composition that is also in between the SOL and EDL. One issue with the results from the FDS experiment, however, is that these tissues were not removed in the same manner as the EDL and SOL and some tissue damage and/or factors involved in removal of the distal femur head may have an effect on the response in this tissue. Further investigation addressing the inherent differences in tissue composition and physiology and their direct impact on GDNF protein expression is warranted.

Another interesting result from this study was identifying the role that the nicotinic acetylcholine receptors (nAChRs) have in the previously mentioned
response to field stimulation. Field stimulation would allow for depolarization of all membranes within the electrical field, thereby causing depolarization of the intact synaptic terminal and subsequent release of neurotransmitter, ACh. Acetylcholine binds to receptors at the skeletal muscle endplate and normally causes a small change in end plate potential that can eventually lead to muscle membrane depolarization and muscle contraction. In the field stimulation experiment, the muscle membrane is directly depolarized and undergoes muscle contraction, but ACh is also released from the terminals, binds to receptors and may activate other chemical messaging pathways in the tissue. By adding the nAChR antagonist \( \alpha \)-bungarotoxin we are able to block the effects of these receptors and still allow for muscle membrane depolarization and the subsequent events that result in skeletal muscle contraction.

We found that the significant decrease of GDNF content in the EDL caused by field stimulation (44% ±19%, n = 7) was attenuated when the tissues were pre-treated with \( \alpha \)-bungarotoxin (125% ±31%, n = 9). However, in the SOL, field stimulation with or without \( \alpha \)-bungarotoxin pre-treatment appeared to cause an increase in GDNF protein content. While the GDNF protein content of the \( \alpha \)-bungarotoxin pre-treated group was not statistically different from control (140% ±16%, n = 6) the discrepancy appears to be due to be a result of an increase in the variability of the data set and may not necessarily be a direct result of a change in physiological response to stimulation. However, in the absence of a direct comparison between the stimulated SOL and the stimulated, \( \alpha \)-bungarotoxin-treated SOL, a definitive conclusion could not be made.
These findings suggest that a change in GDNF protein content in the EDL may be a result of nAChR activation and not a direct result of membrane depolarization or subsequent muscle contraction. The change in GDNF protein content may instead be a result of nAChR activation. For the SOL, the physiological response to stimulation appears to be only slightly affected if at all, by nAChR activity. Instead, these tissues may regulate GDNF protein content in a manner that is different from EDL, possibly as a result of mechanical stimuli, membrane depolarization, changes in intracellular calcium levels, a result of muscle contraction, or a number of other physiological processes that are not a direct result of nAChR activity. While the reason for the change in GDNF protein content in these tissues has not been determined, what has been identified in this study is that these two tissue types differ in physiological response to electrical field stimulation and that these responses also differ in regards to the role of the nAChRs in this response.

Field stimulation after pretreatment with α-bungarotoxin caused a significant decrease in GDNF protein content of the FDS (51% ±15%, n = 9) as compared to the unstimulated, α-bungarotoxin treated control group. This result might suggest that GDNF protein content in this tissue is not affected by blockade of the nAChRs. This response is similar to that of the SOL with respect to the role of the nAChRs but similar to the EDL with respect to direction of change of GDNF protein content. The FDS appears to again respond with characteristics similar to both the EDL and the SOL. However, the result in the FDS might be affected by the methods of the tissue dissection (i.e. presence of bone from distal femur) and should not be considered a conclusive determination of the physiological response of normal tissue.
The effects of passive stretching on GDNF protein content of hind limb skeletal muscles is similar to the effects observed with field stimulation. Like field stimulation, passive stretching caused a significant decrease in GDNF protein content of EDL (66% ±10%, n = 6) compared to unstretched controls. This response also appears to be dependent upon nAChR activation. Pre-treatment of EDL with α-bungarotoxin has no significant change (80% ±9%, n = 6) from control, but attenuates the response to stretch. Chen and Grinnell (1997) have demonstrated that there is a direct, mechanical modulation of the release of transmitter with stretch. These results suggest that the effects of passive stretching of the EDL on GDNF protein content are due primarily to an increase in transmitter release with stretch and subsequent nAChR activation. Blockade of the nAChRs with α-bungarotoxin attenuated the effects of passive stretch on GDNF protein content in the EDL, much as it did with electrical field stimulation.

The response of the SOL to passive stretching was once again different than that of the EDL. Passive stretching had no significant effect on GDNF protein levels as compared to unstretched controls. Passive stretching after α-bungarotoxin treatment caused a significant increase in GDNF protein content (240% ±10%, n = 6) when compared to unstretched, α-bungarotoxin treated controls. This was the largest change in GDNF protein content observed with any of the manipulations tested. In this tissue, mechanical activity mediated through passive stretch might act as a strong signal to increase GDNF protein content. This increase might be reduced by neurotransmitter activity. This would explain why the increase in GDNF protein
content is greatest when nAChRs are blocked via α-bungarotoxin and mechanical activity is initiated with passive stretching. When passive stretching is performed without α-bungarotoxin pre-treatment, mechanical release of neurotransmitter is increased and this increased neurotransmitter activity may be responsible for partially attenuating the effects of passive stretch in the SOL.

In the FDS the response with stretch once again shared similar results with both the EDL and the SOL. Like the SOL, GDNF protein levels were unaffected by stretch alone. However, similar to EDL, when FDS were pretreated with α-bungarotoxin and subsequently stretched there was no significant change from α-bungarotoxin control FDS. This blended response to stretch stimulus further supports the possibility of a skeletal muscle fiber-type specific response to activity, however direct evidence linking specific fiber types to differences in regulation of GDNF protein expression is still needed. These observations only lend support to this hypothesis but do not directly test this phenomenon as whole muscle extracts were used for GDNF determination and did not specifically assay skeletal muscle fiber-type subpopulations.

*Carbachol*

To further investigate the role of the nAChR in GDNF protein expression we used an agonist for these receptors, carbamylcholine or carbachol. We found that similar to both field stimulation and passive stretching, treatment of these tissues with 10 μM carbachol caused a significant decrease in the EDL GDNF protein content. These tissues lacked the coordinated mechanical activity associated with whole
muscle contraction via field stimulation and the mechanical activity associated with
cyclical passive stretching and yet still had significant decreases in GDNF protein
content associated with both of these manipulations. Even though the bath
application of carbachol is not as coordinated as NMJ delivery of ACh, we cannot
rule out the possibility of membrane depolarization and the subsequent processes that
may lead to generation of muscle tension in these bath experiments. We did attempt
to measure tensile force after carbachol application and it was not detectable, even
when the sensitivity was adjusted well beyond that used to record tension from field
stimulation and ACh dosages.

In the SOL, carbachol treatment was only able to cause a slight increase in
GDNF protein content but this response was not statistically significant. These
experiments were performed based on standard experimental values of carbachol for
skeletal muscle; however, future investigations should include the use of tissue
specific concentrations found using dose-response evaluations.
Summary

One common trend throughout all of these experiments is that each muscle type responds in a manner that is correlative to its muscle fiber-type composition. The primarily fast-twitch EDL would only significantly decrease in GDNF protein content in response to bath manipulations. The primarily slow-twitch SOL would only significantly increase in GDNF protein content in response to bath manipulations. However, these observations with skeletal muscle fiber-type composition are only correlative and are not the only possible explanation for differences observed between these skeletal muscles. Furthermore, GDNF values were measured from whole muscle extracts and any number of cell types within the muscle could be responsible solely for, or in conjunction with skeletal muscle cells, to account for changes in the expression of GDNF protein content.

These results do suggest that GDNF protein content of skeletal muscle is regulated via activity and that the nAChRs may play an important role in this response. We have determined that changes in GDNF protein content of different muscles are regulated differently and that it is not adequate to conclude a response to a stimulus like exercise or activity based upon the response of one muscle type as a representative of all skeletal muscle responses.
CHAPTER VII

DISCUSSION AND CONCLUSIONS

Disclaimer and Disclosure

For all studies where GDNF protein content was measured skeletal muscle extracts were prepared from whole muscle preparations. This includes all cell types within the skeletal muscle at the time of extraction including but not limited to; skeletal muscle cells both mature and satellite cells, neuronal fibers, glial cells, smooth muscle cells of skeletal muscle vasculature, endothelia cells of skeletal muscle vasculature, red blood cells, white blood cells, fibroblasts and any other cell that happens to be within the skeletal muscle at the time of extraction. While references in this dissertation are made to changes in GDNF protein content of skeletal muscle we do not imply this to solely mean mature skeletal muscle fibers. When using whole tissue preparations it is important to keep in mind the composition of the tissue examined and the possible sources for GDNF protein content.

Histology

One of the primary results from our histology studies was the localization of GDNF protein expression to the neuromuscular junction (NMJ). Previously, there were conflicting reports citing that GDNF was in the region of the NMJ in human tissue (Suzuki et al. 1998) and another report that did not find GDNF at the NMJ of rat skeletal muscle (Russell et al. 2000). We too were unable to label GDNF at rat
NMJ using standard histological procedures and found that successful labeling was the result of a few years of trial and error troubleshooting.

GDNF is not produced in large amounts in skeletal muscle and when the protein is quantified using ELISA, values of GDNF protein ranged from 2 – 100 pg/mg of tissue. Adding additional difficulty was the fact that GDNF protein is a releasable signaling molecule that is intended to be exported out of the skeletal muscle cells. The best possibility for identifying positive GDNF signal from skeletal muscle would therefore likely occur if GDNF protein expression was concentrated into a focal region, either internally in storage vesicles or externally tagged to the muscle surface. Knowledge about its function and its trophic support for motor neurons subsequently led us to initially investigate the neuromuscular junction.

When successful labeling of GDNF at the NMJ was finally attained we noticed that GDNF labeling appeared in a hazy region associated with the nAChRs labeled by α-bungarotoxin. This hazy appearance may be on the skeletal muscle surface as faint signal sometimes continues along the length of the muscle fiber away from the NMJ and appears between muscle fibers. GDNF has the ability to bind to heparin-sulfate proteoglycans (HSPGs) (Hamilton et al. 2001; Davies et al. 2003; Rider 2003) and it is possible that they may be used to concentrate GDNF protein at the region of the NMJ. HSPGs have been shown to be used as a means to localize the expression of at least one other NMJ-associated, extracellular, signaling protein called neuregulin (Loeb 2003). This might explain the hazy appearance of GDNF immunopositive labeling at the NMJ. Future studies could clarify this implied
association by using commercially available antibodies to determine if GDNF signaling is co-localized to HSPGs.

Another interesting finding was that not every NMJ in rat muscle stained positive for GDNF. This was also in agreement with the previous functional and histochemical studies involving GDNF. It had already been determined that GDNF-deficient and GFRα1-deficient mice lost approximately 22-35% of the motor neurons (Moore et al. 1996; Sanchez et al. 1996; Cacalano et al. 1998; Oppenheim et al. 2000). Furthermore analyses of GFRα1 mRNA and protein found that only approximately 25% of spinal motor neurons contained GFR α1 (Bergman et al. 1999). Our observations visually support these findings. However, GDNF-positive endplate counts were not attempted due to the thickness of the muscle preparations used.

We attempted to only visually compare endplates in the same field of view and in the same plane of depth in the tissue. If whole muscle counts were performed one could not say with certainty that the absence of GDNF immunopositive labeling would be due to a lack of GDNF protein or an inability of the antibody to diffuse into the deeper regions of the muscle preparations. Assessment for GDNF positive-endplates may be possible if the skeletal muscle preparations were thinner and had uniform thickness.

Another interesting finding was that GDNF immunopositive labeling was identified at NMJs of slow-twitch muscle fibers. These results were preliminary in nature and repeated preparations are needed to confirm this initial finding. Additionally an association with slow-twitch muscle fibers does not mean that GDNF
is exclusively expressed in these tissues or even that it is expressed at all slow-twitch NMJs. More information is needed, including labeling of endplates from fast-twitch, fast-intermediate, and fast-oxidative muscle fibers in order to draw further conclusions about GDNF protein expression and skeletal muscle fiber-type.

One skeletal muscle fiber-type that displayed positive GDNF staining within the muscle fibers was the intrafusal muscle fiber type. Labeling of GDNF protein in human intrafusal muscle fibers was demonstrated by Suzuki et. al. (1998). It was later shown that GDNF protein was associated with intrafusal muscle fibers of mice during development (Whitehead et al. 2005). We provided similar evidence for GDNF immunoreactivity in the intrafusal muscle fibers of rat skeletal muscle.

Functional studies from mice demonstrated that it was the motor neuron innervation of these intrafusal muscle fibers that was responding to changes in GDNF gene expression (Whitehead et al. 2005). They claimed that all of the motor neuron loss associated with GDNF heterozygous knockout could be accounted for by the \( \gamma \)-motor neurons that innervate intrafusal muscle fibers. However no direct label for motor neuron subtypes currently exists that selectively identifies \( \gamma \)- versus \( \alpha \)-motor neurons. Additionally they also pointed out that a small portion of extrafusal muscle fibers also expressed positive labeling of GDNF protein in their GDNF \(^{+/\lacZ}\) mice. While GDNF has been shown to be associated with intrafusal muscle fibers by our results and previous studies, their findings and ours suggest that extrafusal muscle fibers also contain GDNF and that expression in these fibers occurs at the NMJ.
Aging

An interesting result when comparing the two aging studies was the observation that GDNF protein content varied significantly when the extraction methods were altered. While methods provided by the manufacturer did not provide a specific protocol for skeletal muscle processing, other published reports used between 1:10 and 1:20 dilutions of tissue weight to processing buffer volume for extracting GDNF protein from skeletal muscle. The protocol in our lab called for a 1:4 dilution. This became problematic because certain samples would become gel-like while others remained in a liquid phase.

The inconsistency of sample processing was then addressed and changes were made to the second study to change the dilution factor to 1:14. The original intent of the second study was to add the results from the new time points to the results attained in the first study. However, in this process vast differences in GDNF protein content made it not possible to directly compare the GDNF protein content values between studies. These apparent differences in extracted GDNF protein changed our current processing protocol to include a higher dilution of skeletal muscle.

This concern was also the reason why in the bath studies, comparisons were not made between content values but rather changes from contralateral, unmanipulated control tissues and expressed as a percent change from control. This ensured that differences in processing of skeletal muscle samples between studies did not directly affect our statistical analyses of GDNF protein content. Rather
significant differences in protein content should then be due to the muscle bath
treatment itself.

We suggest that GDNF protein extraction from skeletal muscle may vary
depending upon the amount of available buffer in relation to the skeletal muscle mass. It is therefore important, when comparing absolute values of GDNF protein content between studies that the dilution factor used should be the same. Additionally there may be a concentration at which GDNF protein content is optimally extracted from skeletal muscles. Future work should include a step-wise dilution analysis to assess this possibility to determine an optimum dilution factor for GDNF protein extraction from skeletal muscle.

While the age-related changes that occurred in skeletal muscle were previously discussed in detail (see chapter 4), interesting comparisons could be made between the different responses of each skeletal muscle with regards to the process of aging. For all tissues GNDF protein content did not significantly change throughout normal adulthood (5 months – 19 months of age). During this time frame, the NMJ is stable and does not undergo significant changes in morphology. Likewise, GDNF protein expression in skeletal muscles does not undergo significant changes. This is the only phenomenon that appeared to occur similarly for all three of the skeletal muscles examined.

Both in early development and in late life GDNF protein content significantly changed for the SOL and the FDS. These tissues followed the same pattern of GDNF protein expression; highest at 1 month, decreased at 10 months, and elevated at 23 months of age. While both of these tissues had significant changes in GDNF protein
content with age, the EDL did not appear to be significantly altered at 1, 10, or 23 months of age. These differences in GDNF protein expression at various time points may be attributed to inherent differences in skeletal muscle fiber-type composition.

At both the 1 month and the 23 month time points neurological changes may be occurring. It has been demonstrated that refinement of the NMJ in rat continues after birth up until 1 month of age at which time the physical properties of the NMJ are established and remain proportionate throughout adulthood (Balice-Gordon and Lichtman 1990). Likewise significant effects of sarcopenia occurs in the last quartile of an organisms life-span (Rosenberg 1997) which would begin at approximately 21 months of age in the rat (Kanda and Hashizume 1998). During these times the GDNF protein content of the primarily slow-twitch SOL muscle was significantly higher than the GDNF content of the primarily fast-twitch EDL muscle. In normal adulthood, when there are no significant neurological changes occurring in skeletal muscles, GDNF protein content of the SOL was not significantly different than the EDL. Together these results may suggest that neurological changes associated with development or aging may have a significant effect on the GDNF protein content of slow-twitch muscle fibers but may not affect fast-twitch muscle fibers in the same manner. Future aging studies should address specifically the neurological changes that may occur at these time points and look for histological evidence of denervation or sprouting that can occur with advanced aging.
Voluntary Running

This study began as a confirmation of methods and in the end generated interesting information about GDNF protein expression in skeletal muscle with exercise. We began our voluntary running study to confirm previous findings that exercise had the ability to alter GDNF protein content in adult rat skeletal muscle. Previously we had used forced treadmill running and wanted to change methods to a voluntary running apparatus to reduce the negative reinforcement associated with forced treadmill running. We found that voluntary running did indeed significantly alter GDNF protein content of the EDL.

The GDNF protein content of the EDL significantly decreased in response to voluntary running. This response due to exercise is opposite the previous result attained with forced treadmill running. With treadmill running GDNF protein content of the SOL significantly increased (Wehrwein et al. 2002). The initial experimental design included the use of the SOL as well as the EDL in the voluntary running study so that direct comparisons of the results from each study could be made. However technical error not identified until after completion of the study, resulted in the subsequent removal of the FDS. Nonetheless interesting comparisons were made between the EDL and the FDS.

The intent of comparing the results between the SOL and EDL was to identify if there were tissue specific responses in GDNF protein content with exercise. However comparisons drawn between the responses of the FDS and the EDL demonstrated this effect. While the EDL had a significant decrease in GDNF protein
content with exercise, the FDS had no significant change. Combined with the previous results that SOL had a significant increase with forced treadmill running this may suggest that exercise-dependent changes in GDNF protein content is skeletal muscle specific. This phenomenon was also observed in the skeletal muscle bath studies which will be discussed later in this chapter.

Results from this study also helped to further characterize the change in GDNF protein content of skeletal muscle in response to exercise. Prior to this study, GDNF protein content of skeletal muscle was analyzed shortly after the last bout of exercise (Wehrwein et al. 2002). In this study our exercise group was not euthanized until one week after the last bout of voluntary running. GDNF protein content of the EDL was still significantly decreased after three weeks of voluntary running and one week of rest when compared to sedentary control animals. This suggests that skeletal muscle changes in GDNF protein content are long-lived. This activity-based, long-lived alteration of neurotrophic factor protein expression by skeletal muscles may have implications on long-term potentiation and learning and memory.

The electrical activity of both neurons and skeletal muscle are measured in milliseconds. The effects that three weeks of exercise had on neurotrophic factor expression, however, lasted at least one week beyond the last exposure to voluntary exercise. This suggests that activity-related skeletal muscle adaptations may have occurred to alter the baseline expression of GDNF. Long-term (24hr) exposure to GDNF at the NMJ enhances transmitter release and Ca^{2+} influx into nerve terminals during evoked transmission in a frequenin-dependent manner (Wang et al. 2001). This phenomenon, in principle, is similar to adaptations that may occur to support
long-term potentiation and learning and memory. If tissues are able to respond to neuronal stimuli and adapt their neurotrophic factor expression, then those neurons will receive continued neurotrophic support beyond the cessation of neuronal stimulation. This prolonged neurotrophic activity may, in turn, affect neurotransmission. Additionally, frequenin is expressed in neurons throughout the brain (Paterlini et al. 2000) and null mutation of frequenin in C. elegans was shown to affect learning and memory (Gomez et al. 2001). However, the NMJ is only one type of synapse and occurs in the peripheral nervous system and therefore the physiological adaptations that occur at the NMJ may not completely apply for synapses of the central nervous system.

Further investigation into the time-course of GDNF expression in skeletal muscle is warranted. Studies should address the following questions; when is GDNF protein content first significantly altered, are changes in GDNF protein content dependent on intensity of activity, do changes in GDNF protein content reach a physiological plateau, and how long after cessation of exercise is GDNF protein content significantly altered?. Our results and the results from these studies may have implications on the enhanced effects of training and neuromuscular facilitation.
Bath Studies

The experiments involving tissue bath studies were intended to determine the physiological cues associated with exercise that may be important for altering GDNF protein content of skeletal muscles. Results from these studies demonstrated that the response of GDNF protein content to various stimuli again appeared to be tissue specific. For the EDL, all changes in GDNF protein content were either significant decreases in GDNF content or no change. Conversely, all responses for the SOL were either significant increases in GDNF protein content or no change. These observations suggest that GDNF protein content is not expressed in the same manner for all skeletal muscles, and therefore a change in GDNF protein content from one skeletal muscle type should not be generalized to apply for all skeletal muscles.

With these unidirectional responses of both the SOL and the EDL one may make a comparison between a change in GDNF protein and skeletal muscle fiber-type composition. When comparing the responses of these tissues from field stimulation, stretch and carbachol treatment the magnitude of the change in GDNF protein content appeared to be greater in the EDL than in the SOL. The EDL, which has 98% fast-twitch muscle fiber composition, has a more uniform fiber-type composition than the SOL, which is only 88% slow-twitch. To truly compare the results according to fiber-type, one might prefer a skeletal muscle with 100% composition of a single fiber-type. However such a skeletal muscle does not exist in the rat hind limb. Alternatively, new methods involving the isolation of skeletal...
muscle fibers and experiments on individual skeletal muscle fibers may prove to be useful in correlating these responses to skeletal muscle fiber-type.

In the EDL, both of the significant decreases associated with field stimulation and stretch were attenuated by pre-treatment of the tissues with α-bungarotoxin. In these tissues it appeared as though activation of the nAChRs caused a significant decrease in GDNF protein content of the EDL. This was not the case for the SOL. In these tissues, significant changes in GDNF protein content from control were only observed in response to field stimulation and stretch after pre-treatment with α-bungarotoxin. This may suggest that in these tissues GDNF protein content is increased by mechanical activity.

Stretch alone had no significant effect on GDNF protein content of the SOL. It has been demonstrated that repetitive cyclical stretch causes an increased release of ACh from nerve terminals (Chen and Grinnell 1997). In this case the increase in GDNF protein content from the mechanical activity of stretch may have been overshadowed by a decrease in GDNF protein expression due to possible nAChR activation associated with increased release of ACh during cyclical stretch. Field stimulation of the SOL also caused nAChR activation (data not shown) but it is possible that the amount of force and mechanical activity associated with depolarizing field stimulation of skeletal muscle may be a strong enough mechanical stimuli to overcome this hypothetical nAChR activity-dependent decline. Again clarification of these results may be attained by performing these experiments on isolated skeletal muscle fibers preparations.
Conclusions

One underlying theme that became apparent as a result of these combined studies is that GDNF protein content is not expressed in the same manner for all skeletal muscles. Results from our investigations in histology, voluntary exercise, aging, and bath studies all suggest that GDNF is expressed differentially in the SOL as compared to the EDL and that skeletal muscle fiber-type composition might be an important factor in GDNF protein regulation. Therefore, a significant change in GDNF protein content from one skeletal muscle should not be generalized to apply for all skeletal muscles. In the future, care should be taken to address these tissue differences in regards to regulation of GDNF protein in skeletal muscle.


Copray, S., R. Liem, N. Brouwer, P. Greenhaff, F. Habens and P. Fernyhough (2000). "Contraction-induced muscle fiber damage is increased in soleus muscle of
streptozotocin-diabetic rats and is associated with elevated expression of
effect-derived neurotrophic factor mRNA in muscle fibers and activated

Corse, A. M., M. M. Bilak, S. R. Bilak, M. Lehar, J. D. Rothstein and R. W. Kuncl
(1999). "Preclinical testing of neuroprotective neurotrophic factors in a model

heparan sulphate modulation of GDNF signalling." Growth Factors 21(3-4):
109-19.

de Graaff, E., S. Srinivas, C. Kilkenny, V. D'Agati, B. S. Mankoo, F. Costantini and
V. Pachnis (2001). "Differential activities of the RET tyrosine kinase receptor

Diamond, S. E., R. A. Spiorici, A. L. Ferry, J. J. Higginson, J. J. McCarthy, D. M.
Locasto, R. Purohit, K. A. Esser, S. Apparsundaram and L. B. Meszaros

Dupont-Versteegden, E. E., J. D. Houle, R. A. Dennis, J. Zhang, M. Knox, G.
Wagoner and C. A. Peterson (2004). "Exercise-induced gene expression in
soleus muscle is dependent on time after spinal cord injury in rats." Muscle

Edstrom, E. and B. Ulfhake (2005). "Sarcopenia is not due to lack of regenerative

Edstrom, L. and E. Kugelberg (1968). "Histochemical composition, distribution of

"Development of cranial parasympathetic ganglia requires sequential actions
of GDNF and neurturin." Development 127(22): 4877-89.

Gissel, H. and T. Clausen (1999). "Excitation-induced Ca2+ uptake in rat skeletal

(1999). "Expression of neurturin, GDNF, and GDNF family-receptor mRNA

Gomez, M., E. De Castro, E. Guarin, H. Sasakura, A. Kuhara, I. Mori, T. Bartfai, C.
I. Bargmann and P. Nef (2001). "Ca2+ signaling via the neuronal calcium


expression in regenerating muscle fibers in neuromuscular diseases."

expression of glial cell line-derived neurotrophic factor in human skeletal


Trupp, M., M. Ryden, H. Jornvall, H. Funakoshi, T. Timmusk, E. Arenas and C. F.
new neurotrophic factor for avian and mammalian peripheral neurons." J Cell

Trupp, M., R. Scott, S. R. Whittemore and C. F. Ibanez (1999). "Ret-dependent and
-independent mechanisms of glial cell line-derived neurotrophic factor

Tsui-Pierchala, B. A., R. C. Ahrens, R. J. Crowder, J. Milbrandt and E. M. Johnson,
Jr. (2002). "The long and short isoforms of Ret function as independent

"Ca(2+) binding protein frequenin mediates GDNF-induced potentiation of
Ca(2+) channels and transmitter release." Neuron 32(1): 99-112.

Mizukami, T. Matsushita, Y. Hanazono, A. Kume, T. Nagatsu, K. Ozawa and
I. Nakano (2002). "Neuroprotective effects of glial cell line-derived
neurotrophic factor mediated by an adeno-associated virus vector in a
transgenic animal model of amyotrophic lateral sclerosis." J Neurosci 22(16):
6920-8.

in an activity-dependent manner in rat skeletal muscle." Muscle Nerve 26(2):
206-11.

line derived neurotrophic factor-dependent fusimotor neuron survival during

anatomical features and adaptations to various forms of increased, or


