6-2015

Regulation of Glial Cell Line-Derived Neurotrophic Factor (GDNF) Production in Voluntary and Involuntary Muscle

John-Mary Vianney
Western Michigan University, srvianney44@yahoo.com

REGULATION OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) PRODUCTION IN VOLUNTARY AND INVOLUNTARY MUSCLE

by

John-Mary Vianney

A dissertation submitted to the Graduate College
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Biological Sciences
Western Michigan University
June 2015

Doctoral Committee:

John M. Spitsbergen, Ph.D., Chair
Christine Byrd-Jacobs, Ph.D.
Cindy Linn, Ph.D.
Damon A. Miller, Ph.D.

REGULATION OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) PRODUCTION IN VOLUNTARY AND INVOLUNTARY MUSCLE

John-Mary Vianney, Ph.D.

Western Michigan University, 2015

Glia cell line-derived neurotrophic factor (GDNF) is a survival factor for sub-populations of neurons, including somatic and autonomic motor neurons. These neurons depend, in part, on GDNF that is synthesized and secreted by their target tissues. It has been shown that a number of tissues in the periphery express GDNF and these target tissues differ in their composition, function, and in the case of different muscle cell types, their contractile characteristics. Whether the processes regulating GDNF production in these different tissues is similar or different is poorly understood. The broad goal of this study is to examine factors that normally regulate GDNF expression in skeletal and cardiac muscles, with an emphasis on comparing the similarities and differences in these voluntary and involuntary muscles in relation to GDNF production. Previous studies with nerve-muscle co-cultures in our laboratory have shown that GDNF protein levels are reduced when skeletal muscle is in contact with cholinergic nerves. Thus, the hypothesis being tested is that cellular activation by neural cells, via neurotransmitter effects, regulates GDNF expression in voluntary and involuntary muscles. Some cultures were electrically stimulated (30min to 48h) to determine whether electrical activity is an important regulator of neurotrophic factor production. Samples of culture medium and cells were collected between 0h and 48h. The results show that acetylcholine inhibits GDNF secretion in both

cell types, while electrical stimulation has opposing effects on GDNF production, where GDNF levels increase with long-term electrical stimulation in skeletal muscle and decrease with long-term electrical stimulation in cardiac muscle. When cardiac muscle cells were tested alone, norepinephrine was found to stimulate production of GDNF but inhibit production of nerve growth factor (NGF). Electrical stimulation had a similar effect on NGF and GDNF production in cardiac muscle cells. This work suggests that GDNF expression may be regulated differently in cardiac and skeletal muscle. Understanding the regulation of GDNF production in these tissues will provide a better understanding of how these processes may be modulated therapeutically.

ACKNOWLEDGEMENTS

I cannot find the words to express all of my gratitude for my research advisor, Dr. John Spitsbergen, for his excellent mentorship during my dissertation research. Without his guidance, persistent help, and support this degree would not have been possible. My wish is to be the kind of mentor to others that he has been to me; by promoting the freedom to explore new questions, challenging students to think in new ways and always extending the kindness that makes everyone feel at home in the lab.

I would like to thank the members of my dissertation committee, Dr. Christine Byrd-Jacobs, Dr. Cindy Linn, and Dr. Damon Miller, for their valued support to help me succeed as a researcher. I also acknowledge the technical support that Dr. Miller provided me tirelessly whenever I needed his help. Special thanks to all members in Dr. Spitsbergen's lab with whom I got an opportunity to work, for creating such a friendly working environment; as an international student, this meant a lot to me.

I would also like to acknowledge the Department of Biological Sciences, the Graduate College, and International Student Office for funding and encouragement in the awards and grants they have awarded to me during my studies at Western Michigan University (WMU).

Equally important, my deepest gratitude goes to Fr. Kenneth Schmidt, the Pastor at St. Thomas More Catholic Student Parish for allowing me to stay in the Parish ground throughout my Master and Doctoral studies. I am grateful to Sr. Sue McCrery and Sr. Pam

Owens for the community life I have shared with them, they made me feel at home. I am also thankful to the entire Pastoral team and Parish staff for the love, care, and support I continuously received that made my studies possible. Their generosity will never be erased from my heart.

To those who inspired, encouraged, and supported, the first step in my higher education journey: the Sisters of St. Therese of the Child Jesus, to The Dominican Sisters of San Rafael, California where the first four years of my higher education journey started; and for all of the start-up support including room and board, and to the faculty members in Science and Mathematics department at Dominican University of California who introduced me to the scientific research world, I deeply appreciate all of the help.

I owe my deepest gratitude to my family and my closest friends for their invaluable support. Special thanks to my two beloved sisters, for their warm words of encouragement felt so close, although I was hundreds of miles from home. Finally, I would like to dedicate this dissertation to my beloved mother, Philomena Kalikwendwa for her love, care, prayer, encouragement, and inspiration for higher education; without her, I would not be where I am today.

I share the credit for my work with you all. Thank you.

John-Mary Vianney

TABLE OF CONTENTS

Table of Contents - Continued

CHAPTER

Table of Contents - Continued

Table of Contents – Continued

CHAPTER

Table of Contents - Continues

CHAPTER

Table of Contents – Continued

CHAPTER

Table of Contents – Continued

CHAPTER

Table of Contents – Continued

CHAPTER

LIST OF TABLES

LIST OF FIGURES

List of Figures – Continued

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

The somatic and autonomic nervous systems are the major branches of the nervous system that operate in the periphery. Both systems consist of motor nerves, but differ in functional characteristics including their origin, efferent impulse transmission pathways, target effectors, and target responses in terms of excitatory or inhibitory effects. Thus, the two systems differ in the type of control they exert. While the somatic system is voluntary and provides conscious control of voluntary (skeletal) muscle, the autonomic nervous system is involuntary and exerts unconscious control of involuntary (cardiac and smooth) muscles. Voluntary and involuntary target muscles differ in their composition, location, and function. In mammals including humans, skeletal muscles are distributed throughout the body, whereas, cardiac muscles are found only in the heart (Sherwood, 2010).

In order to maintain optimal motor movement, nerves, muscles, and other elements at the synapse should all function synergistically. This may include, but is not limited to proper neurotransmitter release, expression of synaptic receptors, and sufficient supply of target-derived neurotrophic factors. The neurotrophic factors are the family of proteins that have been proven to be capable of providing survival and maintenance of neuronal populations (Silva and Wang, 2011). However, little is known about processes that normally regulate levels of expression for muscle target-derived neurotrophic factors.

Moreover, it is not clear whether a given neurotrophic factor is regulated in a similar or different ways within different muscle types.

This study utilizes skeletal muscle and cardiac muscle as models for voluntary and involuntary muscles, respectively. We have examined the synthesis and secretion of glial cell line-derived neurotrophic factor (GDNF) in these two types of muscle. Nerve growth factor (NGF) was also examined in cardiomyocytes. Understanding the similarities and differences in regulation of the neurotrophic factors in different types of targets, in this case, voluntary and involuntary muscles, will give insight into how the neurotrophic factors can be modulated in therapeutic settings.

1.2. NEUROTROPHIC FACTORS

Neurotrophic factors are a family of proteins that are responsible for the growth and survival of developing neurons and the maintenance of mature neurons. Neurotrophic factors are classified in several subgroups including the neurotrophin family and glial cell line-derived neurotrophic factor (GDNF) family.

The neurotrophin family is a group of neurotrophic factors which is comprised of the structurally related proteins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Henderson, 1996; Allen and Dawbarn, 2006). Each member of the neurotrophin family binds with high affinity to the receptor tyrosine kinase of the Trk family, specifically, NGF and NT-3 act via Trk-A and Trk-C, respectively whereas BDNF and NT-4/5 bind to the Trk-B receptor. All neurotrophins also exert trophic action by binding to a common receptor, p75 (Henderson, 1996; Lu, 2003).

Glial cell line-derived neurotrophic factor (GDNF) family ligands are distant members of the transforming growth factor beta (TGF-β) superfamily, since each member in the GDNF family ligand contains seven cysteine repeated amino acids at the same distance as the other members of the TGF-β superfamily (Lin et al., 1993). The GDNF family includes four members: GDNF, neurturin (NTRN), artermin (ARTN), and persephin (PSPN). All members of the GDNF family induce signaling through binding with high affinity to GDNF family receptor alpha (GFR-α1-4). Specifically, GDNF binds to GFR-α1, NTRN binds to GFR-α2, ARTN binds to GFR-α3, and PSPN binds to GFR-α4. Each ligand binds to its receptor anchored to glycosyl- phosphatidylinositol (GPI). The ligand-receptor complex signals through receptor tyrosine kinase RET, to induce neuronal differentiation and survival (Saarma, 2000; Sariola and Saarma, 2003).

1.2.1 General characteristics and functions of neurotrophic factors

Neurotrophic factors are synthesized in neuronal and non-neuronal tissues. Neurotrophic factor localization studies have shown that a target tissue can express more than one type of trophic factor, as seen in skeletal muscle that expresses BDNF, NT-3, and GDNF (Funakoshi et al., 1993; Kingham and Terenghi, 2006). Additionally, a single trophic factor can be synthesized in several different target tissues (Table 1). Furthermore, depending on the demand, expression of neurotrophic factors can be regulated in activity-dependent manner or constitutively secreted (Schinder and Poo, 2000; Lu, 2003). The neurotrophic factors have been found to provide their trophic effect in several ways including axonal guidance during development, neuronal survival during programmed cell death, synaptic transmission and maintenance in adults, and facilitation of nerve regeneration (Houenou et al., 1996). While other neurotrophic factors have been shown to

depend on each other to exert their trophic effects (Aszmann et al., 2002; Gould and Enomoto, 2009), in most cases, a single neurotrophic factor can elicit a neurotrophic effect alone. NGF and GDNF (to be discussed in later sections) are examples of neurotrophic factors that each acts independently to provide trophic effects to the nervous system (Bohn, 2004; Govoni, 2011).

1.3. OVERVIEW OF NEURAL-TARGET INTERACTION

Most, if not all, communications between the nervous system and the periphery are carried out through the interaction between a nerve and its target at the synapse. The peripheral synapse is a critical anatomical structure where, the connection between the nerve and its target occurs and signal transmission takes place. This allows the movements of the body upon which life depends. These bodily movements include muscle contraction, breathing, swallowing, heartbeat, and walking. The efficient communication between a nerve and its target can be disrupted in neurodegenerative diseases, aging, or by nerve damage (Aszmann et al., 2002; Lie and Weis, 1998). However, studies have shown that reinnervation can take place, although it is not common that natural reinnervation can completely restore the original synaptic integrity. Administration of neurotrophic factors have been shown to enhance reinnervation following denervation or nerve damage (Michalski et al., 2008; Zhang et al., 2009). It has been also shown that the process of reinnervation shows similar characteristics to that of neuronal development. These characteristics include the expression of growth associated protein 43 (GAP43), suggesting that dysfunction, aging, or damaged nerve switches from transmitting function to growth mode when making new connections to restore synaptic structure (Fu and Gordon, 1997)

Table 1.1: NGF and GDNF expression in various target and non-target tissues

NERVE GROWTH FACTOR		**GLIAL CELL LINE-DERIVED NEUROTOPHIC FACTORS**	
Tissue	**Citation**	**Tissue**	**Citation**
Tissues/Cells in the CNS and PNS		**Tissues/Cells in the CNS and PNS**	
Cortex and hippocampus	Mufson et al., 1999*	Striatum, substantia nigra	Springer et al., 1995
Glia, ependymal, endothelia	Thoenen et al., 1995*	basal ganglia, purkinje neurons	
Dorsal root ganglia	Hiltunen et al., 2001*	brainstem, cerebellum, cerebral	
Retina and the Schwann cells	Micera et al., 2004*	cortex	Nosrat et al., 1996
of the iris	Rush et al.,1984*	Thalamus	Hase et al., 1999
Smooth muscle cells	Schäper et al., 2009*	Spinal cord	McCollough and
Heart/ heart cells	Hiltunen et al., 2001*		Spitsbergen, 2013
	Kaye et al., 2000	Superior cervical ganglia	Trupp et al., 1995
Epithelialcells, fibroblasts	Schäper et al., 2009*	Dorsal root ganglia	Trupp et al., 1995
Lung	Freund et al., 2004*	Microglia	Matsushita et al., 2008
Colon	Stanzel et al., 2008*	Astrocyte and Schwann cells	Zhao et al., 2004
		Retina	Kretzet et al., 2006
Glands		Nasal cavity (epithelium)	Springer et al., 1995,
Anterior pituitary gland	Calzà et al., 1997*	inner year and year canal	Nosrat et al.,1996
(mammotroph cells)	Missale et al., 1996*	Heart/cardiomyocyte	Martinel et al., 2007
Submaxillary/submandibular		Skeletal muscle	Gyorkos and Spitsbergen, 2014
sublingual glands	Levi-Montalcine, 1951*	Smooth muscle	Nosrat et al., 1996
Granular convoluted tubule cells	Mathison et al.,1995*	Lung	Nagano and Suzuki, 2003
Salivary glands	Nam et al., 2007*	Gastrointestinal tract	Trupp et al., 1995; Moore et al., 1996
Thyroid gland	Van der Laan et al., 1995*		
Pancreas	Miralles et al., 1998*		
Gastro-enteric tract, liver	Govon et al., 2011		
Other areas		**Other areas**	
		Adrenal gland	Trupp et al., 1995
Biological fluids	Govon et al., 2011		Nosrat et al., 1996
(source of GDNF isn't known)		Tangue	Rind et al., 2005
Spleen, prostate, and epidemis	Govon et al., 2011	Teeth	Nosrat et al., 1996
Mast cell, macrophages and	Lambiase et al., 2004*	Uteric buds	Moore et al., 1996
lympocytes	Leon et al., 1994*		Pichel et al., 1996
Reproductive organs	Govon et al., 2011	Spleen, Testes	Suzuki et al., 1998

* Citations are also found in Govoni, G., Pascalea, A., Amadioa, M., Calvillo, L., D'Elia, E., Ceredad, C., Fantuccie, P., Ceronif, M., Vanolih, E., 2011. NGF and heart: Is there a role in heart disease? Pharmacological Res. 63: 266-277.

It can be that suggested understanding of nerve development could provide an alternative way to examine nerve regeneration. Therefore, a better understanding of origin, progenitor cells, trophic effects, and guidance cues in the process of nerve-target formation can add information and give insight into the process of reinnervation.

1.3.1. Somatic nervous system development

Three types of cells are present at the neuromuscular junction (NMJ): motor neuron, muscle fiber, and Schwann cells (Band-Saberi et al., 1996; Hasan, 2013). In early development these cells originate from different types of cell layers but meet to form the NMJ: motor neurons are formed from somata in the neural tube while Schwann cells develop from neural crest cells. Mesodemal cells give rise to myogenic cells, which in turn differentiate into myoblasts; finally the myoblasts fuse to form myotubes (Band-Saberi et al., 1996). Since each myoblast contains a single nucleus, the fusion of myoblasts results in multinucleated myotubes, a specific characteristic of skeletal muscle fiber. A pool of motor neurons originates from the ventral horn of the spinal cord, then the pool of motor neurons converges into a single motor neuron that grows an axon and innervates its appropriate skeletal muscle fibers (Burden, 1998). These observations suggest that there should be guidance cues for axon pathfinding towards appropriate targets. The complex of target-derived GDNF, its receptor GFRα1, and neural cell adhesion molecules (NCAM) was reported to play a role in motor neuron axonal guidance towards appropriate targets (Paratcha et al., 2003; Paratcha and Ledda, 2008). Once a motor axon growth cone contacts a newly formed myotube, differentiation of the presynaptic and postsynaptic apparatus begins. The synaptic transmission molecules include acetylcholine (ACh), acetylcholine

receptors induction activity (ARIA), ion channels, neuregulin, integrins, acetylcholinesterase (AChE), heparan sulfate proteoghylcan, neural cell adhesion molecules (NCAM), rapsyn, muscle-specific kinase (MuSK), acetylcholine receptors (AChRs), sodium channels, and other synaptic molecules (Meier and Wallace, 1998). Interestingly, neurotrophic factors, such as GDNF, were found to be involved in this motor nerve signaling and synaptic transmission (to be discussed in later sections).

1.3.2. Autonomic nervous system development

The development of the heart takes place independently of its innervation and it is the first organ to form and function during development. Studies also have confirmed that heart formation is conserved in all vertebrates (Yutzey and Kirby, 2002). Cardiac progenitor cells have been identified even before gastrulation. In chick embryo, the cardiac progenitors can be seen in the primitive streak as early as 10-12 hours after fertilization and the beating heart tube is apparent within 33-38 hours (Yutzey and Kirby, 2002). In mouse models, mapping studies show the progenitor heart cells as early as embryonic day 10. The p75NTR neutrophin receptor, and vagal nerves can be observed 2-days following heart formation (Hildreth et al., 2008).

The origin of sympathetic and parasympathetic neural cells during development have been examined and extensively reviewed (Hasan et al., 2013; Kimura et al., 2012; San Mauro, 2009; Mitchell, 1953; Olshansky et al., 2008). Briefly, both parasympathetic and sympathetic neurons arise from neural crest cells that migrate from the neural fold and neural tube respectively and subsequently differentiate into mature neurons (Hasan et al., 2013; Kimura et al., 2012; Serbedzija et al., 1990). The neural crest migration, differentiation, early ganglia formation, and axonal projections, depend on guidance cues

including neurotrophic factors, specifically, NGF (Gammill et al., 2005; Hasan, 2013; Kimura et al., 2012; Levi-Montalcini, 1987). However, the arrival of neural crest cells that form parasympathetic nerves precede those that form sympathetic nerves (Hildreth et al., 2008); in other words, the cholinergic inhibition of heart rate begins to function earlier than the sympathetic stimulation of the heart (Hasan, 2013).

The mature sympathetic and parasympathetic pathways contain two neurons, preganglionic and postganglionic. Parasympathetic neurons arise from medial medullary site nuclei: nucleus ambiguous, nucleus tractus solitarius, and dorsal motor nucleus. This vagus nerve extends from the medulla and synapses with postganglionic nerves that innervate the heart via ganglia located in cardiac fat pads in the atria (Olshansky et al., 2008). Sympathetic neurons originate in the spinal cord and emerge in the spinal nerve derived from thoracic (T) regions, T-1 through T-3. Although, preganglionic fibers of both divisions synapse with postganglionic fibers at the ganglia, the location of the ganglia differs, with parasympathetic ganglia located within the heart epicardium and sympathetic ganglia located near the spinal cord (Olshansky et al., 2008).

Parasympathetic and sympathetic preganglionic neurons release ACh at the ganglia. The released ACh binds to nicotinic acetylcholine receptors (nAChRs) located on both postganglionic neurons. Sympathetic postganglionic fibers release norepinephrine (NE) which activates adrenergic receptors. Parasympathetic postganglionic neurons release ACh which interacts with muscarinic acetycholine receptors (mAChRs). Although sympathetic and parasympathetic fibers are distributed in both atria and ventricles of the heart, their innervation density varies from region to region: The conduction system is innervated by both divisions (Crick et al.,1999; Kimura et al., 2012), but in the sinoatrial

and sinoventricular nodes, the cholinergic fibers are more concentrated than the adrenergic fibers (Kawano et al., 2003; Kimura et al., 2012). Likewise, muscarinic type 2 receptors (M_2) are the most abundant receptors in atria, possibly reflecting the abundance of cholinergic fibers (Olshansky et al., 2008). Taken together, compared to somatic neural system innervation, cardiac nervous system innervation is complex. The complexity of innervation of the heart may also suggest the strong influence of the extrinsic nervous system on the heart's performance at the beat-to-beat level (Kimura et al., 2012).

1.4. EFFECT OF DYSFUNCTION OF NEURAL- TARGET INTERACTION

Synaptic transmission depends on the association of the pre- and post-synaptic elements which are also highly regulated to ensure accuracy in information transfer. Dysfunction in nerve-target interaction can result in pathological conditions (Chen et al., 2007; Kimura et al., 2009), including neurodegenerative disorders (Dadon-Nachum et al., 2011). It has been shown that the programmed cell death that occurs during development involves synaptic elimination. It has been further suggested that the cells that survive this period of naturally occurring cell death, do so by competing for target-derived neurotrophic factors (Oppenheim et al., 2000). It can then be hypothesized that the neuronal dysfunction or death that occurs in adults can be caused in part, by the deficiency in target-derived neurotrophic factors.

1.4.1. Effects of dysfunction of motor nerve-skeletal muscle interaction

Motor neurons are the sole means by which the brain can trigger voluntary (skeletal) muscle to contract and produce all movements that occur in the body. Motor neuron diseases affect motor neurons in the central and peripheral nervous system. As a

result, voluntary movements are compromised. There are a number of motor neuron diseases (reviewed in Wijesekera and Leigh, 2009), which include upper and lower motor neurons, the motor neurons whose cell bodies are located in the motor cortex and spinal cord respectively. The pure lower motor neuron diseases include spinal muscular atrophy, a group of genetic disorders that are caused by a loss of motor neurons in the spinal cord and the brainstem. Primary lateral sclerosis mostly affects upper motor neurons and causes stiffness and weakness of the limbs, voice, or swallowing muscles. Amyotrophic lateral sclerosis (ALS) is a common motor neuron disease that affects both the upper and lower motor neurons: the cerebral cortex, the brainstem, and the spinal cord (Wijesekera and Leigh, 2009).

Neuronal cell death is the common feature in neurodegenerative diseases, including those mentioned above. Several investigators use ALS animal models to examine how neurodegeneration occurs. The full pathogenesis of ALS is not well understood; however, several hypotheses have been proposed as key factors for causing neuronal death in ALS (Wijesekera and Leigh, 2009). The point at which degeneration or death of a motor neurons starts in ALS has been the center of investigations. The proposed sites for neuronal death in ALS can be summarized into two major areas, the dying-forward and dying-back hypotheses.

1.4.1.1. The hypotheses in the dying-forward theory

The dying-forward theory assumes that the pathogenesis is initiated at the soma and progresses towards the axons or dendrites. This may include genetic causes and mutations of copper/zinc superoxide dismutase 1 gene (SOD1). Although the SOD1 mutant accounts for only 20% of all familial ALS cases and only 10% of all ALS cases, the

discovery of SOD1 mutation has provided insight into how motor neuron dysfunction may occur. Generally, almost 90% of ALS cases are without genetic linkage and the cause is unknown (Burvill, 2009; Wijesekera and Leigh, 2009). One of the hypotheses is that failure in SOD1 causes glutamate excitotoxicity which contributes to the neuronal death. The physiology behind glutamate toxicity is based on overstimulation of glutamate receptors by glutamate neurotransmitter (Burvill, 2009). Because the SOD1 gene is also involved in glutamate reuptake, the mutation in SOD1 results in accumulation of glutamate at the synapse which causes more damage to neural cells (Orrell, 2010; Shaw, 2005). Another hypothesis is that the mutant SOD1 plays a part in free radical-mediated oxidative stress, although the mechanism of action is not fully understood. Barber et al. suggests that the mutant SOD1 may be toxic through loss of function, inhibition of normal SOD1, or an increase in mutant SOD1 activity leading to increased hydrogen peroxide and hydroxyl free radical levels (Barber et al., 2006). Along with oxidative stress, mitochondrial dysfunction is another proposed factor that may cause neuronal death in a dying forward manner.

The hypothesis that protein misfolding and aggregation is the major cause of neuronal death has been of supported by research results. This is because the misfolded proteins are not only observed in ALS but in other neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Dunnings et al., 2012; Vekrellis et al., 2011). In ALS, the mutant SOD1 aggregates with itself and with other proteins (Burvill, 2009). It is hypothesized that the accumulation of aggregated proteins prevents the neural cells from carrying out their normal activities and eventually the motor neurons die.

1.4.1.2. The hypotheses in the dying-back theory

The dying-back theory has attracted the attention of most investigators studying neurodegeration. Studies have indicated that in early stages of ALS disease, motor nerve terminals are seen partially degraded while the cell bodies in the spinal cord are mostly intact (reviewed in Dadon-Nachum et al., 2011). These observations suggest that axon terminals in ALS degenerate first, followed by progression towards the cell body in the spinal cord, brain stem, or motor cortex (Fischer et al., 2004). It has also been proposed that the neuromuscular junction degenerates first and this degeneration may not only precede, but actively cause the loss of upper and lower motor neurons (Krakora et al., 2012). This hypothesis can also explain other potential mechanisms involved in the dying-back theory. One such mechanism is the disruption of retrograde axonal transport that is observed prior to disease manifestation (Krakora et al., 2012; Parkhouse et al., 2008, Wijesekera and Leigh, 2009). Another major hypothesis in the dying-back theory is the reduction of target-derived neurotrophic factors that have been observed in ALS patients and ALS transgenic animals. These small proteins are responsible for neuroprotection and possess regenerative properties, thus, their deficiencies have been proposed to be involved in motor neurons death in adults (Brujin et al., 2004; Krakora et al., 2012). However, little is known about what normally regulates the production of neurotrophic factors by target tissues.

1.4.1.3. Summary and comments on the neurotrophic factor hypothesis

At this time, there is no exclusive factor that is known to cause motor nerve degeneration. It is not clear whether the mechanisms outlined above all cause the neurodegeneration in ALS. It may be possible that the outlined mechanisms are actually

the results of degeneration itself. Since the dying-back hypothesis assumes that motor neurons die at the synapse first, while the cell bodies are kept intact, therapies providing neuroprotection to axons at the distal end may be the most valuable. However, gaps in knowledge about regulation of neurotrophic factors still exist. These gaps may include understanding a) Which factors normally regulate secretion and release of a neurotrophic factor from target tissues? b) Which is the optimal level of activation of a target tissue that results in maximal production of neurotrophic factor? c) What is the physiological significance of the differential response of each motor nerve to a neurotrophic factor in the motor neuron pool? Understanding neurotrophic factor physiology will enhance the therapeutic intervention for peripheral nervous system disorders.

1.4.2. Dysfunction in cardiac nervous system

As stated earlier, the mammalian heart is extensively innervated by both extrinsic and intrinsic nerves that compose the cardiac nervous system. The intrinsic ganglia of the adult mammalian heart contain approximately 43,000 neurons (Pauza et al., 2000). This local circuit within the heart not only acts as a relay system but gives the heart the intrinsic ability to independently function without extrinsic neural input (Vasegh and Shivkumar, 2008). However, the rate and rhythm of the heart are under control of the extrinsic nervous system. Thus, sympathetic or parasympathetic dysfunction may result in cardiac disorders.

1.4.2.1. Effect of altered communication between sympathetic nerves and cardiac muscle

The primary role of the sympathetic nervous system in the heart is to accelerate the rate and force of contraction of cardiomyocytes. Studies have shown that cardiovascular diseases may be caused by the dysfunction of sympathetic neurons (Chidsey et al., 1963; Himura et al., 1993; Meredith et al., 1991). Increased heart rate in pathological heart has

been associated with sympathetic overactivity, which in turn has been linked with increased in NE release (Esler, 2011; Kimura et al., 2012). Also, NE administration has been shown to affect NGF protein expression levels in vivo and in vitro (Esler, 2011; Kimura et al., 2012). This may indicate that elevated NE release in heart diseases may impact the trophic effects of NGF on sympathetic neurons. Studies aimed at determining the regulation of NE in normal and pathological heart may give insight into the relationship between neurotransmitter effects and neurotrophic factors expression in the heart.

1.4.2.2. Effect of altered communication between parasympathetic nerves and cardiac muscle

An interesting question may be whether the overactivation of the sympathetic nervous system in cardiac pathophysiology is a result of failure in the parasympathetic control. The involvement of parasympathetic innervation in heart failure was tested in early studies conducted by Eckberg et al. (1971). Parasympathetic effects were blocked with atropine following adrenergic blockade with propranolol in patients with heart disease and in normal individuals. Results from these early studies showed that atropine significantly elevated heart rate in normal subjects compared to individuals with heart disease in which the effect of atropine was low (Eckberg et al., 1971). In humans, with the chronic heart failure the degree of parasympathetic dysfunction was found to be related to the severity of left ventricular dysfunction (Nolan et al., 1992). However, identifying the mechanism underlying the pathology has been challenging. In dogs with left ventricle dysfunction, but no heart failure, indices of parasympathetic control decreased significantly after four days of pacing, whereas, in dogs with fully developed heart failure, both vagal and sympathetic contributions were small. These observations suggest that changes in vagal control of heart

rate become apparent at a very early developmental stage of left ventricle dysfunction (Kinugawa et al., 1995). Thus, it can be further hypothesized that parasympathetic dysfunction may account for the elevated heart rate.

1.4.2.3. Potential mechanism underlying the decrease in vagal control

Although the mechanism underlying the loss in vagal control is not clear, several potential mechanisms related to ACh neurotransmitter have been proposed. A decrease in acetylcholinesterase, the enzyme responsible for ACh breakdown, has been reported in the sinoatrial node with heart failure, suggesting reduced cholinergic neurotransmission (Dunlap et al., 2003). Bibvski and Dunlap (2004) suggested that the reduction in the parasympathetic control in heart failure occurs due to the decrease in presynaptic transmission in parasympathetic ganglia which is mediated by nicotinic acetylcholine receptors (nAChRs). Reduced expression of vesicular acetylcholine transporter has been observed in heart failure (Lara et al., 2010***).*** Recently, it was found that cardiomyocytes also secrete ACh. This suggests that non-neuronal sources of ACh may be induced to boost cholinergic signaling to counterbalance sympathetic overactivity (Rana et al., 2010; Roy et al., 2013). Thus, therapies aimed at parasympathetic neuroprotection such as enhancing parasympathetic survival and/or activation (Rana et al., 2011), may have positive effects on patients with cardiac disorders displaying sympathetic overactivation.

1.5. TARGET-DERIVED NEUROTROPHIC FACTORS FOR AUTONOMIC AND MOTOR NEURONS

As described above, some neurotrophic factors exert their trophic effects in a synergistic way. When tested as a single neurotrophic factor BDNF, NT-3, or NT-4/5 failed to rescue granule neurons in the dentate gyrus and the cerebellum, while the combination

of BDNF and NT-3 rescued the neurons from programmed cell death (Gould and Enomoto, 2009). In the periphery, administration of a combination of BDNF and GDNF has been shown to enhance motor neuron survival after brachial plexus lesion, while BDNF or GDNF alone was shown to have no effect (Aszmann et al., 2002). However, in most cases preferential utilization of a single neurotrophic factor by neuronal subpopulation has been observed. NGF exclusively has protective effects for cardiac sympathetic neurons (Levi-Montalcini et al., 1951), whereas, GDNF is known as a potent neurotrophic factor for somatic motor neurons (Bohn, 2004; Henderson et al., 1994). Heart-derived GDNF has been shown to give trophic support to parasympathetic fibers (Enomoto et al., 2002; Hasan, 2013; Rana et al.,2011) and developing sympathetic fibers (Martinelli et al., 2002; Miwa et al., 2013).

1.5.1. Nerve growth factor

NGF was the first growth factor to be identified in the neurotrophin family. Subsquently, NGF signaling and function have been studied extensively. The trophic factor was purified from the submaxillary glands of male mice and used as a survival factor for neurons in culture (Levi-Montalcini, 1951). NGF was shown to support the growth of axons of sympathetic and sensory neurons in vivo and in vitro, but had little or no effect on motor neurons (Albers et al., 1994; Glebova and Ginty, 2004; Levi Montalcini, 1951). NGF was also shown to play a significant role in the development, differentiation, survival, and maintenance of both sympathetic and sensory neurons (Levi-Montalcini, 1951, 1987). In addition to its role as a survival factor, NGF is involved in synaptic transmission. Lockhart et al. found that application of exogenous NGF in vitro produced a pronounced, reversible enhancement of synaptic strength, whereby the effect of NGF on synaptic

transmission was shown to be concentration-dependent (Lockhart et al., 1997). The broad range of effects of NGF is summarized in Table 1.

1.5.1.1. Heart-derived NGF and the sympathetic nervous system

A number of studies have shown that NGF is required for cardiac sympathetic innervation (Glebova and Gity, 2004). Studies have also shown that the levels of NGF expressed in innervated heart correspond approximately to the density of sympathetic innervation (Heumann et al., 1984; Shelton and Reichardt, 1984). Overexpression of NGF in the heart results in hyperinnervation, suggesting that NGF prevents programmed cell death in cardiac sympathetic neurons (Glebova and Ginty, 2004). The survival effect of NGF on sympathetic neurons was confirmed in NGF knockout mice. The number of sympathetic ganglion neurons was significantly reduced in mice lacking genes for NGF or for its receptor TrkA. Similar results were obtained in mice treated with NGF neutralizing antibodies (Angeletti and Levi-Montalcini, 1997; Crowley et al., 1994; Govoni et al., 2011; Snider, 1994). NGF has also been shown to enhance cardiac reinnervation of surgically denervated canine heart (Kaye et al., 1979), suggesting a strong dependence on NGF for sympathetic innervation.

1.5.1.2. Challenges with heart-derived NGF

Since its discovery, a number of studies have demonstrated the importance of NGF in the regulation of sympathetic neuronal development and innervation (Levi-Montalcini, 1987; Govoni et al., 2011). However, there are some discrepancies in the results describing changes in NGF levels in diseased heart. First, some studies have reported unusual sympathetic nerve sprouting in infarcted hearts, which was correlated with overexpression of NGF, and was responsible for increased sympathetic effects in heart

failure. However, other studies have reported the constant reduction of NGF and a decrease in sympathetic innervation in congestive heart failure (Cao et al., 2000; Chen et al., 2007; Cohn et al., 1984; Hassankhan et al., 1995; Kaye et al., 2000; Kimura et al., 2007; Oh et al., 2006). A second challenge is the regulation of NGF levels with NE administration. While several studies have reported an increase in NGF levels with NE treatment, others have shown a constant reduction in NGF concentration with NE treatment (Govoni et al., 2011; Kaye et al., 2000). Similarly, expression of NGF receptor TrkA and its mRNAs decreased in dogs treated with NE (Govoni et al., 2011; Qin et al., 2002). Third, the function of NGF synthesized in various glands, including salivary glands, are yet to be determined (Govoni et al., 2011). These challenges indicate that more studies are needed to better understand NGF regulation and function in the mature cardiac sympathetic system.

1.5.2. Glial cell-derived neurotrophic factor

Glial cell line–derived neurotrophic factor was purified from B49 glial cells and was first identified as a potent survival factor for dopaminergic neurons in the CNS (Lin et al., 1993). Later, GDNF was reported to be a trophic factor for other populations of neurons in the peripheral nervous system (PNS) including spinal motor neurons (Bohn, 2004; Henderson et al., 1994; Kannings et al., 2010), sensory neurons and autonomic neurons (Buj-Bello et al, 1995; Matrinelli et al., 2002; Enomoto et al., 2000; Hashino et al., 2001). Like other members of the GDNF family, GDNF induces its trophic effects via GPI-anchored GFR-α1. The complex of GDNF-GFR-α1-GPI activates autophosphorylation of the receptor tyrosine kinase, RET, which in turn triggers downstream signaling cascades including phosphatidylinositol 3-kinase (PI3-kinase), extracellular regulated kinase

(ERK), and mitogen-activated protein kinase (MAPK: Soler et al., 1999). In the absence of RET, neural cell adhesion molecule (N-CAM) was reported to be the alternative signal transducing receptor for GDNF- GFRα-1 complex (Paratcha et al 2003; Paratcha and Ledda, 2008). Like other members of the GDNF family, GDNF is synthesized in a precursor form, pre-pro-GDNF (Airaksinen and Saarma, 2002). The two mature forms, GDNF 633 and GDNF 555 are obtained after cleavage during secretion (Springer et al., 1995; Airaksinen and Saarma, 2002).

The important question is why we should care about GDNF? This trophic factor is important for many reasons. Specifically, GDNF is a potent survival factor for motor neurons, so it has the potential to be a therapeutic agent for neurodegenerative diseases such as Parkinson's and ALS. The roles played by GDNF in development and maintenance of the sympathetic and parasympathetic innervation of the heart could allow GDNF to be used to counteract an overstimulated cardiac sympathetic system by increasing parasympathetic effects. Moreover, GDNF has a broad range of mechanisms of action, plays a major role in synaptic plasticity and acts indirectly by interacting with serval other pre-and postsynaptic molecules to exert trophic effects. Therefore, it is clearly possible that other, currently unidentified roles for GDNF exist which could lead to more therapeutic applications.

1.5.2.1. GDNF is a potent survival factor for motor neurons.

Although GDNF was first discovered as a potent survival factor for dopaminergic neurons (Lin et al., 1993) and later as a survival factor for other neuronal populations (Buj-Bello et al., 1995), it has also been shown to be a potent survival factor for motor neurons during development and in adulthood (Bohn, 2004; also reviewed in

Vianney et al., 2013). When compared with other neurotrophic factors, GDNF was found to be more potent in preventing programmed cell death in cultured motor neurons. GDNF prevents the death of nearly 100% of motor nerves in neonatal rats that have been deprived contact with their targets. (Henderson et al., 1994; Yan et al., 1995; Houenou et al., 1996). In addition, there was a significant loss of motor neurons in GDNF knockout mice (Moore et al., 1996). It was also reported that the survival of motor neurons during programmed cell death depends on GDNF secreted by skeletal muscle (Angka et al., 2008). These results indicated that GDNF secreted by skeletal muscle is sufficient to provide a survival effect to the motor nerve innervating muscle during development; however recent studies have shown that within motor neuron pools, gamma and alpha-motor neurons are dependent on the trophic effects of GDNF (Gould and Enomoto, 2009; Kannings et al., 2010; Shneider et al., 2009). Nerve injury studies have also revealed the trophic importance of GDNF to motor neurons in adults. GDNF has been regarded as a potential therapy for spinal cord injury (Cote et al., 2011, also reviewed in Vianney et al., 2013). From this, it can be suggested that motor neurons depend on GDNF as a trophic factor and that GDNF may be useful as a potential therapeutic candidate for motor neuron diseases, including ALS, Parkinson's disease, and following spinal cord injury.

1.5.2.2. GDNF in the heart

In the heart, the preferential utilization of neurotrophic factors by sympathetic and parasympathetic systems has been suggested. While NGF has been shown to be mainly associated with sympathetic fibers (Heumann et al., 1984; Habecker et al., 2008), two of the GDNF family members, GDNF and neurturin (NRTN), were shown to be mainly associated with the parasympathetic system (Hiltunen et al., 2000; Hiltunen and

Airaksinen, 2004; Hoover et al., 2004; Mabe and Hoover, 2006, 2009). Enomoto and co-investigators found that both GDNF and NRTN are required for cranial parasympathetic ganglia development. Interestingly, the two closely related trophic factors differ in terms of the time at which they exert their trophic effects. While GDNF was found to be critical for early cellular proliferation and migration, NRTN exerts its effect later and is required for further development and maintenance of these neurons. This switch in ligands may correspond to a switch in availability of receptors. While GDNF receptor (GFRα-1) is predominant in early stages, NRTN receptor (GFRα2) dominates after ganglion formation (Enomoto et al., 2000). Interestingly, unlike NRTN which is mainly associated with parasympathetic fibers, GDNF provides the trophic effect in both sympathetic and parasympathetic fibers in the heart. GDNF was shown to be required for the survival of sympathetic neurons (Martinelli et al., 2002). It is maximally expressed during embryonic stage and is critical for sympathetic innervation of neonatal heart rats (Miwa et al., 2010). The same authors also found that GDNF provided a greater degree of support for sympathetic neurite growth and cardiac innervation than NGF (Miwa et al., 2013). These reports suggest that GDNF is also critical for the development and innervation of the heart and possibly for reinnervation after myocardial injury. A better understanding of processes regulating GDNF expression in the heart may help to understand the potential roles of GDNF in development of sympathetic and parasympathetic nervous system in healthy, diseased, or aging heart.

1.5.2.3. GDNF: a therapeutic candidate for neurodegeneration

Most current treatments for neurodegenerative diseases are focused on altering neurotransmitters expression or effects. Such treatments target increasing dopamine in

Parkinson's disease (Poewe et al., 2010), suppressing glutamate in ALS (Burvill, 2009), or blocking norepinephrine action in heart disease (Aggarwal et al., 2001; Liang et al., 2003). However, these pharmacological agents do not prevent continued neurodegeneration or neural dysfunction. Therefore, treatment with neurotrophic factors is a promising therapy for nerve restoration. In particular, GDNF is regarded as therapeutic candidate for neurodegenative motor neuron diseases. Treatments using direct neurotrophic delivery into target sites have been developed and clinical trials are underway. Methods of GDNF delivery include, but are not limited to, biodegradable microspheres (Gabayo et al., 2009), gene therapy (Fletcher et al., 2011), stem cell therapy (Pastor et al., 2012), and GDNF-Liposome complex technique (Wu et al., 2014). Although results from these studies have been promising, there are some accompanying challenges discussed in Kannings et al., (2010).

Interestingly, other treatments or mechanisms that have been utilized or suggested for neuroprotection have been shown to indirectly act through GDNF. Riluzole, currently the only drug approved for ALS treatments, was shown to increase GDNF production, even though its mechanism of action was designed to suppress the action of glutamate neurotransmitter (Burvill, 2009). The anti-dementia drug, FK960 [N-(4-acetyl-1piperazinyl)-p-fluorobenzamide monohydrate] also upregulates GDNF expression in spite of the fact that the drug was originally identified as a cholinergic agonist (Koyama et al., 2004; Matsuoka and Aigner, 1997). Furthermore, studies including those in our laboratory have shown that exercise training elevates muscle-derived GDNF protein in rat and mouse models (Cote et al., 2011; Gyorkos et al., 2014; McCullough et al., 2011; Wehrwein et al., 2002). These observations may help explain why neurotrophic factors

could be some of the best therapeutic agents for maintaining and restoring dysfunctional nerves.

1.5.2.4. GDNF potential mechanisms of Action: GDNF acts on synaptic plasticity

Administration of GDNF causes axonal growth (Wang et al., 2002) and the number of axons innervating skeletal muscle depends on the concentration of GDNF protein available (Nguyen et al., 1998). Overexpression of GDNF causes hyperinnervation in muscle fibers (Keller-Peck et al., 2001; Zwick et al., 2001), suggesting that GDNF prevents motor neurons from undergoing apoptosis. GDNF acts on both presynaptic and postsynaptic components during synaptic transmission increases both spontaneous and evoked neurotransmitter release (Ribchester et al., 1998; Wang et al., 2001). Changes in endplate morphology following exercise correlate with an increase in GDNF at the NMJ (McCullough et al., 2011; Gyorkos et al., 2014). These results suggest that GDNF likely plays a role in synaptic maintenance and remodeling at the NMJ.

1.5.2.5. GDNF potential mechanisms of action: Interaction with targets at NMJ

GDNF interacts or synergizes with other molecules at the soma, pre-synaptic, and post-synaptic levels to induce its trophic effect. In the cell body, muscle-derived GDNF increases neuregulins (NRG), an isoform of acetylcholine receptor-induced activity (ARIA). Neuregulins are expressed in motor neurons and are anterogradely transported to the NMJ where they increase the expression of acetylcholine receptors (AChRs: Loeb and Fischbach, 1997). Muscle-derived GDNF also controls cell body positioning, dendrite patterning, assembly of sensory-motor reflex circuitry, and muscle innervation, by triggering the expression of ETS (E26 transformation-specific) transcription factor Pea3 in the spinal cord (Haase et al., 2002; Vrieseling and Arber, 2006). GDNF interacts with the

neural adhesion molecule (NCAM 140 isoform) in stimulating neurite outgrowth in both, central and peripheral nervous system (Paratcha et al., 2003; Paratcha and Ledda, 2008). GDNF also mediates axon-glial interaction by inducing proliferation of Schwann cells (Allodi et al., 2012). Of all neurotrophic factors tested, only GDNF specifically enhances expression of frequenin, an N-type calcium binding protein, thereby facilitating calcium influx into the presynaptic nerve terminal (Wang et al., 2001). Unlike NT-3/4, target-derived GDNF regulates NMJ transmission by stimulating expression of large-conductance Ca^{2+} activated K^{+} channels (KCa^{2+}) in developing chick lumbar motoneurons (Martin-Caraballo and Dryer, 2002). In the postsynaptic cell, GDNF was shown to increase the size of AChR aggregates (Wang et al., 2002) and cause insertion of ACh receptors (Yang and Nelson, 2004). Taken together, it can be suggested that GDNF promotes axon branching and innervation, enhances synaptic formation, maintains synaptic connection, and modulates mammalian NMJ directly and indirectly through interactions with molecules of both presynaptic and postsynaptic structures.

1.5.2.6. GDNF potential mechanisms of GDNF action: Synthesis and transport modes

GDNF is widely distributed in various tissues of the central and peripheral nervous system, suggesting the importance of its neuroprotective trophic effect. Surprisingly, the synthesized and released GDNF is transported in different ways. Table 1 briefly summarizes the cells and tissues that have been confirmed to express GDNF. In addition to being secreted in various tissues within and outside the nervous system, GDNF reaches its target in variety of ways, including retrograde and anterograde transport and paracrine or autocrine modes of action on tissues. GDNF was shown to be transported via axons to cell bodies through a receptor-mediated process, in a retrograde fashion (Nguyen

et al., 1998; Rind et al., 2005; Vrieseling and Arber, 2006). Zhao et al. showed that GDNF is secreted by astrocytes of the CNS and provides its effects on peripheral nervous system by anterograde transport (Zhao et al., 2004). In the paracrine mode of action, muscle-derived GDNF induces the Schwann cell proliferation at the NMJ (Allodi et al., 2012). GDNF increases AChR density in the absence of innervation, indicating an autocrine action, since both the ligand and its receptor are expressed in skeletal muscle (Yang and Nelson, 2004; Vianney and Spitsbergen unpublished data). Results of a study conducted by Li et al. (2007), using both myoGDNF mice and GFAP-GDNF mice, showed that the classical retrograde transport may have more physiological effects for treatment of amyotropic lateral sclerosis (ALS) compared to anterograde transport. Due to the widespread expression in different regions of the body and the various modes of action, GDNF has been shown to be a very important molecule that performs multiple functions to support cellular function and survival.

1.6. AIM OF STUDY

The broad goal of this study is to examine factors that normally regulate GDNF expression in skeletal muscle and cardiac muscle, with an emphasis on comparing the similarities and differences in these voluntary and involuntary muscles. Previous studies with nerve-muscle co-culture in our laboratory have shown that GDNF protein levels are reduced when skeletal muscle is in contact with cholinergic nerves (Vianney and Spitsbergen, 2011). The hypothesis being tested is that cellular activation by neural cells, via neurotransmitter release, regulates GDNF expression in voluntary and involuntary muscles. Although the survival effect of exogenous GDNF on neurons has been extensively

investigated (Henderson et al., 1994; Keller-Peck et al., 2001; Angka et al., 2008; Dudanova et al., 2010), little is known concerning the normal regulation of expression in muscle-derived GDNF. Understanding the normal factors that regulate GDNF expression in voluntary and involuntary muscles will provide a better understanding of how these processes may be modulated therapeutically.

1.7. REFERENCES

Aggarwal, A., Esler, M.D., Socratous, F., Kaye, M.D., 2001. Evidence for functional presynaptic alpha-2 adrenoceptors and their down-regulation in human hear. Cardiol. 37, 1246–1251.

Airaksinen, M. S., Saarma, M., 2002. The GDNF family: signalling, biological functions and therapeutic value. Nat. Rev. Neurosci. 3, 383–394.

Albers, K.M., Wright, D. E., Davis, B. M., 1994. Overexpression of nerve growth factor epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. J. Neurosci. 14, 1422–1432.

Allen, S. J., Dawbarn D., 2006. Clinical relevance of the neurotrophins and their receptors. Clin. Sci. (Lond.) 110, 175–191.

Allodi, I., Udina, E., Navarro, X., 2012. Specificity of peripheral nerve regeneration: interactions at the axon level. Prog. Neurobiol. 98, 16–37.

Angeletti, P.U., Levi-montalcini, R., 1997. Growth regulation of the sympathetic nervous system: immunosympathectomy and chemical sympathectomy. The saga of the nerve growth factor: pp. 244–252. doi: 10.1142/9789812830319_0017

Angka, H.E., Geddes, A.J., Kablar, B., 2008. Differential survival response of neurons to exogenous GDNF depends on the presence of skeletal muscle. Dev. Dyn. 237, 3169–3178.

Aszmann, O.C., Korak, K.J., Kropt, N., Fine, E., Aevescher, P., Frey, M., 2002. Simultaneous GDNF and BDNF application leads to increased motoneuron survival and improved functional outcome in an experimental model for obstetric brachial plexus lesions. Exp. 110:1066–1072.

Barber, S.C., Mead, R.J., Shaw, P.J., 2006. Oxidative stress in ALS: A mechanism of neurodegeneration and a therapeutic target. Biochemica et Biophysica Acta 1762, 1051–1067.

Brand-Saberi, B., Wilting, J., Ebensperger, C., Christ, B., 1996. The formation of somite compartments in the avian embryo. Int. J. Dev. Biol. 40, 411–420.

Bibevski, S., Dunlap M.E., 2004. Prevention of diminished parasympathetic control of the heart in experimental heart failure. Am. J. Physiol. Heart Circ. Physiol. 287, H1780–H11785.

Bohn, M.C., 2004. Motoneurons crave glial cell line-derived neurotrophic factor. Exp. Neurol. 190, 263–275.

Bruijin, L. I., Miller, T.M., Cleveland, D.W., 2004. Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annu. Rev. Neurosci. 27, 723-49.

Buj-Bello, A., Buchman, V.L., Horton, A., Rosenthal, A., Davies, A. M., 1995. GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron. 15, 821–828.

Burden, S.J., 1998. The formation of neuromuscular synapses. Gene Dev. 12, 133–148.

Burvill, E., 2009. Focus on: Glutamate neuropharmacology. The current understanding of motor neuron disease. Curr. Anaesthesia & Critical Care 20, 236–239.

Cao, J-M., Chen, L.S., KenKnight, B.H., Ohara, T., Lee, M-H., Tsai, J. Lai, W.W., Karagueuzian, H.S., Wolf, P.L., Fishbein, M.C., Chen, P-S., 2000. Nerve sprouting and sudden cardiac death. Circ. Res. 86, 816–821.

Chen, L.S., Zhou, S., Fishbein, M.C., Chen, P-S., 2007. New perspectives on the role of autonomic nervous system in the genesis of arrhythmias. J. Cardiovasc. Electrophysiol. 18, 123–127.

Chidsey, C.A., Kaiser, G.A., Sonnenblick, E.H., Spann, J.F., Braunwald, E., 1963. Cardiac norepinephrine stores in experimental heart. J. Clin. Invest. 43, 2386–2393.

Cohn, J.N., Levine, T.B., Olivari, M.T., Garberg, V., Lura, D., Francis, G.S., Simon, A.B., Rector, T., 1984. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N. Engl. J. Med. 311, 819–23.

Cote, M.P., Azzam, G.A., Lemay, M.A., Zhukareva,V., Houle´, J.D., 2011. Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury. J.Neurotraum.28, 299–309.

Crick, S.J., Sheppard, M.N., Ho, S.Y., Anderson, R.H., 1999. Localisation and quantitation of autonomic innervation in the porcine heart I: conduction system. J. Anat. 195, 341–357.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K. S., Pitts-Meek, S., Armanini, M, P., 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76, 1001–11.

Dadon-Nachum, M., Melamed, E., Offen, D., 2011. The “dying-back” phenomenon of motor neurons in ALS. J. Mol. Neurosci. 43, 470–477.

da Silva, S., Wang, F., 2011. Retrograde neural circuit specification by target-derived neurotrophins and growth factors. Curr. Opinion in Neurobiol. 21, 61– 67.

Dudanova, I., Gatto, G., Klein, R., 2010. GDNF acts as a chemoattractant to supportephrin A-induced repulsion of limb motor axons. Curr. Biol. 20, 2150–2156.

Dunlap, M.E., Bibevski, S., Rosenberry, T.L., Ernsberger, P., 2003. Mechanisms of altered vagal control in heart failure: influence of muscarinic receptors and acetylcholinesterase activity. Am. J. Physiol. Heart Circ. Physiol. 285, H1632–H1640.

Dunning, C.J.R., Reyes, J.F., Steiner, J.A., Brundin, P., 2012. Can Parkinson's disease pathology be propagated from one neuron to another? Prog. Neurolobiol. 97, 205–219.

Eckberg, D. L., Drabinsky, M., Braunwald, E., 1971. Defective cardiac parasympathetic control in patients with heart disease. N. Engl. J. Med. 285, 877–883.

Enomoto, H., Heuckeroth, R.O., Golden, J. P., Johnson, E.M., Milbrandt, J., 2000. Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. Dev. 127, 4877–4889.

Esler, M., 2011. The sympathetic nervous system through the ages: from Thomas Willis to resistant hypertension. Exp. Physiol. 00.00 pp 1–12.

Fischer, L.R., Culver, D.G., Tennant, P., Davis, A.A., Wang, M., Castellano-Sanchez, A., Khan, J., Polak, M.A., Glass, J.D., 2004. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. Ex. Neurol. 185, 232–240.

Fletcher, A.M., Kowalczyk, T.H., Padegimas, L., Cooper, M.J., Yurek, D.M., 2011. Transgene expression in the striatum following intracerebral injections of DNA nanoparticles encoding for human glial cell line-derived neurotrophic factor (hGDNF). Neurosci. 194, 220–226.

Fu, S.Y., Gordon, T., 1997. The cellular and molecular basis of peripheral nerve regeneration. Mol. Neurobiol. 14, 67–116.

Funakoshi, H., Frisen, J., Barbany, G., Timmusk, T., Zachrisson, O., Verge, V.M.K., Persson H., 1993. Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J. Cell Biol. 123, 455–465.

Gabayo, E., Montero-Menei, C.N., Ansorena, E., Lanciego, J.L., Aymerich, M.S., Blanco-Prieto, M.J., 2009. Effective GDNF brain delivery using microspheres – A promising strategy for Parkinson's disease. J controlled release 135, 119–126.

Gammill, L.S., Conzalez, C., Gu, C., Bronner-Fraser, M., 2005. Guidance of trunk neural crest migration requires neuropilin 2/semaphoring 3F signaling. Dev. 133, 99–106.

Glebova, N.O., Ginty, D.D., 2004. Heterogeneous reguirement of NGF for sympathetic target innervation in vivo. J. Neurosci. 24, 743–751.

Gordon, T., 2009. The role of neurotrophic factors in nerve regeneration. Neurosurg. Focus 26, 1–9.

Gould, T.W., Enomoto, H., 2009. Neurotrophic modulation of motor neuron development. Neuroscientist 15, 105–116.

Govoni, G., Pascalea, A., Amadioa, M., Calvillo, L., D'Elia, E., Ceredad, C., Fantuccie, P., Ceronif, M., Vanolih, E., 2011. NGF and heart: Is there a role in heart disease? Pharmacol. Res. 63, 266–277.

Gyorkos, A.M., McCullough, M.J., Spitsbergen, J.M., 2014. Glial cell line-derived neurotrophic factor (GDNF) expression and nmj plasticity in skeletal muscle following endurance exercise. Neurosci. 257, 111–118.

Haase, G., Dessaud, E., Garces, A., Bovis, B., Girling, M-C., Fillipi, P., Schmalbruch, H., Arber, S., de Lapeyriere, O., 2002. GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. Neuron 35, 893–905.

Habecker, B.A., Bilimoria, P., Linick, C., Gritman, K., Lorentz, C.U., Woodward, W., Birren, S.J., 2008. Regulation of cardiac innervation and function via the p75 neurotrophin receptor. Auton. Neurosci 140, 40–48.

Hase, A., Suzuki, H., Arahata, K., Akazawa, C., 1999. Expression of human GDNFα-1 (GDNF receptor) at the neuromuscular junction and myelinated nerves. Neurosci. Lett. 269, 55–57.

Hasan, W., 2013. Autonomic cardiac innervation. Development and adult plasticity. Organogenesis 9, 176–193.

Hashino, E., Shero, M., Junghans, D., Rohrer, H., Milbrandt, J., Johnson E.M., 2001. GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development. Dev. 128, 3773–3782.

Hassankhan, A., Steinhelper, M.K., Snoopaa, M.H., Katz, E.B., Taylor, D.A., Andrade-Rosental, A., Factor, S.M., Sterinberg, J.J., Field, L.J., Federoff, H.J., 1995. Overexpression of NGF within the heart of transgenic mice causes hyperinnervation, cardiac enlargement, and hyperplasia of ectopic cells. Dev. Biol. 169, 309–321.

Henderson, C.E., Phillips, H.S., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M., Simpson, L.C., Moffet, B., Vandlen, R.A., Koliatsos, V.E., Rosenthal, A.,

1994. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266, 1062–1064.

Henderson, C.E., 1996. Role of neurotrophic factors in neuronal development. Curr. Opin. Neurobiol. 6, 64–70.

Heumann, R., Korsching, S., Scott, J., Thoenen, H., 1994. Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. Embo Journal. 3, 3183–3189.

Hildreth, V., Anderson, R.H., Henderson, D.J., 2009. Autonomic innervation of the developing heart. Clinical Anat. 22, 36–46.

Hiltunen, J.O., Laurikainen, A., Airaksinen, M.S., Saarma, M., 2000. GDNF family receptors in the embryonic and postnatal rat heart and reduced cholinergic innervation in mice hearts lacking ret or GFRα2. Dev. Dynamics 219, 28–39.

Hiltunen, P.H., Airaksinen, M.S., 2004. Sympathetic cholinergic target innervation requires GDNF family receptor GFRα2. Mol. Cell Neurosci. 26, 450-457.

Himura, Y., Felten, S.Y., Kashiki, M., Lewandowski, T.J., Delehanty, J.M., Liang, C.S., 1993. Cardiac noradrenergic nerve terminal abnormalities in dogs with experimental congestive heart failure. Circ. 88, 1299–1309.

Hoover, D.B., Ganote, C.E., Ferguson, S.M., Blakely, R.D., Parsons, R.L., 2004. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc. Res. 62, 112–121.

Houenou, L.J., Oppenheim, R.W., Li, L., Lo, A.C., Prevette, D., 1996. Regulation of spinal motoneuron survival by GDNF during development and following injury. Cell Tissue Res. 286, 219–223.

Kanning, K. C., Kaplan, A., Henderson, C. E. 2010. Motor neuron diversity in development and disease. Annu. Rev. Neurosci, 33, 409–440.

Kawano, H. Okada, R., Yano, K., S., 2003. Histological study on the distribution of autonomic nerves in the human heart. Heart Vessels 18, 32–39.

Kaye, M.P., Wells, D.J., Tyce, G.M., 1979. Nerve growth factor – enhance reinnervation of surgically denervated canine heart. Am. J. Physiol. 236, H624–H628.

Kaye, D.M., Viddadi, G., Gruskin, S.L., Du, X-J., Esler M.D., 2000. Reduced myocardial nerve growth factor expression in human and experimental heart failure. Circ. Res. 86, e80–e84.

Keller-Peck, C.R., Feng, G., Sanes, J.R., Yan, Q., Lichtman, J.W., Snider, W.D., 2001. Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. J. Neurosci. 21, 6136–6146.

Kimura, K., Ieda, M., Knazawa, H., Yagi, CT., Tsunoda, M, Ninomiya, S., Kurosawa, H., Yoshimi, K., Mochizuki, H., Yamazaki, K., Ogawa, S., Fukuda, K., 2007. Cardiac sympathetic rejuvenation: a link between nerve function and cardiac hypertrophy. Circ. Res. 100, 1755–64.

Kimura, K., Kanazawa, H., Ieda, M., Manabe-Kawaguchi, H., Miyake, Y., Yagi, T., Arai, T., Sano, M., Fukuda, K., 2010. Norepinephrine-induced nerve growth factor depletion causes cardiac sympathetic denervation in severe heart failure. Autonomic Neurosci. : Basic and Clinical 156, 27-35.

Kimura, K., Ieda, M., Fukuda, K., 2012. Development, maturation, and transdifferentiation of cardiac sympathetic nerves. Circ. Res. 110, 325–336.

Kingham, P.J., Terenghi, G., 2006. Bioengineered nerve regeneration and muscle reinnervation. J. Anat. 209, 511-526.

Kinugawa, T., Dibner-Dunlap M.E., 1995. Altered vagal and sympathetic control of heart rate in left bentricular dysfunction and heart failure. Am. J. Physiol. 268, R310–R316.

Koyama, Y., Egawa, H., Osakada, M., Baba, A., Matsuda, T., 2004. Increase by FK960, a novel cognitive enhancer, in glial cell line-derived neurotrophic factor production in culture rat astrocytes. Biochem. Pharmacol. 68: 275-282.

Krakora, D., Macrander, C., Suzuki, M., 2012. Neuromuscular junction protection for the potential treatment of amyotrophic lateral sclerosis. Neurol. Res. Intl. doi:10.1155/2012/379657.

Lara, A., Damasceno, D.D., Pires, R., Gros, R., Gomes, E.R., Gavioli, M., Lima, R.F., Guimaraes, D., Lima, R., Bueno, C.R., Vasconcelos, A., Roman-Campos, D., Menezes, C.A.S., Sirvente, R.A., Salemi, V.M., Mady, C., Caron, M.G., Ferreira, A.J., Brum, P.C., Resende, R.R., Cruz, J.S., Gomez, M.V., Prado, V.F., de Almeida A. P., Prodo, M.A.M., Guatimosim, S., 2010. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol. Cellular Biol. 30, 1746–1756.

Levi-Montalcini, R., 1987. The nerve growth: thirty-five years later. The EMBO J. 6, 1154-1154.

Li, W., Brakefield, D., Pan, Y. C., Hunter, D, Myckatyn T M, Parsadanian, A., 2007. Muscle-derived but not centrally derived transgene GDNF is neuroprotective in G93A-SOD1 mouse model of ALS. Exp. Neurol. 203, 457–471.

Liang, C-S., Yatani, A., Himura, Y., Kashiki, M., Stevens, S.Y., 2003. Desipramine attenuates loss of cardiac sympathetic neurotransmitters produced by congestive heart failure and ne infusion. Am. J. Physiol. Heart Circ. Physiol. 284, H1729 – 1736.

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

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

Lockhart, S.T., Turrigiano, G.G., Birren, S.J. 1997. Nerve growth factor modulates synaptic transmission between sympathetic and cardiac myocytes. J. Neurosci. 17, 9573–9582.

Loeb, J.A., Fischbach, G.D., 1997. Neurotrophic factors increase neuregulin expression in embryonic ventral spinal cord neurons. J. Neurosci. 17, 1416–1424.

Lu, B., 2003. BDNF and activity-dependent synaptic modulation. Learn. Mem. 10, 86–98.

Mabe, A.M., Hoard, J. L., Doffourc, M.M., Hoover, D.B., 2006. Localization of cholinergic innervation and neurturin knockout mice. Cell Tissue Res. 326, 57–67.

Mabe, A.M., Hoover, D.B., 2009. Structural and functional cardiac cholinergic deficits in adult neurturin knockout mice. Cardiovasc. Res. 82, 93–99.

Martin-Caraballo, M., Dryer, S.E., 2002. Glial cell line-derived neurotrophic factor and target-dependent regulation of large-conductance Kca2+ channels in developing chick lumbar motoneurons. J. Neurosci. 22, 10201–10208.

Martinelli, P.M., Camargos, E.R.S., Morel, G., Ravares, C.A.P., Nagib, P.R.A., Machado, C.R.S., 2002. Rat heart GDNF: effect of chemical sympathectomy. Histochem. Cell Biol. 118, 337–343.

Matsuoka, N., Aigner, T., 1997. FK960 [N-(4-Acetyl-1-piperazinyl)-p-fluorobenzamide Monohydrate], a Novel Potential Antidementia Drug, Improves Visual Recognition Memory in Rhesus Monkeys: Comparison with Physostigmine. J. Pharmacol. Exp. Therapeutics 280, 1201–1209.

Matsushita, Y., Nakajima, K., Tohyama, Y., Kurihara, T., 2008. Activation of Microglia by endotoxin suppresses the secretion of glial cell line-derived neurotrophic factor (GDNF) through the action of protein kinase Cα (PKCα) and mitogen-activated protein kinases (MAPKs). J. Neurosci. Res. 86: 1959–1971.

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

Meier, T., Wallace, B.G., 1998. Formation of the neuromuscular junction: molecules and mechanisms. BioEssays 20, 819–829.

Meredith, I.T., Esler, M.D., Cox, H.S., Lambert, G.W., Jennings, G.L., Eisenhofer, G., 1991. Biochemical evidence of sympathetic denervation of the heart in pure autonomic failure. Clin Auton Res. 1, 187-94.

Michalski, B., Bain., J. R., Fahnestock., M., 2008. Long-term changes in neurotrophic factor expression in distal nerve stump following denervation and reinnervation with motor or sensory nerve. J. Neurochem, 105, 1244–1252.

Mitchell, G.A.G., 1953. The innervation of the heart. Br Heart J. 15, 159–171.

Miwa, K., Lee, J-K., Takagishi, Y., Opthof, T., Fu, Kodama, I., 2010. Glial cell line-derived neurotrophic factor (GDNF) enhances sympathetic neurite growth in rat hearts at early developmental stages. Biomedical Res. 31, 353–361.

Miwa, K., Lee, J-K., Takagishi, Y., Opthof, T., Fu, X., Hirabayashi, M., Watabe, K., Jimbo, Y., Kodama, I., Komuro I., 2013. Axon guidance of sympathetic neurons to cardimyocytes by glial cell line-derived neurotrophic factor (GDNF). PLoS ONE 8: e65202.

Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L.F., Ryan, A.M., Carver-Moore, K., Rosenthal, A., 1996. Renal and neuronal abnormalities in mice lacking GDNF. Nature 382, 789–792.

Mufson, E.J.I., Kroin, J.S., Sendera, T.J., Sobreviela, T., 1999. Distribution and retrograde transport of trophic factors in the central nervous system: functional implications for the treatment of neurodegenerative disease. Prog. Neurobiol. 57, 451–84.

Nagano, M., Suzuki, H., 2003. Quantitative analyses of expression of GDNF and neurotrophins during postnatal development in rat skeletal muscles. Neurosci. Res. 45, 391–399

Nguyen, Q.T., Parsadanian, A.S., Snider, W.D., Lichtman, J.W., 1998. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science 279, 1725–1728.

Nolan, J., Flapan, A.D., Capewell, S., MacDonald, T.M., Neilson, J.M.M., Ewing, D.J., 1992. Br. Heart J. 67, 482–5.

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

Oh, Y.S., Jong, A.Y., Kim, D.T., Li, H., Wang, C., Zemljic-Harpf, A., Ross, R. S. Fishbein, M.C., Chen, P.S., Chen, L.S., et al., 2006. Spatial distribution of nerve sprouting after myocardial infarction in mice. Heart Rhythm 6, 728–736.

Olshansky, B., Sabbah, H.N., Hauptman, P.J., Colucci, W.S., 2008. Parasympathetic nervous system and heart failure: Pathophysiology and potential implications for therapy. Circ. 118, 863–871.

Oppenheim, R., Houenou, L.J., Parsadanian, A.S., Prevette, D., Snider, W.D., Shen, L., 2000. Glial cell line-derived neurotrophic factor and developing mammalian

motoneurons: regulation of programmed cell death among motoneuron subtypes. J. Neurosci. 20, 5001–5011.

Orrell, R.W., 2010. Motor neuron disease: systematic reviews of treatment for ALS and SMA. British Medical Bulletin 93, 145–159.

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

Paratcha, G., Ledda, F., 2008. GDNF and GFRalpha: a versatile molecular complex for developing neurons. Cell 31384–391.

Parkhouse, W.S., Cunningham, L., Mcfee, I., Miller, J.M., Whitney, D., Pelech, S.L., Krieger, C., 2008. Neuromuscular dysfunction in the mutant superoxide dismutase mouse model of amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. 9, 24–34.

Pastor, D., Viso-Leon, M.C., Jones, J., Jaramillo-Merchan, J., Toledo-Aral., J. J., Moraled, J. M., Martinez, S. 2011. Comparative effects between bone marrow and mesenchymal stem cell transplantation in GDNF expression and motor function recovery in a motorneuron degenerative mouse model. Sterm Cell Rec. and Rep. 8, 445–458.

Pauza, S.H., Skripka, V., Pauziene, N., Stropus, R., 2000. Morphology, distribution, and variability of the epicardiac neural ganglionated subplexuses in the human heart. Anat. Rec. 259, 353–382.

Pichel, J.G., Shen, L., Sheng, H.Z., Granholm, A-C., Drago, J., Grinberg, A., Lee, E.J., Huang, S.P., Saarma, M., Hoffer, B.J., Sariola, H., Westphal, H., 1996. Defects in enteric innervations and kidney development in mice lacking GDNF. Nature 382, 73–76.

Poewe, W., Antonini, A., Zijlmans., J.C.M., Burkhard, P.R., Vingerhoets, F., 2010. Levodopa in the treatment of Parkinson's disease: an old drug still going strong. Clinical Interventions in Aging 5, 229–238.

Qin, F., Vulapalli R.S., Stevens, S.Y., Ling, C-S., 2002. Loss of cardiac sympathetic neurotransmitters in heart failure and NE infusion is associated with reduced NGF. Am. J. Physiol. Heart Circ. Physiol. 282, H363–H371.

Rana, O.R., Schauerte, P., Kluttig, R., Schröder, J.W., Koenen R.R.,Weber, C., Nolte, K. W., Weis, J., Hoffmann, R., Marx, N., Saygili, E. 2010. Acetylcholine as an age-dependent non-neuronal source in the heart. Autonomic Neurosci: Basic and Clinical. 156, 82–89.

Rana, O.R., Saygili, E., Gemein, C., Zink M. D. H., Buhr, A., Saygili, E., Mischke K., Nolte, K. W., Weis, J., Weber, C. Marx, N., Schauerte, P., 2011. Chronic electrical neuronal stimulation increases cardiac parasympathetic tone by eliciting neurotrophic effects. Circ. Res. 108, 1209–1219.

Ribchester, R. R., Thomson, D., Haddow, L. J., Ushkaryov, Y. A., 1998. Enhancement of spontaneous transmitter release at neonatal mouse neuromuscular junctions by the glial cell line-derived neurotrophic factor (GDNF). J. Physiol. 512, 635–641.

Rind, H.B., Butowt, R., von Bartheld, C.S., 2005. Synaptic targeting of retrogradely transported trophic factors in motoneurons: comparison of glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, and cardiotrophin-1 with tetanus toxin. J. Neurosci 25, 539–549.

Roy, A., Fields, W.C., Roche-Resende, C., Resende, R. R., Guatimosim, S., Prado, V.F., Gros, R., Prado, M.A., 2013. Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. FASEB J. 27, 5072–82.

Saarma, M., 2000. GDNF – a stranger in the TGF-β superfamily? Eur. J. Biochem. 267, 6968–6971.

San Mauro, M.P., Patronelli, F., Spinelli, E., Cordero, A.,Covello, D., Gorostiaga, J.A., 2009. Nerves of the heart: a comprehensive review with a clinical point of view. Eissn 1303–1775. Pissn 1303–1783.

Sariola, H., Saarma, H., 2003. Novel functions and signaling pathways for GDNF. J. Cell Science 116, 3855–3862.

Schäper, C.1., Gläser, S., Groneberg, D.A., Kunkel, G., Ewert, R., Noga, O., 2009. Nerve growth factor synthesis in human vascular smooth muscle cells and its regulation by dexamethasome. Regul. Pept. 157, 3–7.

Serbedzija, G.N., Fraser, S.E., Bronner-Fraser, M., 1990. Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. Dev. 108, 605–612.

Shaw, P.J., 2005. Molecular and cellular pathways of neurodegeneration in motor neurone disease. J. Neurol. Neurosurg. Psychiatry 76, 1046–1057.

Shelton, D.L., Reichardt, L.F., 1984. Expression of the beta-nerve growth factor gene correlates with the density of sumpathetic innervation in effector organs. Proc. Natl. Acad. Sci. USA 80, 7951–5.

Sherwood, L., 2010. Human physiology: From cells to systems, seventh ed. Cengage Learning.

Shneider, N. A., Brown, M. N., Smith, C. A., Pickel, J., Alvarez, F. J., 2009. Gamma motor neurons express distinct genetic markers at birth and require muscle spindle-derived GDNF for postnatal survival. Neural Dev. 4, 42.

Snider, W.D., 1994. Functions of the neurotrophins during nervous system development: What knockouts are teaching us. Cell 77, 627–638.

Soler, R. M., Dolcet, X., Encinas, M., Egea, J., Bayascas, J. R., Comella, J. X., 1999. Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. J. Neurosci. 19, 9160–9169.

Springer, J.E., Seeburger, J.L., He, J., Gabrea, A., Blankenhorn, E.P., 1995. cDNA sequence and differential mRNA regulation of two forms of glial and rat skeletal muscle. Exp. Neurol. 131, 47–52.

Suzuki, H., Hase, A., Kim, B.Y., Miyata, Y., Ikuya, N., Arahata, K., Akazawa, C., 1998. Up regulation of glial line-derived neurotrophic factor (GDNF) expression in regeneration muscle fibers in neuromuscular diseases. Neurosci. Lett. 257, 165–67

Trupp, M., Rydén, M., Jörnvall, H., Funakoshi, H., Timmusk, T., Arenas, E., Ibáñez, C. F., 1995. Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J. Cell Biol. 130, 137–148.

Vaseghi, M., Shivkumar, K., 2008. The role of the autonomic nervous system in sudden cardiac death. Prog. Cardiovasc. Dis. 50, 404–419.

Vekrellis, K., Xilouri, M., Emmanoulidou, E., Rideout, H.J., Stefanis, L., 2011. Pathological roles of α-synuclein in neurological disorders. Lancet Neurol. 10, 1015–25.

Vianney, J.-M., Spitsbergen, J.M., 2011. Cholinergic neurons regulate secretion of glial cell line- derived neurotrophic factor by skeletal muscle cells in culture. Brain Res. 1390, 1–9.

Vianney, J-M., McCullough, M.J., Gyorkos, A.M., Spitsbergen, J.M., 2013. Exercise-dependent regulation of glial cell line-derived neurotrophic factor (GDNF) expression in skeletal muscle and its importance for the neuromuscular system. Front. Biol. 8, 101–108.

Vrieseling, E., Arber, S., 2006. Target-induced transcriptional control of dendritic patterning and connectivity in motor neurons by the ETS gene Pea3. Cell 127, 1439–52.

Wang, C.-Y., Yang, F., He, X.-P., Je, H.-S., Zhou, J.-Z., Eckermann, K., Kawamura, D., Feng, L., Shen, L., Lu, B., 2002. Regulation of neuromuscular synapse development by glial cell line-derived neurotrophic factor and neurturin. J. Biol. Chem. 277, 10614–10625.

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

Wijesekera, L.C., Leigh, P.N., 2009. Amyotrophic lateral sclerosis. Orphanet J. Rare Disease 4, 3.

Wu, A., Ying, Z., Gomez-Pinilla, F., 2008. Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. Neurosci. 155, 751–759

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

Yang, L.-X., Nelson, P.G., 2004. Glia cell line-derived neurotrophic factor regulates the distribution of acetylcholine receptors in mouse primary skeletal muscle cells. Neurosci. 128, 497–509.

Yutzey, K.E., Kirby, M.L., 2002. Wherefore heart thou? Embryonic origins of cardigenic mesoderm. Dev. Dyn. 223–307.

Zhang, L., Ma, Z., Smith, G.M., Wen, X., Pressman, Y., Wood, P.M., Xu, X.M., 2009. GDNF-enhanced axonal regeneration and myelination following spinal cord injury is mediated by primary effects on neurons. Glia 57, 1178–1191.

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

Zhao, Z. Q., Alam, S., Oppenheim, R. W., Prevette, D. M., Evenson, A., Parsadanian, A., 2004. Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. Exp. Neurol. 190, 356–372.

Zwick, M., Teng, L., Mu, X., Springer, J.E., Davis, B.M., 2001. Overexpression of GDNF induces and maintains hyperinnervation of muscle fibers and multiple end-plate formation. Exp. Neurol. 171, 342–350.

CHAPTER 2

EXPERIMENTAL DESIGN, MATERIALS, AND METHODS

A: EXPERIMENTAL DESIGN - RATIONALE AND DEVELOPMENT

2. 1. SPECIFIC AIMS

The broad goal of this study was to examine factors that normally regulate glial cell line-derived neurotrophic factor (GDNF) expression in skeletal and cardiac muscles, with an emphasis on comparing the similarities and differences in these voluntary and involuntary muscles in relation to GDNF production. The hypothesis tested was that cellular activation by neural cells, via neurotransmitter release, regulates GDNF expression in voluntary and involuntary muscles. This hypothesis was addressed in the following specific aims:

2.1.1. Specific Aim #1

The regulation of expression of GDNF is different in voluntary and involuntary muscles: Studies under this aim characterized GDNF synthesis and secretion in skeletal muscle and cardiac muscle and examined the similarities and differences in production of GDNF in these voluntary and involuntary muscles. GDNF protein content in the muscle was analyzed by enzyme-linked immunosorbant assay (ELISA), immunocytochemical techniques, and western blot. Since nerve growth factor (NGF) is a major trophic factor for the autonomic nervous system, the same procedure was performed with NGF to compare its synthesis and secretion with that of GDNF in cardiac muscles. Results from these studies

confirmed the presence and examined the patterns of production of the growth factors at the cellular level.

2.1.2. Specific Aim # 2

Neurotransmitters found in somatic motor neurons, sympathetic neurons, or parasympathetic neurons are involved in regulation of GDNF production by skeletal and cardiac muscle: Studies under this aim were focused on understanding the cellular processes controlling GDNF production at the level of the cell. The hypothesis tested was that neural cells innervating muscle regulate GDNF expression via neurotransmitter release. The study examined the presence, distribution, and localization of neurotransmitter receptors on differentiated skeletal muscle using immunocytochemical methods. Specificity of effects of a neurotransmitter on GDNF secretion was examined by studying effects of specific blocking agents. Results from this study determined that neurotransmitter-receptor interactions are involved in regulating GDNF production by muscle cells.

2.1.3. Specific Aim #3

Direct electrical stimulation affects GDNF expression in skeletal muscle and cardiac muscle: Studies under this aim examined the effect of direct electrical stimulation on GDNF secretion by skeletal muscle and cardiac muscle. The hypothesis was that there is a relationship between muscle contraction and GDNF production in both voluntary and involuntary muscles. Various frequencies and durations of stimulation were examined to determine the optimal stimulation pattern for eliciting changes in GDNF levels. The study

also aimed to give insight into potential benefits of the use of electrical stimulation on motor and autonomic nervous systems in regards to enhancing the trophic effect of GDNF.

2.2. INNOVATIONS

2.2.1. Idea/Hypothesis

This study examines whether neural cells regulate their own supply of GDNF produced by voluntary and involuntary muscle cells. As mentioned earlier, there are a number of studies examining the effects of exogenous GDNF on neurons and target tissues (Henderson et al., 1994; Houenou et al., 1996; Nguyen et al., 1998; Keller-Peck et al., 2001; Wang et al., 2002; Angka et al., 2008; Dudanova et al., 2010); however, to our knowledge there are no studies examining and comparing how GDNF production is normally regulated at the cellular level in skeletal and cardiac muscle. Thus, we believe that the results from these studies are the first to report that GDNF production is differentially regulated in voluntary and involuntary muscles and the nervous system may be involved in the regulation process.

2.2.2. Materials

This research involves the use of a novel cell line (HL-1) derived from mouse atrial cells. HL-1 cells retain ultrastructural characteristics of adult atrial cardiac cells, including the ability to contract spontaneously in culture (Claycomb, 1998). To our knowledge this study is the first to evaluate GDNF production in HL-1 cells. Thus, results from this study suggest that HL-1 cells are a viable model for studying the synthesis and secretion of GDNF in the heart.

2.2.3. Methods

These studies use an electrical stimulation apparatus that maintains a controlled environment for cultured cells; this includes placing cells in a standard cell incubator for long-term stimulation. This method diverges from Marotta et al., (2004) by utilizing a custom-made interface box instead of using two cards that are placed adjacent to the short axis of a 6-well plate. Within the interface box a unity gain voltage buffer maintained the shape of the voltage pulse while providing current to up to six electrodes. Also, stainless steel electrodes are used instead of platinum electrodes.

2.3. PRELIMINARY STUDIES

The following section describes the preliminary studies that were performed using these cell lines. Material and methods involved in the preliminary studies are briefly explained as the full description of methods is found in the material and methods section.

2.3.1. Do C2C12 skeletal muscle cells or HL-1 cardiac muscle cells express GDNF?

Previous studies have shown target tissues of the peripheral nervous system, including skeletal and cardiac muscle, express GDNF (Suzuki et al., 1998; Martinelli et al., 2002). However, the regulation of GDNF production in these various tissues is still to be determined. To address the question whether these voluntary and involuntary cell lines (C2C12 and HL-1, respectively), express GDNF, cells were grown according to the protocol for each cell type. Localization of GDNF and myosin proteins were examined by immunocytochemical techniques. GDNF content in cell and levels of secretion were evaluated using ELISA. Results from these preliminary studies served as part of specific

aim one. Figure 2.1 shows that GDNF could be detected in both types of cells. Unlike skeletal myotube, GDNF in cardiac muscle cells seems to be concentrated in the nucleus.

GDNF in skeletal and cardiac muscles cells

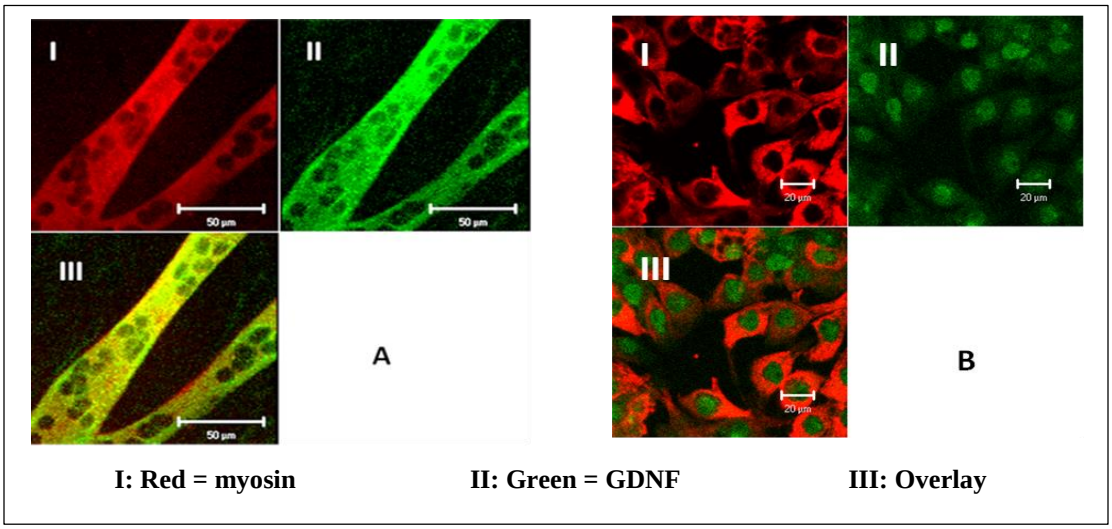


Figure 2.1. Localization of GDNF and myosin in skeletal and cardiac muscle cells. Skeletal and cardiac muscle cells were fixed with paraformaldehyde. A. C2C12 myotubes and B. HL-1 cardiac muscle cells. For both cell types: Panel I, cells were immunolabeled with mouse anti-myosin (sarcomeric myosin MF-20 for cardiac), followed by secondary antibody conjugated with Alexa Fluor 568 (red). Panel II, rabbit anti-GDNF followed by secondary antibody conjugated with Alex Fluor 488 (green). Panel III, overlay. Images were captured using a laser scanning confocal microscope.

Figure 2.2 below shows that the amount of GDNF secreted by cardiac muscle cells was higher than that secreted by skeletal muscle cells. This observation suggests that GDNF secretion processing in these types of muscle cells may differ.

Comparison of GDNF secretion content in skeletal and cardiac muscle cells

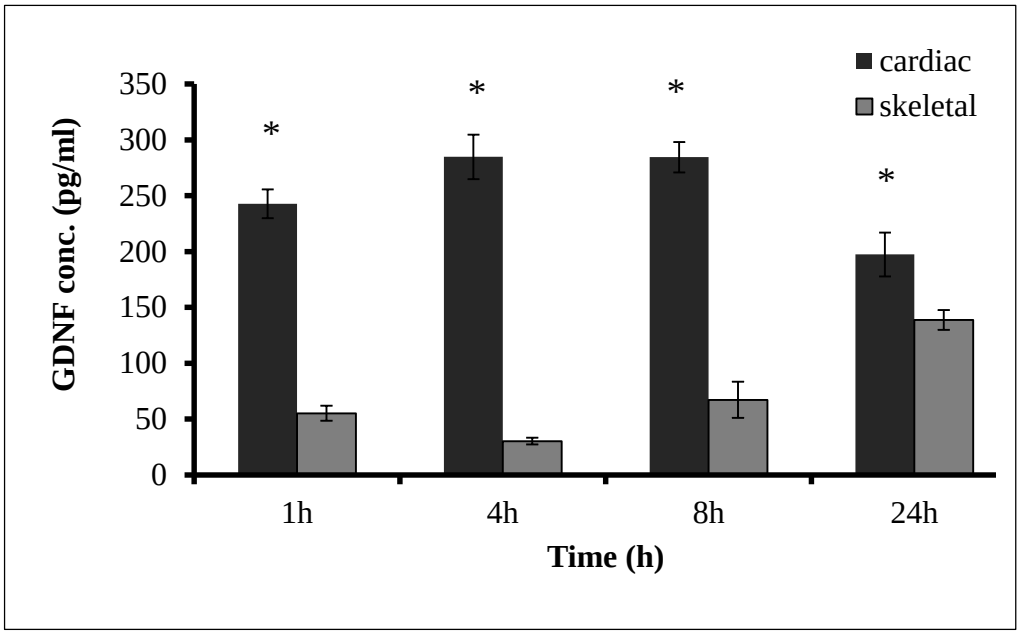


Figure 2.2. GDNF secretion levels in skeletal and cardiac muscle cells. Cells were grown and allowed to differentiate. Samples were collected from 5-day-old cardiac muscle cells and 7-day-old myotubes. Cardiac muscle cells secrete more GDNF than skeletal muscle myotubes. GDNF protein concentration was determined by ELISA. Values are presented as mean ± S.E.M. and Asterisk (*) indicates significance ($P \leq 0.05$). N = 8.

2.3.2. Are somatic motor neurons involved in regulation of GDNF production by skeletal muscle?

The goal in this study was to determine whether neural cells are involved in regulation of GDNF production in the target tissues. Other studies have shown that the survival of peripheral neurons depends on neurotrophic factors supplied by peripheral target tissues (Hassankhani et al., 1995; Martinelli et al., 2002, Nosrat et al., 1996). GDNF is now known as a neurotrophic factor for motor neurons (Henderson et al., 1994), which is secreted by skeletal muscle (Angka et al., 2008; Lie and Weis, 1998) and provides trophic support to the spinal motor neurons via a retrograde mode of action (Nguyen et al., 1998). If this is the case, it can be assumed that a strong relationship should exist between the nerve and its target in regulating the levels of a neurotrophic factor needed for survival and maintenance by the nerve. If this hypothesis were true, then the neurons innervating target tissue should be involved in regulating GDNF production. To test this hypothesis, our laboratory conducted *in vitro* studies (Vianney and Spitsbergen, 2011), aimed at creating neuromuscular junctions in culture. To do this, co-culture studies using skeletal muscle and neural cells were initiated (Fig. 2.3A). It was observed that differentiated neural cells, do not contain detectable levels of GDNF protein (data not shown). Figure 2.3B shows western blot analysis confirming that differentiated NG108-15 do not produce GDNF. Surprisingly, when neural cells and skeletal myotubes were grown together in the nerve-muscle co-culture system, GDNF was localized in both the neural cells and the skeletal muscle myotubes (Fig. 2.4A-C). Moreover, GDNF protein content was significantly reduced in nerve-muscle co-cultures compared to skeletal myotubes grown alone (Fig. 2.5). These results raised the question of how neural cells might be regulating GDNF production.

This question is addressed as specific aim two, and was examined in chapters three and four.

Nerve-muscle in culture

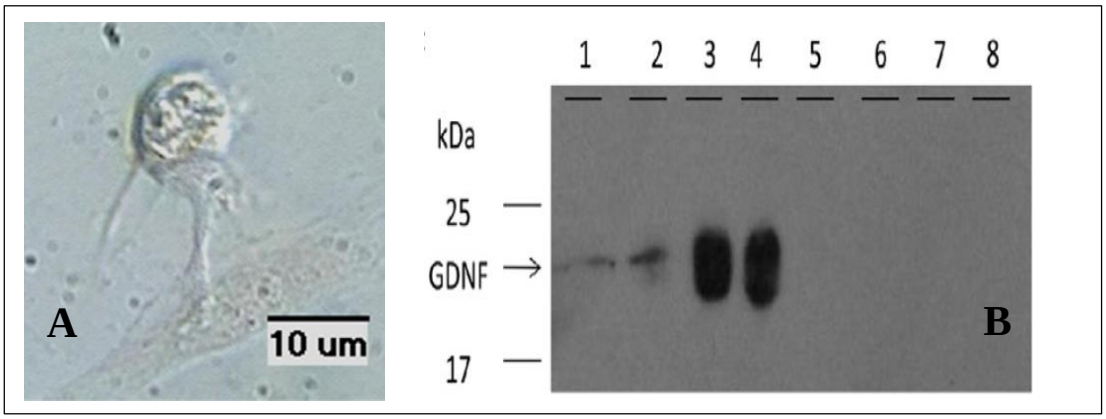


Figure 2.3. Nerve-muscle co-culture system and GDNF molecular sizes. NG108-15 neural cells were added to 7-day-old myotubes and were maintained for 24-36 h to allow neuromuscular contact to form. Panel A shows a nerve-muscle connection. Panel B shows western blot results, GDNF protein was found in C2C12 myotubes but not in NG108-15 cells. Lane 1 and 2, GDNF secreted by myotubes in culture medium and Lane 3 and 4, intracellular GDNF. No GDNF protein was found in culture medium (Lane 5 and 6) or in cells (Lane 7 and 8) from NG108-15 culture.

GDNF localization

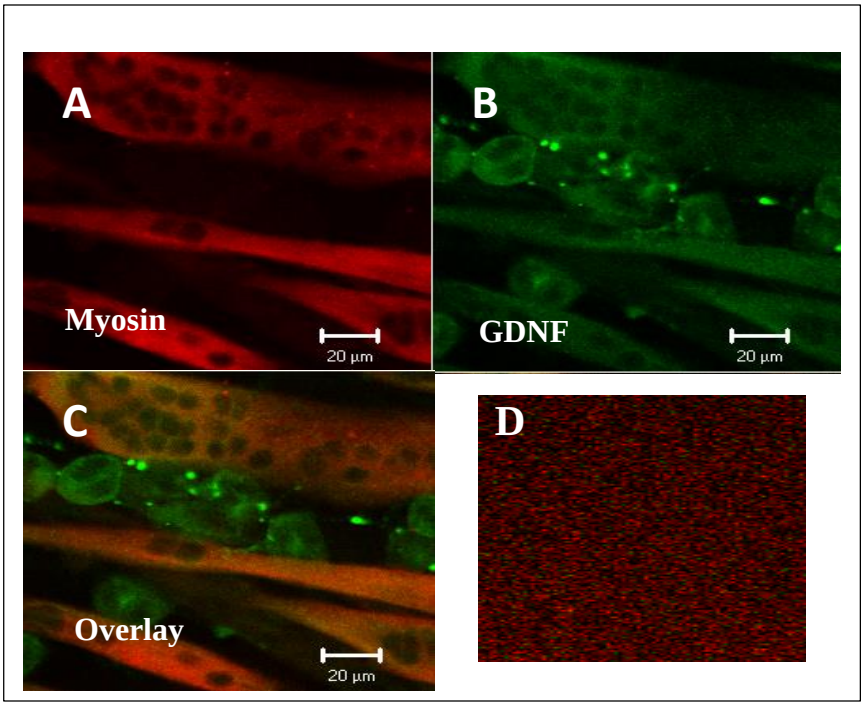


Figure 2.4. GDNF localization in nerve-muscle co-culture system. A-C. Co-cultures of C2C12 cells and NG108-15 cells were cultured on coverslips in DMEM supplemented by 10% horse serum. The cells were fixed in 4% paraformaldehyde and mouse anti-myosin followed by donkey anti-mouse conjugated to Alexa Fluor 568 was added to localize myosin. Also, rabbit anti-GDNF followed by donkey anti-rabbit conjugated to Alexa Fluor 488 was added to localize GDNF. **A**. Myosin (red), **B.** GDNF (green), and **C.** Overlay, and **D**. Negative control. GDNF was observed in both NG108-15 cells and myotubes. All images were captured by Zeiss laser scanning confocal microscope.

Measurement of GDNF protein concentration by ELISA

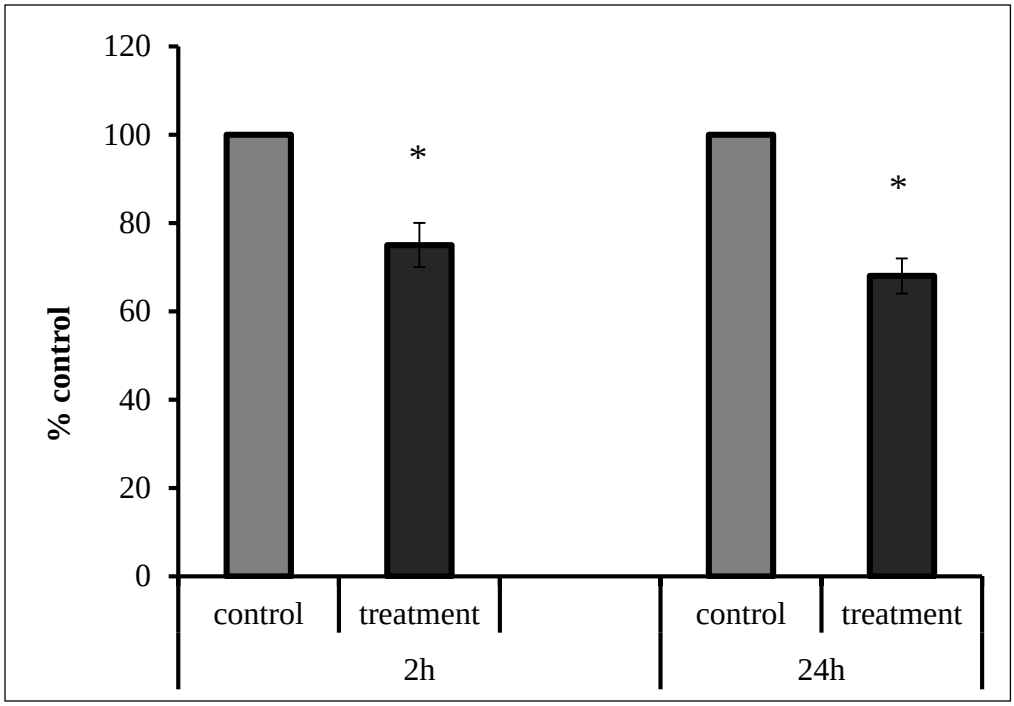


Figure 2.5. Quantification of GDNF protein secretion in nerve-muscle co-culture. Nerve-muscle co-cultures were grown as in previous studies. ELISA results show that GDNF secretion is inhibited in nerve-muscle co-culture (= treatments). Values are presented as Mean ± S.E.M. Asterisk (*) indicates significance from control, ($P \leq 0.05$), N = 4.

2.3.3. Does electrical stimulation affect GDNF production by skeletal muscle cells?

Electrical stimulation has been shown to induce muscle contraction and protein sysnthesis (Donnelly et al., 2010). Preliminary studies on electrically stimulated cells were initiated in our laboratory to test whether electrical impulses affect GDNF production in C2C12 myotubes. Cells were stimulated at the frequency of 1Hz for 90min as previously decribed in Marrota et al. (2004). Cells were also stimulated at the same frequency for 30min as short-term duration so as to compare with the 90min stimulation. The results showed that intracellular levels of GDNF produced and GDNF that were secreted both were significantly reduced when cells were stimulated at 1Hz for 30 and 90 minutes (Fig. 2.6A-B). However, there was a significant difference between GDNF secreted when cells were stimulated for 90min with that stimulated for 30min. The inhibitory effect seemed to be reduced at 90min stimulation (Fig. 2.6A), but no difference in GDNF protein levels with intracellular GDNF (Fig. 2.6B).

Effect of electrical stimulation on GDNF production in skeletal muscle cells

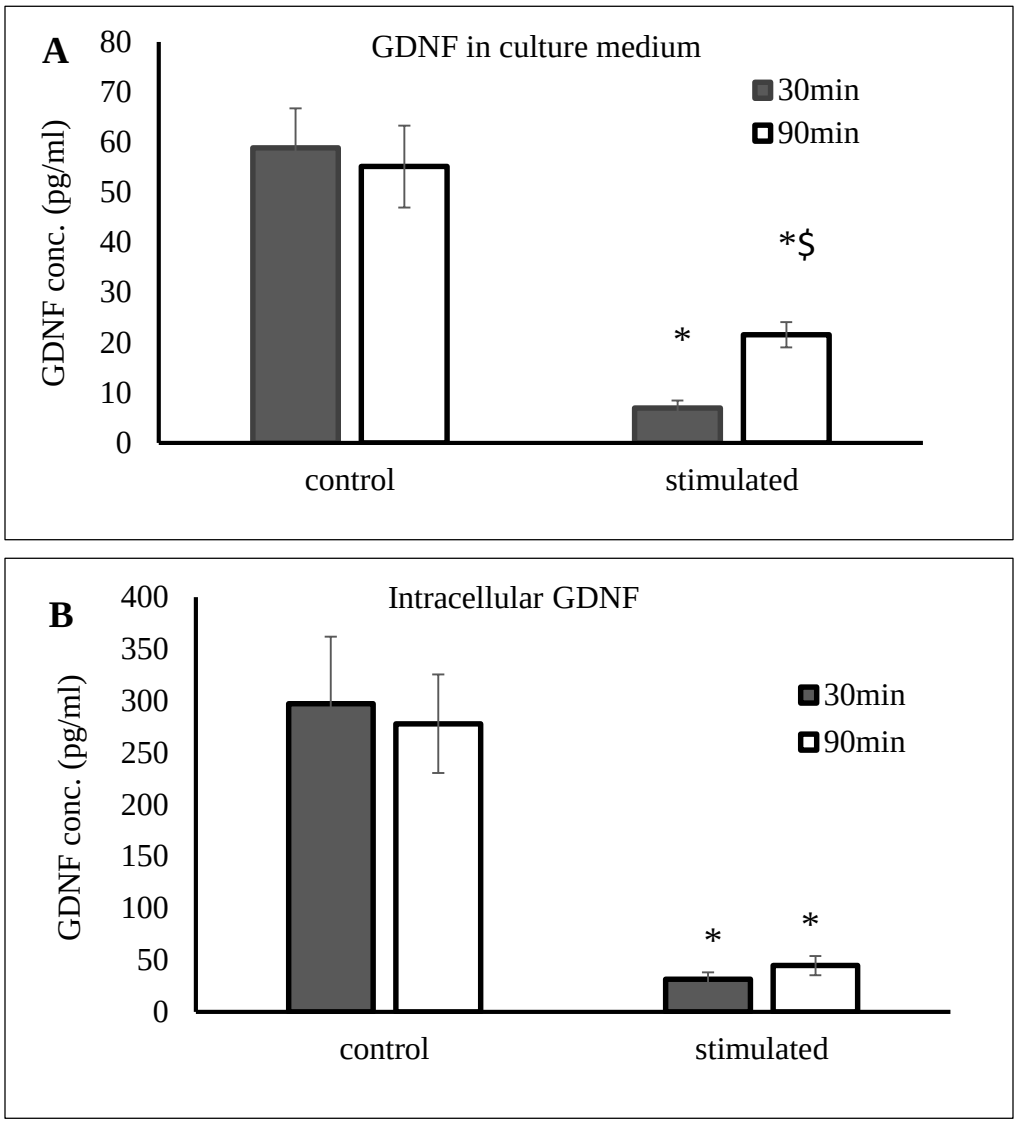


Figure 2.6. GDNF production in electrically stimulated myotubes. Day 7 or 8 skeletal myotubes were stimulated at 1Hz for 30 or 90min. A & B, GDNF production is inhibited. However, the inhibitory effect is reduced on GDNF secretion with a 90min stimulation, A. but no effect was observed in intracellular GDNF, B. Values are presented as Mean ± S.E.M. Asterisk (*) indicates significance from control and ($) indicates significance between the groups, ($P \leq 0.05$), N = 6.

2.3.4. Does electrical stimulation alter myosin heavy chain in C2C12 myotubes?

Since electrical stimulation can induce phenotypic changes in myotubes resulting in changes to myosin heavy chain from fast to slow myosin (Bayol et al., 2005), we tested whether stimulation cells at 1Hz could induce a visible change in myosin. The question was whether the changes in GDNF production that were observed could possibly be accompanied by changes in myosin expression. However, results from western blot analysis (Fig. 2.7A-B) and immunocytochemistry (Fig. 2.8A-B) did not show significant differences in the amount of slow myosin versus fast myosin between controls and electrical stimulated groups.

Fast and slow myosin in myotubes

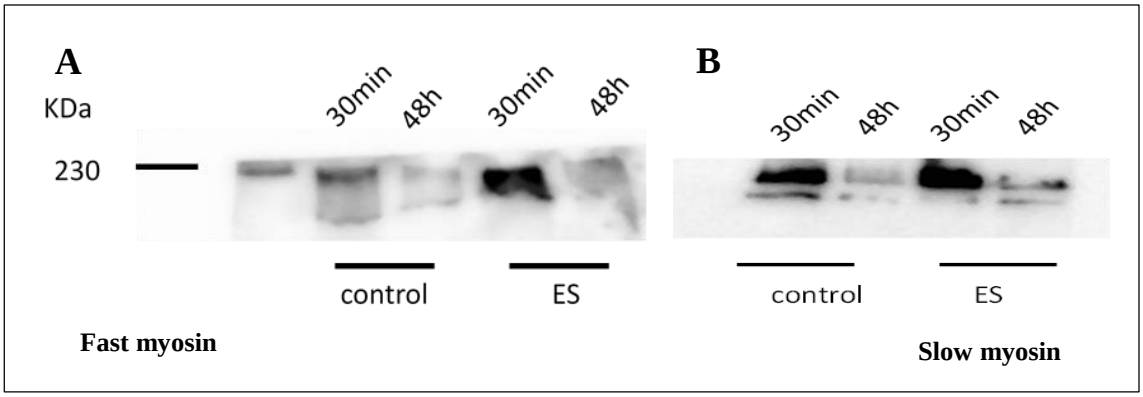


Figure 2.7. Fast and slow myosin in electrically stimulated C2C12 myotubes. Myotubes were stimulated at 1Hz for 30min or 48 hours. Westen blot analys show that fast myosin (**A**) and slow myosin (**B**) do not seem to change with 1Hz electrical stimulation.

Fast and slow myosin in myotubes

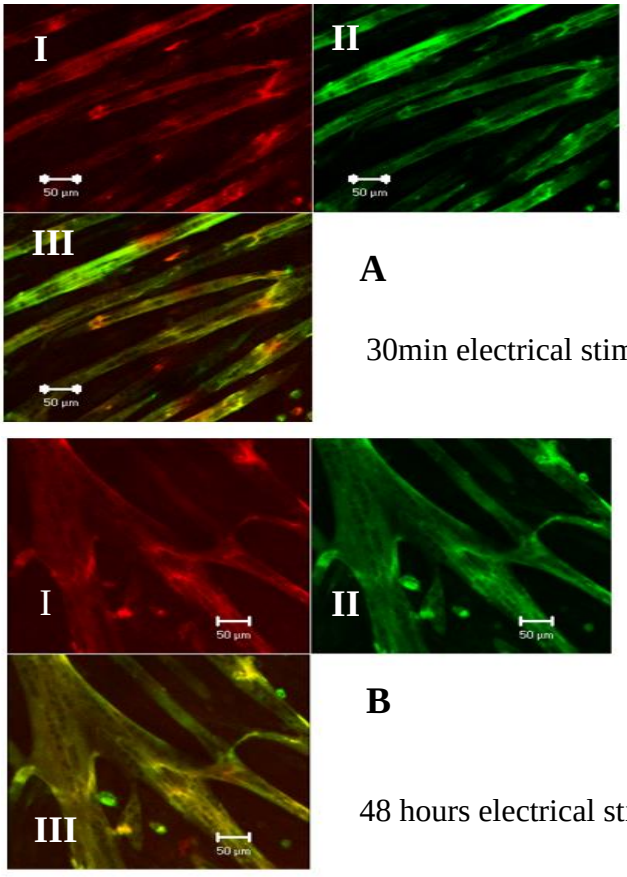


Figure 2.8. Localization of fast and slow myosin in C2C12 myotubes. Cells were fixed in 4% paraformaldehyde and mouse anti-slow myosin followed by donkey anti-mouse conjugated to Alexa Fluor 568 was added to localize slow myosin. Also, rabbit anti-fast myosin followed by donkey anti-rabbit conjugated to Alexa Fluor 488 was added to localize fast myosin. For both A and B, slow myosin (I), fast myosin (II), overlay (III). The results show that fast and slow myosin do not seem to change electrical stimulation. All images were captured by Zeiss laser scanning confocal microscope.

With a significant decrease in GDNF following 30 minutes of electrical stimulation, next we examined whether GDNF receptors (GFRα-1) on myotubes are affected by this short-term electrical stimulation. Western blot results did not show significant differences in concentrations of GFRα-1 between control group and electrically stimulated myotubes.

GDNF receptors on myotubes

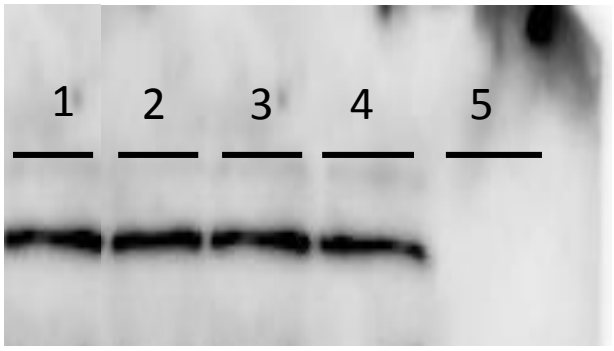


Figure 2.9. GFRα-1 receptors on C2C12 myotubes. Myotubes were stimulated at 1Hz for 30 minutes. Lane 1&2: controls Lanes 3 & 4: Stimulated Lane 5: protein ladder.

Preliminary results suggest that neural cells utilize GDNF secreted by skeletal muscle and inhibit further secretion of GDNF by skeletal muscle (Vianney and Spitsbergen, 2011). Early studies by others have also shown increased GDNF levels in denervated muscle compared to the muscle with normal innervation (Lie and Weis, 1998). This suggests that innervation status may be one determinant of GDNF levels secreted by a target tissue; which in turn, may suggest that the neural cells regulate their own supply of neurotrophic factors. Studies described in Chapters 3 and 4 utilized the treatment with neurotransmitters known to be released by motor neurons, sympathetic neurons, and

parasympathetic neurons, to test the hypothesis that neural cells regulate GDNF production by skeletal muscle and cardiac muscle via neurotransmitter release. For these studies, skeletal muscle cells and cardiac muscle cells were treated with acetylcholine (ACh) and/or norepinephrine (NE). Neurotransmitter receptor blocking agents were also utilized to help determine which type of receptors were being activated.

In other studies, cells were electrically stimulated to test the hypothesis that electrical activity is an important regulator of GDNF production.

B. MATERIALS AND METHODS

2.4. MATERIALS

2.4.1. C2C12 skeletal muscle cell line

C2C12 is a mouse myoblast cell line. The cells were originally obtained through serial passage of myoblasts cultured from the crush injury thigh muscle of 2- month-old normal mouse of C3H strain. These cells are capable of differentiation into skeletal muscle myotubes (Yaffe and Saxel, 1977). C2C12 cells have been a useful model for studying skeletal muscle biology and physiology. This includes processes involved in the differentiation of myoblasts (Yaffe and Saxel, 1977) and the regulation of apoptosis in myoblasts (Schöneich et al., 2014). C2C12 cells are also used to study myogenesis (Burattini et al., 2004), metabolism (Marotta et al., 2004), muscle diseases (Yaffe and Saxel., 1977), and diabetes (Dymkowska et al., 2014). This skeletal muscle cell line resembles its in vivo counterparts by expressing proteins that are found in mammalian skeletal muscle (Burattini et al., 2004; Thelen et al., 1997). The cells express extracellular matrix proteins (Park et al., 2008), contractile proteins including actin and myosin

(Burattini et al., 2004), ion channels (Dymkowska et al., 2014), muscle receptors, and enzymes including acetylcholinesterase (Siow et al., 2002; Vianney and Spitsbergen, 2014). C2C12 cells have also been used in electrical stimulation studies for calcium imaging (Ishibashi et al., 2009) and exercise induced-glucose uptake (Marotta et al., 2004; Nedachi et al., 2008). In the current study, C2C12 cells were used to study production of the neurotrophic factor GDNF and possible regulation by electrical activities or neurotransmitters.

2.4.2. HL-1 cardiac muscle cell line

HL-1 cells were derived from mouse atria. The cells retain ultrastructural characteristics in vitro of adult atrial cells, including the ability to contract spontaneously in culture (Claycomb, 1998). A number of investigators have utilized HL-1 cells for studying various aspects of cardiac muscle physiology, including apoptosis (Carlson et al., 2002), cell cycle (Zandstra et al., 2003), electrophysiology (Claycomb et al., 1998), oxidative stress (Kitta et al., 2001), signal transduction (Chaudary et al., 2002), transcriptional regulation (Kitta et al., 2001), and cellular transplantation (Watanabe et al., 1998). In this study we have utilized HL-1 cells to examine regulation of expression of GDNF and NGF by electrical activity and autonomic neurotransmitters.

2.5. METHODS

2.5.1. Cell culture

Mouse skeletal muscle cells (C2C12), glioma×neuroblastoma hybrid cells (NG108-15), and culture medium were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Culturing procedures were performed according to the ATCC

protocols. C2C12 myoblasts, undifferentiated skeletal muscle cells, were initially seeded on a 100-mm plate (Falcon) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Mediatech, Manassas, VA) and 1% antibiotic–antimycotic (Invitrogen-GIBCO). Cells were incubated at 37 °C in a water-saturated atmosphere of 95% air and 5% CO2. The myoblasts were subcultured (transferred to new plates) after 2 days. Differentiation of myoblasts to myotubes was induced by replacing the growth medium with DMEM supplemented with 10% horse serum and 1% antibiotic–antimycotic. The medium was renewed every 1 to 2 days.

For nerve-muscle co-culture, NG108-15 cells were first cultured on 100-mm culture dishes in DMEM supplemented with 10% fetal bovine serum, 2% HAT supplement, a mixture of hypoxanthine, aminopterin, and thymidine (Invitrogen-GIBCO), and 1% antibiotic–antimyocotic. Differentiation of NG108-15 cells was enhanced by switching from regular medium to a serum free medium. The medium was renewed every 2 to 3 days. Nerve–muscle co-culture procedure was performed as it was first described by Chen et al. (2005) and Ling et al. (2005). Briefly, myoblasts were induced to differentiate and fuse into myotubes. Approximately 1.0×105 NG108-15 cells were plated onto 10-day-old myotube cultures. Co-cultured cells were maintained at 37 °C in a water-saturated atmosphere of 95% air and 5% CO2.

HL-1cells were cultured using the procedure of Dr. William Claycomb (Claycomb et al. 1998; White et al. 2004). Cells were initially grown in 100 mm culture dishes for 2 to 3 days, then were transferred to 6-well plates for electrical stimulation or 12-well plates for treatment with neurotransmitters.

2.5.2. Treatment with cholinergic and adrenergic agonists and antagonists

All studies in myotubes were performed on 6 or 7 day- old- myotubes. On the day of experiment, old medium was removed and fresh medium containing 0.1 - 100µM acetylcholine (ACh) was added. Treatments of HL-1 cells were performed on day 4- or 5-day-old cells. ACh (0.1µM, 1µM and 100µM) or norepinephrine (NE: 0.1mM and 1mM) were added. Conditioned culture medium and harvested cell samples were taken between 0 and 24 hours following treatment. Control plates were processed in the same manner except that no treatments were made.

Treatments with antagonists: On the day of the experiment, old medium was removed and cells were treated with fresh medium containing 200nM unlabeled alpha-bungarotoxin (α-BTX: Biotium, CA) for 25 min. Following incubation the culture medium containing blocking agents was removed and fresh medium containing (0.1µ -100µM) ACh was added. Samples were collected at intervals of 0h, 30min, 2h, 4h, and 24 h. The same procedure was performed for cardiac cells except 0.1µM-3µM atropine or propranolol concentrations (Sigma Aldrich) were used for blocking muscarinic ACh recptors (mAChRs) and α-adrenergic receptors, respectively (Chaote and Feldman, 2003; Wang et al., 2001). Control plates included: non-treated plates, plates treated with ACh only, and plates treated with blocking agents only.

2.5.3. Electrical stimulation

The overall approach of Marotta et al. (2004) inspired the electrical stimulation apparatus outlined in Figure 2.10A. This included its use of switched semicircular electrodes as part of a culture dish lid, a capacitor to block direct currents, and current

sampling resistors. Following Donnelly et. al., (2010) stainless steel electrodes were used instead of platinum electrodes. These electrodes were formed from 19 gauge stainless steel wire (ACE Hardware #5037023). Electrodes were secured to the 6-well culture dish lid with bolts which also provided convenient wire connection points using wires with spade lugs. A Grass Technologies S88 stimulator provided voltage pulses to a custom made interface box (Figure 2.10 B-C). Within the interface box a unity gain voltage buffer maintained the shape of voltage pulses while providing current to up to six electrodes. The voltage buffer used Texas Instruments OPA544 High-Voltage High-Current Operational Amplifier with unity feedback (Franco, 2002) powered by a Tektronix PS280 power supply set to +/-29V. Switches SW1-SW6 enabled each pair of electrodes to be individually selected. Capacitor C1 blocked direct current to reduce medium electrolysis and electrode oxidation (Donnelly et al., 2010). The voltage across each electrode was available at banana plug jacks (e.g. A1 and B1, Figure 2.9A) and measurable using two oscilloscope channels in differential voltage mode. The current through each electrode pair could be measured using a single oscilloscope channel connected across the corresponding current sampling resistor R1-6 (e.g. banana jack B1, Figure 2.9A). This required scaling the voltage waveform by the inverse resistance value. Resistors R1-6 was set to approximately 100Ω. Electrical safety precautions were observed including use of components with sufficient voltage and power ratings. Fuses limited power supply and electrode currents. The PS280 power supply current limit feature was also used as a safety precaution.

Three wells were stimulated simultaneously using these apparatus, whereas the other three wells served as controls. Cells were stimulated at approximately 1Hz or 5Hz

with an approximate 24V 30ms pulse for 30min – 48hours. All cultures were maintained at 37°C in water-saturated incubator of 95% air and 5% CO_2 during stimulation periods.

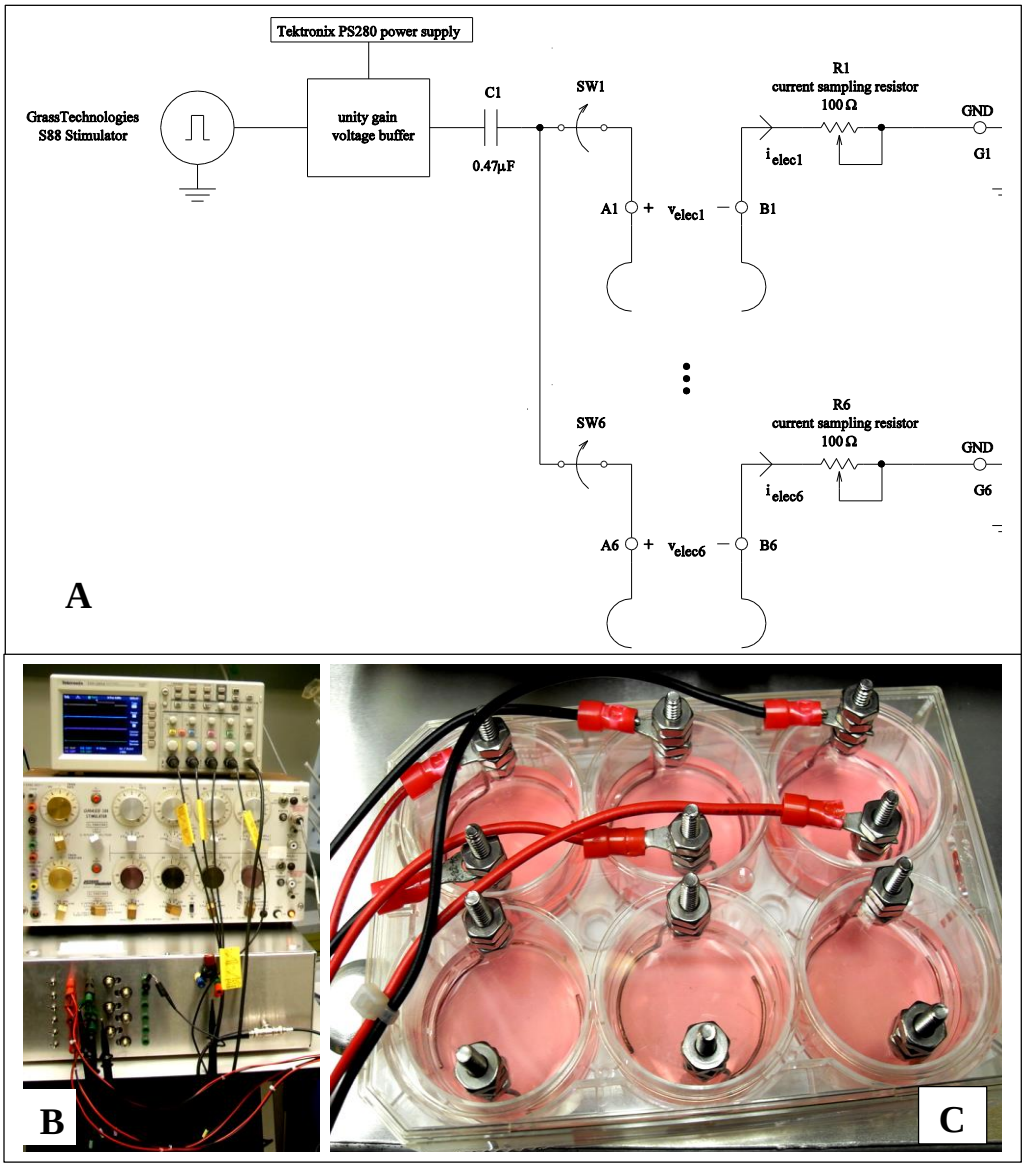


Figure 2.10. Electrical stimulation instruments. **A**. Electrical stimulation apparatus block diagram. Overall approach inspired by Marotta et al. (2004). Use of stainless steel electrodes based on Donnelly et al. (2010). **B**. Pulses were generated by a Grass S88

stimulator and applied via a custom-made voltage buffer circuit. The buffer circuit was capacitively coupled to the electrodes to reduce electrolysis. The myotubes were stimulated directly using stainless steel wire electrodes. The electrodes were bent into half circles and were integrated into a lid of a 6-well plate as shown in **C**. In all electrical stimulation studies, three wells were stimulated simultaneously, whereas the other three wells served as controls. Cells were stimulated at 1Hz or 5Hz with an approximate 24V 30ms pulse for 30min or 90min. All cultures were maintained at 37°C in water-saturated incubator of 95% air and 5% CO_2 during stimulation period.

2.5.4. GDNF protein isolation

At the completion of each experiment, samples of conditioned medium and cells were collected. The time interval for sampling ranged between 0h (right after experimentation) to 24hours. Cardiac cell samples were collected at day 5, whereas skeletal myotube samples were collected at day 6, 7, 8, or 14 depending on the type of experiment immunocytochemistry, chemical or electrical stimulation, or co-culture studies, respectively. For each experiment, a 1-ml sample of culture medium was collected from each culture dish. To remove cells on dishes, culture medium was removed followed by washing with calcium/magnesium-free saline buffer. Then, 1ml of sample buffer (a mixture of phosphate buffered saline, 0.005% Tween-20, 0.5% bovine serum albumin, 0.1 mM benzethonium chloride, 2mM benzemidine, 0.4 M NaCl, 2 mM EDTA and 164 μl/100 ml aprotinin) was added to each culture dish containing cells. The cells were scraped from the dish using a cell lifter (Costar®, Corning Inc., NY). Cells were spun in a cold centrifuge at

13.5 x g and supernatant was removed and stored. All collected samples were stored at −20 °C until GDNF protein content was measured by enzyme-linked immunosorbent assay (ELISA) or GDNF protein molecular weights were measured by western blot analysis.

2.5.5. Enzyme-linked immunosorbant assay

GDNF primary antibody (1 µg/ml: R& D Systems) was added to 96-well ELISA plates, and then incubated overnight at room temperature. Plates were blocked with Phosphate Buffer Saline (PBS) containing 1% Bovine Serum Albumin (BSA: Fisher Scientific) and sucrose 5% (MP Biomedicals, LLC). Plates were rinsed three times with PBS, then GDNF standard (R&D Systems) or samples (conditioned culture medium or harvested cells) were added to each well, and plates were incubated for two hours at room temperature. The wells were washed with PBS and 100ng/ml anti-GDNF secondary antibody conjugated to biotin (R& D Systems) was added and incubated for two hours at room temperature. Following incubation the wells were washed three times with PBS and beta-galactosidase conjugated to streptavidin (Molecular Probes) was added and the plates were incubated for 20 minutes at room temperature. The wells were washed three times and 1mg/ml chlorophenol red- β-D galactopyranoside (CPRG: Roche Diagnostics GmbH) was added and incubated at room temperature. Plate readings were taken every two hours until the standard curve was obtained. Absorbance was measured at 575nm using an absorbance plate reader (Gen5, BioTek). For each assay, a standard curve was calculated from known GDNF concentrations.

2.5.6. Immunocytochemistry

For detection of myosin, GDNF, and NGF, both cell types were allowed to grow on coverslips up to 5-day-old for cardiac cells and 6-day-old or 7-day-old for myotubes. Cells were washed with PBS and fixed with 2.4% (cardiac cells) or 4% (myotubes) paraformaldehyde for 15 min at room temperature. Cells were washed three times for 5 minutes in PBS. Plates were blocked for one hour at room temperature. Following blocking, cells were incubated in primary antibodies against GDNF, NGF, or myosin (for cardiac cells, sarcomeric myosin heavy chain antibody FM-20 from Developmental Studies Hybridoma Bank was used) at 4°C overnight. Cells were washed again with PBS. Next, donkey anti-mouse or donkey ant-rabbit conjugated with Alexa Fluor 488, Alexa Fluor 568 or Fluor 647, were added for two hours at room temperature. Concentrations of each antibody or chemical were used according to manufacturer protocols based on the particular experiment.

Procedures described by Yang and Nelson (2004) were used to examine GDNF receptors (GFRα-1) and acetylcholine receptors (AChRs) on myotubes. Primary GDNF receptors polyclonal mouse anti-GFRα-1 (Santa Cruz Biotechnologies) and alpha bungarotoxin (α-btx) conjugated Alexa Fluor 488 (Molecular Probes) were used for GFRα-1 and AChRs, respectively. Concentrations of each antibody or chemical were used according to manufacturer protocols. The cells were maintained in medium containing antibodies or antagonists at 37°C in a standard incubator for 1hour. Cells were washed with PBS and fixed with 2.4% (cardiac cells) 4% (myotubes) paraformaldehyde for 15 min at room temperature. PBS containing 1% bovine serum albumin and 0.1% triton X-100 were added to permeablize the cells for and block non-specific sites. Cells were washed in PBS, and bound with secondary antibodies conjugated to AlexaFluor 488®, AlexaFluor 568®,

or AlexaFluor 647®, for 2 hours at room temperature. Negative controls consisted of cultures without primary antibodies added. Cells were washed with PBS and the coverslip with cells were mounted on a glass slide with 50% glycerol/50%PBS and sealed. Images were viewed using a Zeiss laser scanning confocal microscope (Zeiss LSM 510).

2.5.7. Western blot

The amount of GDNF in culture medium and in cells was determined by western blot as previous described (Vianney and Spitsbergen, 2011). To examine GDNF protein, culture medium samples or cell samples were loaded with Laemmli 2× loading buffer to make a final volume of 20µl. Controls consisted of a protein ladder (New England BioLabs), GDNF protein (positive control) and NGF protein (negative control). For detection of other proteins, positive control was always the protein of interest, and the negative control was other protein. All samples were boiled for 5 min and loaded into a 15% polyacrylamide gel. The gel was submerged and was run in separating buffer at two different voltages (100V followed by 150V). The transfer of protein from the gel to the polyvinylidene difluoride (PVDF; Invitrogen) membrane was performed at 12 V for 1 h. The PVDF membrane was blocked with I-Block (Tropix) for 1 h at 4 °C on a shaking platform. The membrane is then incubated with a primary antibody against GDNF (Santa Cruz Biotechnologies) in I-Blocking buffer overnight at 4 °C on a rotating platform. The membrane was washed 3 times, for 5, 10, and 20 min while shaking. The membrane was then incubated with a HRP-conjugated secondary antibody (ECL; GE Healthcare) in I-Blocking buffer for 1 h at room temperature, while shaking. GDNF protein was detected with chemiluminescence and is visualized on BioMax XAR film (Kodak) with exposure

times from 30seconds to 15 min, or protein detection was performed using Gel Logic 2200 Pro (Carestream, Molecular imaging).

2.5.8. Statistical analysis

Statistical analysis was performed using a student's t-test and analysis of variances (ANOVA). P values ≤ 0.05 were considered statistically significant. All data values are reported as the mean ± standard error of the mean (SEM).

2.6. REFERENCES

Angka, H.E., Geddes, A.J., Kablar, B., 2008. Differential survival response of neurons to exogenous GDNF depends on the presence of skeletal muscle. Dev. Dyn. 237, 3169–3178.

Bayor, S., Brownson, C., Loughna, P.T., 2005. Electrical stimulation modulates IGF binding protein transcript levels in C2C12 myotubes. Cell Biochem. and Function 23, 361–365.

Burattini, S., Ferri, P., Mattistelli, M., Curci, R., Luchetti, F., Falcieri, E. 2004. C2C12 murine myoblasts as a model of skeketal muscle development: morpho-functional characterization. European J. Histochem. 48, 223–-234.

Carlson, D.L., Lightfoot, E. Jr., Bryant, D.D., Haudek, S. B., Maass, D., Horton, J., Giroir, B.P., 2002. Burn plasma mediates cardiac myocytes apoptosis via endotoxin. Am. J. Physiol. Heart Circ. Physiol. 282, H1907– H1914.

Chaudary, N., Shuralyova, I., Liron, R., Sweeney, G., Coe, I.R., 2002. Transport characteristics of HL-1 cells: a new model for the study of adenosine physiology in cardiomyocytes. Biochem. Cell Biol. 80, 6555–6565.

Choate, J.K., Feldman, 2003. Neuronal control of heart rate in isolated mouse atria. Am.

J. Physiol Heart Circ. Physiol. 285, H1340–H1346.

Chen, S.-S., Lin, C.-H., Chen, T.-J., 2005. Lead-induced attenuation in the aggregation of acetylcholine receptors during the neuromuscular junction formation. Toxicol. Lett. 159, 89– 99.

Claycomb, W.C., Lanson, N.A Jr., Stallworth, B.S., Egeland, D.B., Delcarpio, J.B., Bahinski, A., Izzo, N.J Jr., 1998. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc. Natl. Acad. Sci. 95, 2979–2984.

Donnelly, K., Khodabukus, A., Philip, A., Deldicque, L., Dennis, R.G., Baar, K., 2010. A Novel bioreactor for stimulating skeletal muscle in vitro. Tissue Eng. 16, 711–18.

Dymkowska, D., Drabarek, B., Jakubczyk, J., Wojciechowska, S., Zablocki, K., 2014. Potassium channel openers prevent palmitate-induced insulin resistance in C2C12 myotubes. Arch. Biochem. Biophys. 41, 47–52.

Franco, S., 2002. Design with operational amplifiers and analog integrated circuits, third ed. McGraw Hill.

Hassankhan, A., Steinhelper, M.K., Snoopaa, M.H., Katz, E.B., Taylor, D.A., Andrade Rosental, A., Factor, S.M., Sterinberg, J.J., Field, L.J., Federoff, H.J., 1995. Overexpression of NGF within the heart of transgenic mice causes

hyperinnervation, cardiac enlargement, and hyperplasia of ectopic cells. Dev. Biol. 169, 309–321.

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

Houenou, L.J., Oppenheim,R.W., Li, L., Lo, A.C., Prevette., D. 1996. Regulation of spinal motoneuron survival by GDNF during development and following injury. Cell Tissue Res. 286, 219–223.

Ieda, M., Fukuda, K., 2009. Cardiac innervation and sudden cardiac death. Curr Cardiol Rev 5: 289-295.

Ishibashi, T., Hoshino, Y., Kaji, H., Kanzaki, M., Sato, M., Nishizawa, M., 2009. Localized electrical stimulation to C2C12 myotubes cultured on a porous membrane-based substrate. Biomed. Microdevices 11, 413-419.

Keller-Peck, C.R., Feng, G., Sanes, J.R., Yan, Q., Lichtman, J.W., Snider, W.D. 2001. Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. J. Neurosci. 21, 6136–6146.

Kitta, K., Day, R.M., Ikeda, T., Suzuki, Y.J., 2001. Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis. Free Radic. Biol. Med. 31, 902–910.

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

Ling, K.K.Y., Siow, N.L., Choi, R.C.Y., Tsim, K.W.K., 2005. ATP potentiates the formation of AChR aggregate in the co-culture of NG108-15 cells with C2C12 myotubes. FEBS Lett. 579, 2469–2474.

Marotta, M., Bragos, R., Gómez-Foix, M., 2004. Design and performance of an electrical stimulation for long-term contraction of culture muscle cells. Bio Techniques 36, 68-73.

Martinelli, P.M., Camargos, E.R.S., Morel, G., Ravares, C.A.P., Nagib, P.R.A., Machado, C.R.S., 2002. Rat heart GDNF: effect of chemical sympathectomy. Histochem. Cell Biol.118, 337–343.

Nedachi, T., Fujita, H., Kanzaki, M., 2008. Contractile C2C12 myotubes model for studying exercise-inducible responses in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 295, 1191–1204

Nguyen, Q.T., Parsadanian, A.S., Snider, W.D., Lichtman, J.W., 1998. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science 279, 1725–1728.

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

Park., H., Bhalla, R., Saigal, R., Radisic, M., Watson, N., Langer, R., Vunjak-Novakovis, G., 2008. Effects of electrical stimulation in C2C12 muscle constructs. J Tissue Eng Regen Med 2, 279 – 287.

Schöneich, C., Dremina, E., Galeva, N., Sharov, V., 2014. Apoptosis in differentiating C2C12 muscle cells selectively targets Bcl-2 deficient myotubes. Apopstosis 19, 42–57.

Siow, N.L., Choi, R.C.Y., Cheng, A.W.M., Jiang, J.X.S., Wan, D.C.C., Zhu, S.Q., Tsim, K.W.K., 2002. A cyclic AMP-dependent pathway regulate expression of acetylcholinesterase during myogenic differentiation of C2C12 cells. J. Biol. Chem. 277, 36129–36136.

Suzuki, H., Hase, A., Miyata, Y., Arahata, K., Akazawa, C., 1998b. Prominent expression

of glial cell line-derived neurotrophic factor in human skeletal muscle. J Comp. Neurol. 402, 303–312.

Thelen, M.H.M., Simonides, W.S., van Hardeveld, C.,1997. Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca2+-ATPase gene. Biochem. J. 321, 845–848.

Vianney, J.-M., Spitsbergen, J.M., 2011. Cholinergic neurons regulate secretion of glial cell line-derived neurotrophic factor by skeletal muscle cells in culture. Brain Res. 1390, 1–9.

Wang, H., Han, H., Zhang, L., Shi, H., Schram, G., Nattel, S., Wang, Z., 2001. Expression of multiple subtypes of muscarinic receptors and cellular distribution in the human heart. Mol. Pharmacol. 59, 1029–1036.

Wang, C.-Y., Yang, F., He, X.-P., Je, H.-S., Zhou, J.-Z., Eckermann, K., Kawamura, D., Feng, L., Shen, L., Lu, B., 2002. Regulation of neuromuscular synapse development by glial cell line-derived neurotrophic factor and neurturin. J. Biol. Chem. 277, 10614–10625.

Watanabe, E., Smith, D. M., Delcarpio, J. B., Sun, J., Smart, F. W., Van Meter, C. H. Jr., Claycomb, W. C., 1998. Cardiomyocyte transplantation in a porcine myocardial

infarction model. Cell Transplant 7, 239–246.

White, S.M., Constantin, P.E., Claycomb, W.C., 2004. Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. Am. J. Physiol. Heart Circ. Physiol. 286, H823–H829.

Yafel, D., Saxel, O., 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270, 725–727.

Yang, L.-X., Nelson, P.G., 2004. Glia cell line-derived neurotrophic factor regulates the distribution of acetylcholine receptors in mouse primary skeletal muscle cells. Neurosci. 128, 497–509.

Zandstra, P.W., Bauwens, C., Yin, T., Liu, Q., Schiller, H., Zweigerdt, R., Pasumarthi, K.B., Field, L.J., 2003. Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng. 9, 767–778.

CHAPTER 3

EFFECT OF ACETYLCHOLINE AND ELECTRICAL STIMULATION ON GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR PRODUCTION IN SKELETAL MUSCLE CELLS

3.1. INTRODUCTION

Glia cell line-derived neurotrophic factor (GDNF) was first purified by Lin et al. (1993) as a survival factor for dopaminergic neurons. GDNF is widely distributed in neuronal and non-neuronal tissues (Springer et al., 1995). GDNF exerts its survival effects on other subpopulations of neurons in the central and peripheral nervous systems (Henderson et al., 1994; Moore et al 1996; Trupp et al., 1995). Specifically, GDNF is characterized as a survival factor for spinal motor neurons (Henderson et al., 1994). The trophic factor is synthesize and release by skeletal muscle, and acts as a muscle-derived neurotrophic factor for spinal motor neurons (Suzuki et al., 1998a). During development, GDNF rescues motor neurons from programmed cell death (Oppenheim et al., 1995), acts as a chemoatractant, and assists with motor axonal guidance to motor neuron target tissues (Dudanova et al., 2010; Kramer et al., 2006). GDNF facilitates synaptic transmission (Wang et al., 2001), maintains synaptic activity (Zwick et al., 2001), plays a role in enhancing nerve recovery after injury (Cote et al., 2011; Dupont-Versteegden et al., 2004; Hashimoto et al., 2005; Houenou et al., 1996; Naveilhan et al., 1997; Oppenheim et al., 1995; Zhang et al., 2009) and muscle overexpressing GDNF displays hyperinnervation of endplates (Nguyen et al., 1998). These findings support the hypothesis that motor neurons

depend on GDNF as a target-derived neurotrophic factor and GDNF secreted by skeletal muscle may be important for motor neurons survival (Angka et al., 2008; Bohn, 2004).

Although much is known about the effects of GDNF on motor neurons, little is known about factors regulating GDNF synthesis and release by skeletal muscle. Denervation of sketal muscle causes an increase in GDNF expression (Suzuki et al., 1998b; Lie and Weis, 1998), while muscle cells co-cultured with neural cells in vitro secrete less GDNF (Vianney and Spitsbergen, 2011). These findings suggest that the innervation status of skeletal muscles plays a role in regulating the amount of GDNF produced by muscle.

In the present study the effect of the cholinergic agonists, acetylcholine (ACh) and carbachol (CCh), on GDNF production by skeletal muscle were examined. The question of whether electrical stimulation has a similar effect to that of the cholinergic agonists was also investigated. The results suggest that treatment with ACh and/or short-term electrical stimulation reduces GDNF secretion, while treatment with CCh or long-term electrical stimulation enhances GDNF production by skeletal muscle.

3.2. EXPERIMENTAL PROCEDURE

3.2.1. Cell culture procedure

Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The C2C12 mouse skeletal muscle cell line was purchased from American Type culture collection (ATTC: Manassas, VA, USA). The C2C12 cell line was extracted from 2-month old mouse thigh muscle and these cells have been used by researchers because of their in vivo skeletal muscle phenotype, including expression of contractile proteins (Ling et al., 2005; Yafel and Saxel, 1997). Culturing procedures were

performed according to the ATCC protocols and as described by Vianney and Spitsbergen (2011). Briefly, C2C12 myoblasts were initially seeded on 100-mm plates and maintained in Dulbecco's Modified Eagle's medium (DMEM: ATCC) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Life Technologyies, Carlsbad, CA, USA). Cells were incubated at 37°C in water-saturated atmosphere of 95% air and 5% CO2. For experiments, myoblasts cells were seeded in 6-well plates (USA Scientific, Inco. Ocala, FL, USA). Differentiation of myoblasts to myotubes was induced by replacing the growth medium with DMEM supplemented with 10% horse serum and 1% antibiotic-antimyocotic. The medium was renewed every one to two days. All experiments were run on myotubes that had been maintained in culture for 6 to 8 days.

3.2.2. Sample collection and cell harvesting

To determine GDNF protein concentration in culture medium, a 1 ml sample of medium was collected from each culture dish at 2, 4, and 24 h following treatment and kept at -20°C. To harvest cells, culture medium was removed, cells were washed with calcium/magnesium-free buffer and 1 ml of sample buffer (a mixture of phosphate buffered saline, 0.005% Tween-20, 0.5% bovine serum albumin, 0.1 mM benzethonium chloride, 2 mM benzemidine, 0.4 M NaCl, 2 mM EDTA and 164µl/100 ml aprotinin) was added to each culture dish. The cells were scraped from the dish using a cell lifter. To examine intracellular GDNF, cells were spun in a cold centrifuge at 13.5g and supernatant was removed and stored at -20°C. GDNF protein concent in each experiment was measured by an enzyme-linked immunosorbent assay (ELISA) as described below.

3.2.3. GDNF protein detection by ELISA

Determination of GDNF protein content in culture medium and cell supernatant was measured by ELISA. Briefly, GDNF primary antibody (1 mg/ml:R&D Systems, Minneapolis, MN,USA) was added to 96-well ELISA plates and then incubated overnight at room temperature. Plates were blocked with phosphate buffer saline (PBS) containing1% bovine serum albumin (BSA: Thermo Fisher Scientific, Waltham, MA, USA) and sucrose (5%). Plates were rinsed three times with wash buffer (a mixture of PBS and 0.05% Tween-20), then GDNF standard (R&D Systems) or samples (conditioned culture medium or harvested cells) were added to each well, and plates were in cubated for two hours at room temperature. Plates were washed and 100ng/ml anti-GDNF secondary antibody conjugated to biotin (R&D Systems) was added and incubated for two hours at room temperature. Following incubation plates were washed and betagalactosidase conjugated to streptavidin (Life Technologies) was added and incubated for 20 min at room temperature. Plates were washed and 1mg/ml chlorophenolred-β-D galactopyranoside (CPRG: Roche Diagnostics GmbH, Indianapolis, IN, USA) was added and incubated at room temperature. Plate readings were taken every two hours until the standard curve was developed. For each assay a standard curve was calculated from known GDNF concentrations.

3.2.4. Detection of acetylcholine receptors using alpha-bungarotoxin

Procedures were as previously described in Vianney and Spitsbergen (2011), with minor modification. Briefly, cells were grown on cover slips pre-treated with 0.1% gelatin (Thelen et al., 1997). Cells were maintained at 37°C in a standard incubator and allowed to differentiate into myotubes. To examine whether myotubes express AChRs, live cells

were treated with alpha bungarotoxin (α-BTX) using a procedure adopted from Yang and Nelson (2004). Briefly, myotubes were treated with fresh medium containing 200 nM α-BTX conjugated to Alexa-FluorR 488 (Life Technologies) and maintained at 37°C in a standard incubator for 1 h. Cells were washed and fixed with 4% paraformaldehyde for 30 min, were washed with PBS and the coverslip with cells was mounted on a glass slide with 50% glycerol/50%PBS. Images were captured using a Zeiss laser scanning confocal microscope.

3.2.5. Treatment with acetylcholine or carbachol

Cells were grown as previously described. On the day of the experiment, fresh medium containing acetylcholine or carbachol (ACh or CCh, respectively; 0.1 µM, 1 µM, and 100 µM) was added to myotubes. Samples of conditioned culture medium and harvested cells were taken after 2, 4, and 24 h. Control plates were processed in the same manner except that no ACh or CCh was added. In a separate experiment, CCh was added to myotubes for 5 min then removed and fresh culture medium was added. Samples of conditioned culture medium and harvested cells were collected after 30 min. Both cells and culture medium samples were stored at -20°C. GDNF protein content was measured by ELISA.

3.2.6. Block of acetylcholine receptors with alpha-bungarotoxin

C2C12 myoblasts were grown on 12-well plates and allowed to differentiate into myotubes. Myotubes were treated with fresh medium containing 200 nM unlabeled α-BTX (Biotium, Hayward, CA, USA) for 25 min. Following incubation with α-BTX the cells were washed twice with fresh culture medium and fresh medium containing 100 nM ACh

or 100 nM CCh was added. Controls consisted of plates without treatments and plates that were treated with 100 nM ACh or 100 nM CCh without α-BTX. Samples were collected at intervals of 2, 4, and 24 h. GDNF protein content was measured by ELISA.

3.2.7. Electrical stimulation of skeletal muscle cells

The approach of Marotta et al.,(2004) inspired the electrical stimulation apparatus used in this work, including its use of switched semicircular electrodes as part of a culture dish lid, a series coupling capacitor (0.47 μF rather than the 220 μF) and current sampling resistors (approximately100 Ω). Following Donnelly et al. (2010) stainless steel electrodes were used instead of platinum electrodes used by Marotta et al., (2004) and Thelen et al. (1997). A Grass Technologies S88 stimulator provided voltage pulses to a custom interface box. Electrodes were secured to the culture dish lid with bolts, which also provided convenient wire connection points using wires with spade lugs. Within the interface box a unity gain voltage buffer provided current to up to six pair of electrodes connected to six switches, which enabled each pair of electrodes to be individually selected. Stimulator pulse characteristics were experimentally selected to cause noticeable contraction of the muscle cells. Cells were stimulated at 1Hz or 5Hz for 30 min, 90min, 12h, 24h, and 48h.Typical pulse amplitudes were in the range of 20–25 V, with approximate 30ms widths at the voltage buffer output. Using this apparatus three wells were stimulated simultaneously and three wells served as controls. All cultures were maintained at 37 °C in water saturated incubator of 95% air and 5% CO2 during stimulation periods. GDNF protein content was determined by ELISA.

In order to mimic the motor nerve firing, a separate set of experiments were performed according to Eftmie et al. (1991). In these experiments, cells were stimulated at

100Hz trains of pulses; 1s-duration applied once every 100s. The pulses strength were 10-15mA and duration of 0.5 ms. Cells were stimulated for 30 min while being maintained in the incubator as described above. Samples were taken between 0h and 48h. GDNF protein content was determined by ELISA.

3.2.8. Block of voltage-gated sodium channels

In order to test whether voltage-gated sodium channels were important for the effect of electrical stimulation on GDNF production, channels were blocked using tetrodotoxin (TTX). For each experiment, fresh medium containing 100µM tetrodotoxin (TTX) was added to myotubes and cultures were maintained for 30 min before electrical stimulation. Cells were electrically stimulated and samples of conditioned culture medium and harvested cells were collected at 0, 2, and 24 h following electrical stimulation.

3.2.9. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's test, or Student's t-test. P values ≤ 0.05 were considered statistically significant. All data values ae reported as the mean ± standard error of the mean (SEM).

3.3. RESULTS

3.3.1. Effect of acetylcholine on GDNF production by skeletal muscle cells

In previous studies we showed that cholinergic neurons play a role in regulating GDNF synthesis and release by skeletal muscle (Vianney and Spitsbergen, 2011). Here, we sought to determine whether cholinergic neurons exert their effect via neurotransmitter release. First, cell staining was performed to ensure that the myotubes express ACh

receptors (AChRs). Fig. 3.1A shows that C2C12 skeletal muscle cells stain positively using α-BTX conjugated to AlexaFluor 488, suggesting that the skeletal muscle cells express AChRs. Exposure of myotubes to ACh inhibited GDNF protein secretion. GDNF levels secreted at 2 h were reduced to 70% of control but no effect of treatment with ACh was observed following 24 h of treatment (Fig. 3.1B). Also, treatment with ACh had no effect on intracellular levels of GDNF protein at 2 h (Fig. 3.1C) and 24h (data not shown).

The next experiments were designed to determine whether or not ACh induced its inhibitory effects via AChRs. In this set of experiments, treatment with ACh reduced the secretion of GDNF in culture medium to around 60% of control and pretreatment with α-BTX blocked the inhibitory effects of ACh on GDNF secretion (Fig. 3.1D). Treatment with ACh and α-BTX had no effect on GDNF production at 24 h (data not shown).

Acetylcholine Treatments

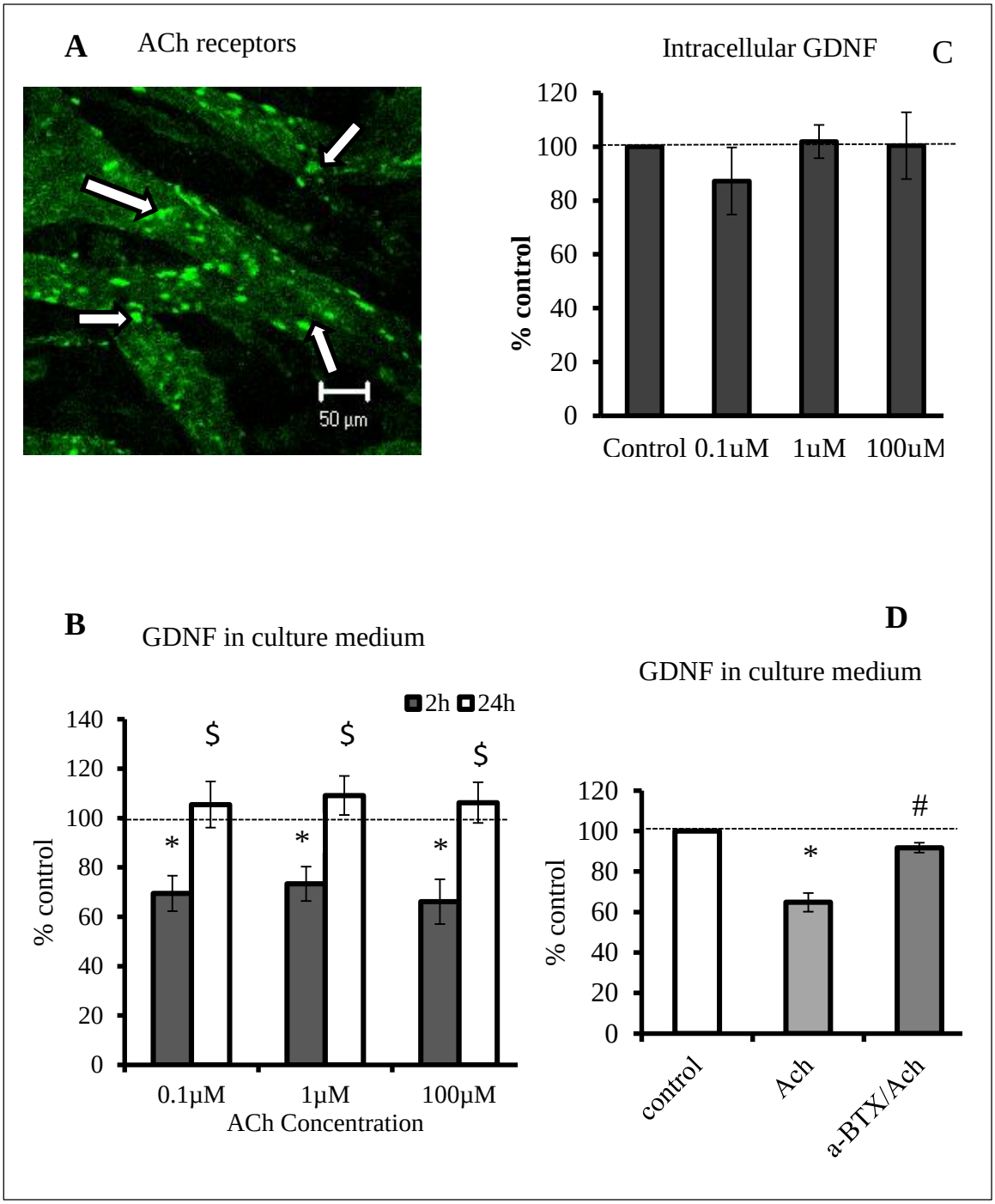


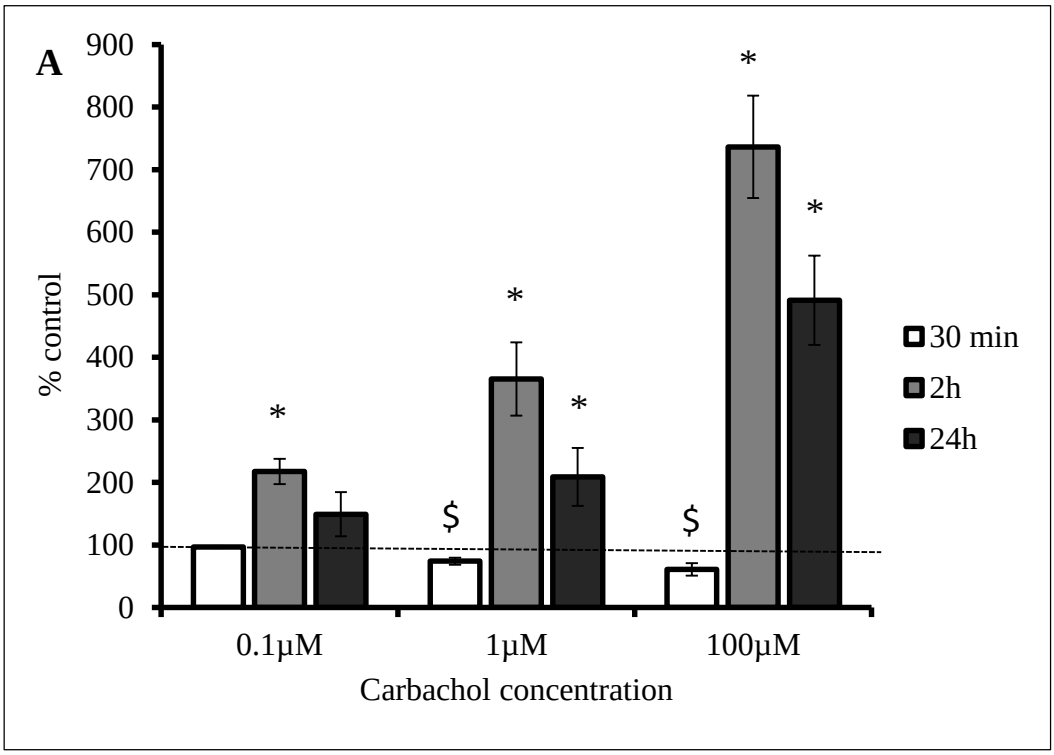
Figure. 3.1 Effect of ACh on GDNF production by skeletal muscle cells. **A.** Acetylcholine receptors on skeletal muscle cells (myotubes). Myoblast cells were grown and allowed to differentiate into myotubes. Culture medium containing α-BTX (200nM) conjugated to Alexa Fluor 488 (green) was added to myotubes and incubated for 1h in a standard incubator. Following 1h of treatment cells were fixed with 4% paraformaldehyde and viewed on a confocal microscope. White arrows show AChRs on myotubes in culture. Effect of ACh on GDNF production by skeletal muscle cells. Myoblast cells were grown and allowed to differentiate into myotubes. **B–C**, 7-day-old myotubes were treated with culture medium containing ACh at concentrations of 0.1 µM, 1 µM, and 100 µM. Conditioned culture medium and cells were collected at 2 h and 24 h. **B.** ACh inhibits GDNF secretion following 2 h but not 24 h of exposure. **C**. ACh had no effect on intracellular GDNF content. **D.** Blocking AChRs with α-BTX prevented the effects of ACh on GDNF secretion at 2 h. An asterisk (*) indicates a significant decrease from control, dollar sign ($) indicates a significant difference in GDNF levels between samples collected at 2 h and that collected after 24 h, pound sign (#) indicates a significant difference in GDNF levels between cells treated with or without α-BTX. Values are presented as means ± S.E.M, $P \leq 0.05$, N=6.

3.3.2. Effect of carbachol on GDNF production by skeletal muscle cells

Since ACh is quickly degraded by acetylcholinesterase (AChE), we sought to test whether CCh, a chemical that mimics ACh effects but is not broken down by AChE, would exert similar effects to those of ACh. Interestingly, ACh and CCh had different effects on

GDNF secretion by muscle cells following 2 h and 24 h of exposure. While ACh inhibited GDNF secretion following 2h exposure and had no effect after 24 h, CCh increased GDNF secretion at both time points (Fig. 3.2A). However, when CCh was added to the cells for only 5 min and then removed, a significant decrease in GDNF secretion was observed 30 min following treatment (Fig.3.2A). Similar to what was observed for ACh, CCh had no effect on intracellular GDNF protein content (data not shown). Pretreatment with α-BTX abolished the action of CCh on GDNF secretion (Fig.3.2B).

Carbachol Treatments



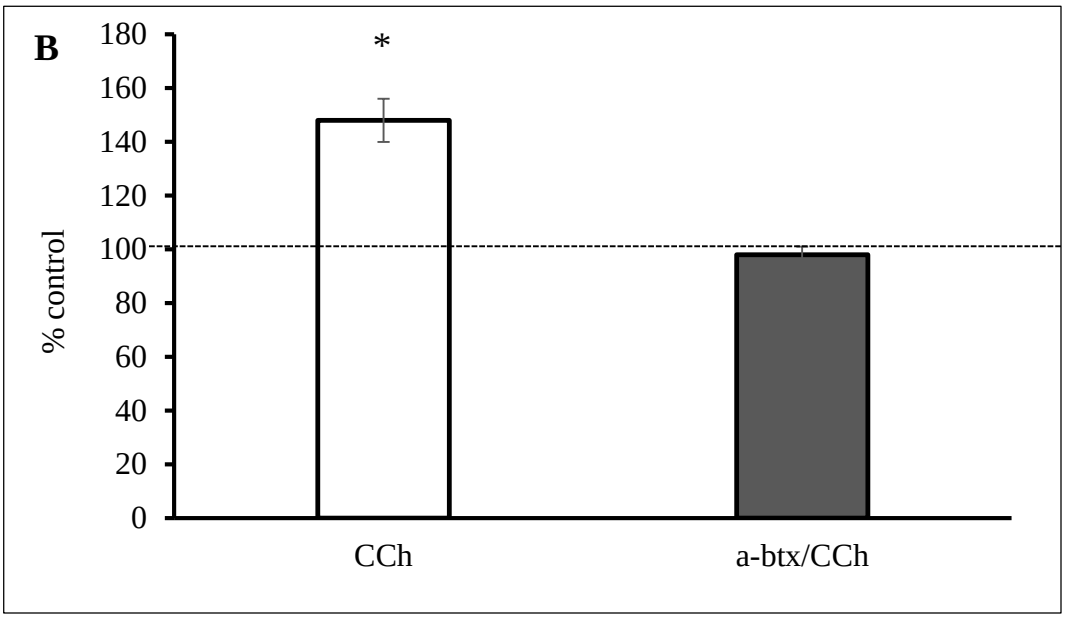
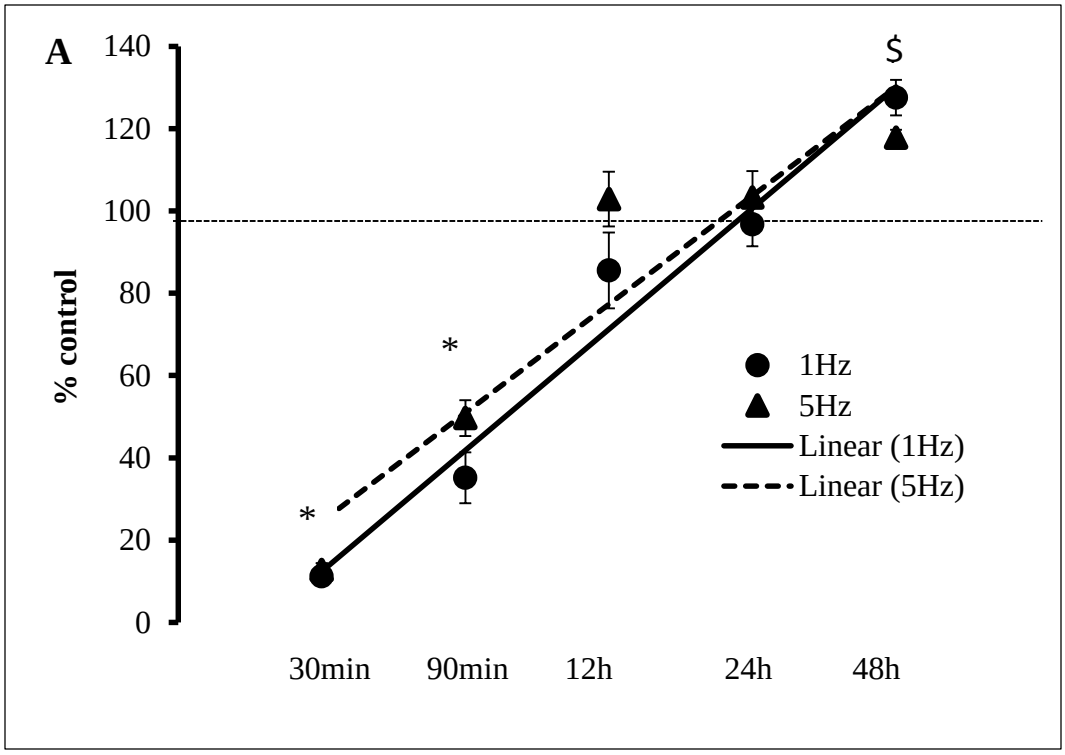


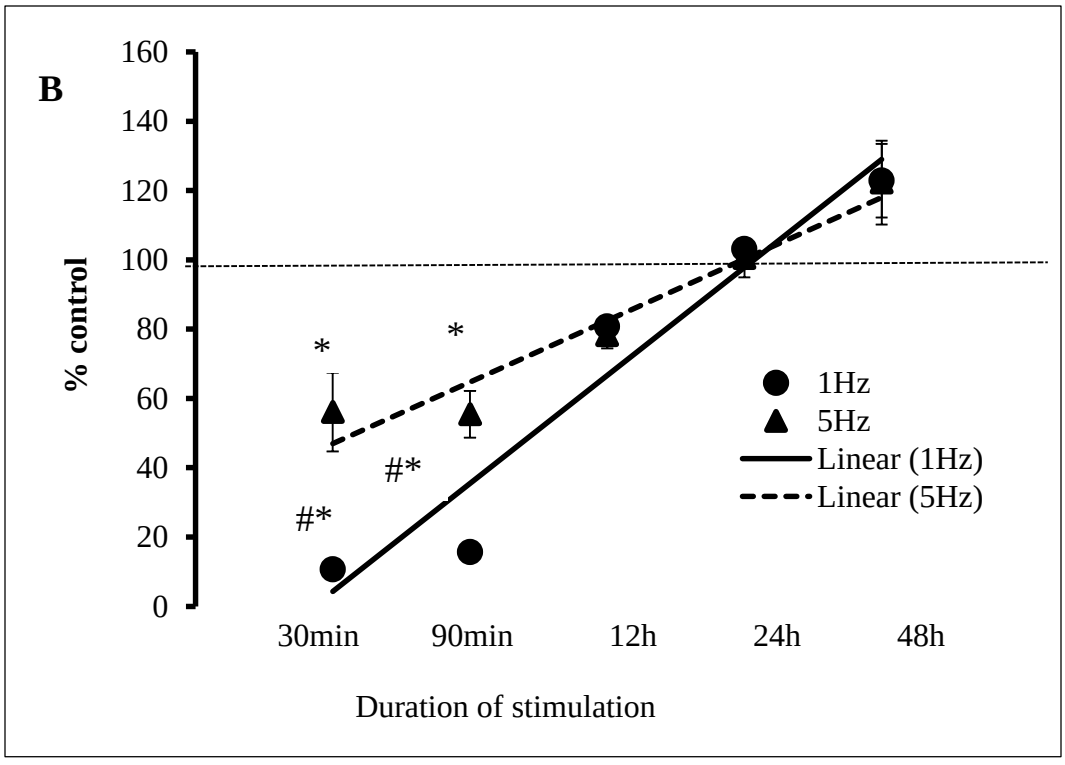
Figure. 3.2. Effect of carbachol on GDNF production by skeletal muscle cells. Seven-day-old myotubes were treated with culture medium containing CCh at concentrations of 0.1 µM, 1 µM, and100 µM. Cells were either treated with CCh for only 5 min, followed by removal of treated medium and replacement with fresh culture medium, or the exposure to CCh was prolonged up to 24 h. Conditioned culture medium and cells were collected at 30 min for a short-term exposure, and 2 h and 24 h for a long-term exposure. **A**. GDNF secretion decreases in the sample collected 30 min following a 5 min exposure to CCh, but increases 2 h and 24 h following exposure to CCh. **B.** Blocking AChRs with α-BTX prevented the action of CCh. An asterisk (*) indicates a significant increase from control. A dollar sign ($) indicates a significant decrease from control. Values a represented as mean ± S.E.M and $P \leq 0.05$, N=4.

3.3.3. Effect of electrical stimulation on GDNF production by skeletal muscle cells

Direct electrical stimulation can be used to elicit muscle contraction, bypassing the effect of ACh. Electrical stimulation has also been shown to alter protein expression in skeletal muscle (Bayol et al., 2005; Donnelly et al., 2010; Thelen et al., 1997). In this study, cells were exposed to 24 V pulses of 30 ms duration, applied at frequencies of 1 or 5Hz. With electrical stimulation, myotubes contracted synchronously at a rate similar to the stimulation frequency. Cells viewed after the stimulation period were found to be intact following all stimulation protocols. For these studies, 30 and 90 min were regarded as short-term stimulation and 12-48 h of stimulation were regarded as long-term stimulation. Fig. 3.3 summarizes the changes in GDNF production by skeletal muscle cells following short-term and long-term electrical stimulation. Thirty minutes of electrical stimulation at either 1 or 5Hz caused a significant inhibition in GDNF secretion by muscle cells, with no difference in effect being observed between stimulation frequencies (Fig. 3.3A). Unlike the effects observed following treatments with ACh or CCh, electrical stimulation also altered intracellular GDNF protein content. Although stimulation at 1Hz and 5Hz significantly decreased GDNF protein levels in cells, the effect following stimulation at 1Hz was more marked than that observed following stimulation at 5Hz (Fig. 3.3B). As the duration of stimulation increased, the inhibitory effect on GDNF production was diminished and changed to a stimulatory effect following 48 h of stimulation (Fig. 3.3A and 3.3B). GDNF was also inhibited when cells were stimulated with 100Hz trains of pulses for 30min (Fig. 3C).

Electrical Stimulation





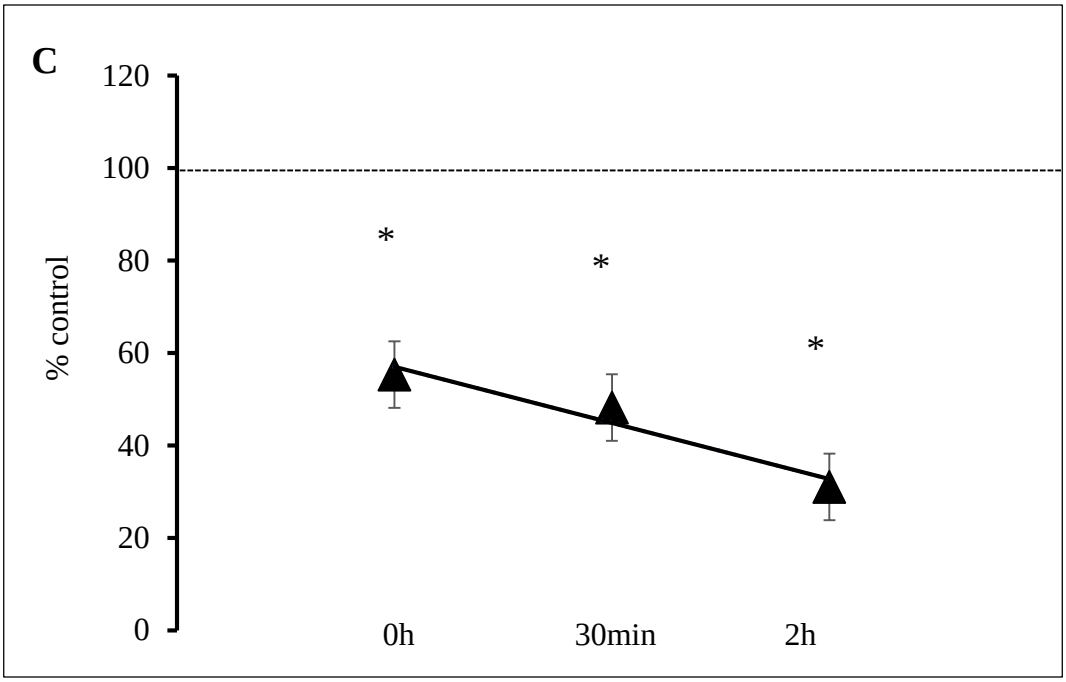
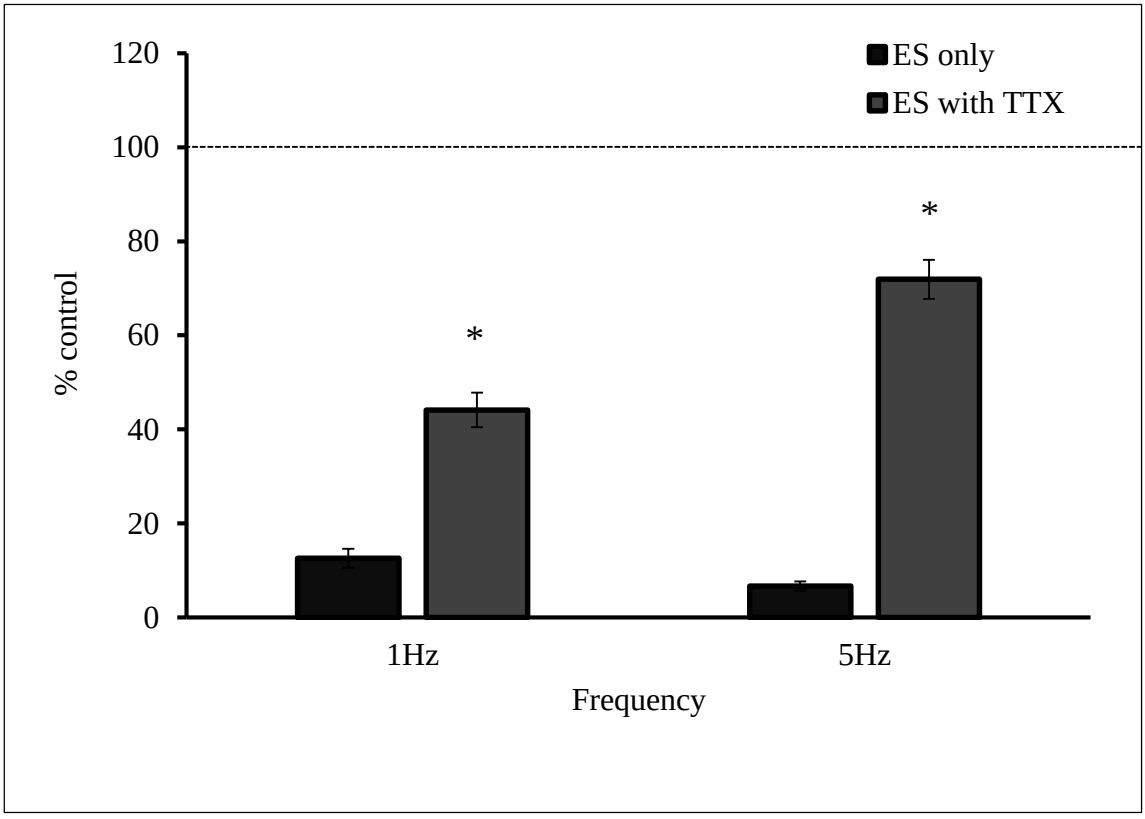


Figure. 3.3. Effects of electrical stimulation on GDNF production. Short-term electrical stimulation decreases GDNF secretion **(Panel A**) and GDNF content in muscle cells **(Panel B)**. The inhibitory effect of electrical stimulation is reduced as the duration of stimulation is increased. Electrical stimulation for 24 and 48h shifts the inhibitory effect of electrical stimulation to a stimulatory effect leading to an increase in GDNF production. Stimulation of the cells with 100Hz trains of pulses also had an inhibitory effect on GDNF secretion **(Panel C).** An asterisk (*) indicates a significant decrease from control. A dollar sign ($) indicates a significant increase from control. A pound sign (#) indicates a significant difference on GDNF levels between 1Hz and 5Hz groups. Values are presented as mean ≤ S.E.M.

3.3.4. Role of voltage-gated sodium channels

In order to test whether the effects that were seen with electrical stimulation on GDNF production involved ion channels, we examined voltage-gated sodium channels with electrical stimulation on GDNF production. Voltage-gated sodium channels were blocked using tetrodotoxin (TTX). Thirty minutes of stimulation in the presence of TTX was chosen because we sought to examine whether TTX could reverse the inhibitory effect of electrical stimulation on GDNF production. Although GDNF production was still inhibited following electrical stimulation, the results showed that the inhibitory effect was significantly reduced following TTX treatment, both for GDNF secretion (Fig. 3.4A) and GDNF content within myotubes (Fig. 3.4B).



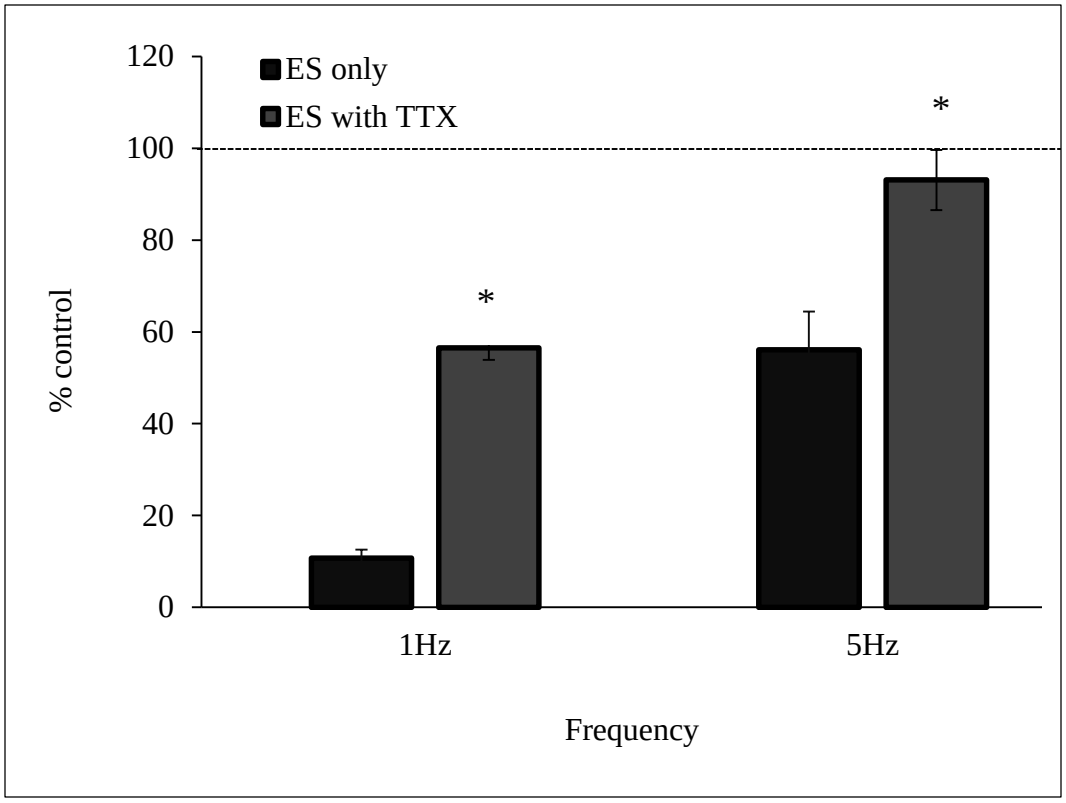


Figure. 3.4. Effect of blocking voltage-gated sodium channels in C2C12 myoubes. Cells were electrically stimulated in the presence or absence of tetrodotoxin (TTX) for 30min. The inhibitory effect on GDNF production caused by electrical stimulation was reduced in cells exposed to TTX. **A**. GDNF secreted into culture medium and **B**. Intracellular GDNF. An asterisk (*) indicates a significant decrease from control and a dollar sign ($) indicates a significant difference on GDNF levels between TTX-treated and non-treated groups. Values are presented as mean ± S.E.M. ($P \leq 0.05$). N= 4

3.4. DISCUSSION

The goal of the current study was to determine whether motor neurons may regulate their own supply of neurotrophic factors produced by skeletal muscle via effects of the neurotransmitter ACh and whether the regulatory effects of ACh are dependent on electrical activity in the muscle tissues. The major findings suggest that: 1) Both ACh and short-term electrical stimulation inhibited GDNF production by skeletal muscle. 2) Carbachol, a chemical that mimics ACh action, inhibited GDNF production at early times following exposure, while increasing GDNF production at later times following exposure. 3) The results suggest that both ACh and CCh act via AChRs, as blocking the receptors prevented the action of both chemicals. 4) Blocking voltage-gated sodium channels with TTX reduced the effect of electrical stimulation on GDNF production. 5) Finally, the magnitude and direction (inhibition vs. excitation) of the effect of electrical stimulation was dependent on the duration of stimulation, where short-term stimulation inhibited GDNF production and prolonged stimulation enhanced GDNF production.

The inhibition of GDNF production caused by short-term depolarization may explain results of previous studies which show that GDNF production is inhibited when skeletal muscle is co-cultured with cholinergic neurons (Vianney and Spitsbergen, 2011). The inhibitory effect of ACh or short-term electrical stimulation may also help explain why GDNF mRNA levels increase in skeletal muscle following denervation (Lie and Weis,1998), and why ACh inhibits extra cholinergic nerve branching during development (An et al.2010). Taken together it can be suggested that signaling pathways activated

following skeletal muscle innervation, treatment with exogenous ACh, or short-term direct electrical stimulation, inhibit GDNF production by skeletal muscle.

The inhibitory effect of short-term electrical stimulation appears to involve activation of voltage-gated sodium channels, as blocking these channels with TTX partially reversed the inhibitory effect. It is interesting to note that TTX was less effective at blocking the effects of electrical stimulation on intracellular GDNF, possibly suggesting that intracellular GDNF content may be regulated differently than secreted GDNF.

Unlike effects observed following treatment with ACh, exposure to CCh increased GDNF secretion by the muscle. C2C12 skeletal myotubes used in the current study express AChE (Choi et al., 2003; Lee et al.,2004; Siow et al.,2002; Tung et al.,2004), thus, these differences may be due in part to differences in the way these molecules are degraded (Taylor and Brown,1999). Unlike ACh, CCh is not broken down by AChE (Jankovic et al., 1998). Because CCh is resistant to AChE, long-term exposure to CCh increases muscle contraction (Protas et al., 1998) and increases the time-course of other cellular responses (Jankovic et al.,1998, Longmore et al.,1986). Therefore, we suggest that the opposite response of ACh compared to CCh, on GDNF expression by myotubes, may be due to the differences in metabolism of the two drugs, as a very short-term exposure to CCh exerts similar effects to that of ACh. If CCh continuously activates the muscle this may signal the muscle to increase GDNF secretion.

A similar phenomenon was observed with short-term vs. long-term electrical stimulation, where the inhibitory effect of electrical stimulation was diminished following increased duration of stimulation, converting to a stimulatory effect following 24–48 h of stimulation. These observations suggest that following prolonged treatment the effect of

electrical activity on GDNF production changes from inhibition to stimulation. A stimulatory effect following prolonged treatment, either with exposure to CCh or long-term electrical stimulation, may help explain the increase in GDNF content found in skeletal muscle following exercise training (Wehrwein et al.,2002, McCullough et al., 2011).

The results show that treatment with ACh, brief exposure to CCh, or short-term electrical stimulation all inhibit GDNF secretion by muscle cells, while longer term exposure to CCh or increased duration of electrical stimulation increases GDNF secretion by muscle cells. The similarities in the pattern of response, early inhibition followed by late stimulation, may suggest that both treatment modalities are altering GDNF secretion via similar mechanisms. However, the observation that CCh can enhance GDNF secretion following as little as 2h of exposure, while electrical stimulation does not increase GDNF secretion until 48h of stimulation, may suggest that different signaling mechanisms are involved. The observation that electrical stimulation alters both intracellular GDNF content and secretion, while treatment with cholinergic agonists only affects GDNF secretion, also supports the notion that different signaling mechanisms may be involved. Additional studies are needed to elucidate the signaling pathways by which electrical and chemical treatments alter GDNF secretion by skeletal muscle.

Previous work has demonstrated that fast and slow type skeletal muscles display different changes in GDNF protein content in response to low intensity exercise and to low frequency field stimulation (McCullough et al., 2011). Walk training or low frequency field stimulation (0.1Hz) of skeletal muscle decreases GDNF protein content in extensor digitorum longus, a muscle comprised primarily of fast-type muscle fibers, while these stimuli increase GDNF protein in soleus muscle, a muscle comprised primarily of slow-

type muscle fibers (McCullough et al.,2011). A switch from fast to slow muscle fiber-type has been observed following electrical stimulation of C2C12 myotubes (Nedachi et al., 2008); and these changes alter physiological demands of the cells in vivo and in vitro (Williams and Neufer, 2011; Zebedin et al., 2004). Thus changes in muscle fiber-type could also help to explain the change in response following long-term stimulation.

These findings, and results of studies by Xie et al. (1997), show that different families of neurotrophic factors may be regulated differently by muscle depolarization. Xie et al. showed that neurotrophin 3 expression in skeletal muscle increased with electrical stimulation or treatment with ACh (Xie et al., 1997), while our result show that GDNF production is inhibited by ACh or short-term electrical stimulation. Our observations that a sizeable pool of intracellular GDNF is maintained in skeletal muscle and that intracellular GDNF content is regulated differently than secreted GDNF may suggest that muscle retains an intracellular store of GDNF that may be released under certain circumstances. This has been shown to be the case with the neurotrophins (Poo, 2001), where these neurotrophic factors appear to be synthesized, stored and released upon demand.

In summary, these results suggest that GDNF production in skeletal muscle cells is regulated in an activity-dependent manner and can be modulated by chemical or electrical stimulation. Furthermore, the results demonstrate that the regulatory effect may change from an inhibitory effect on GDNF production at low levels of stimulation to an excitatory effect with long-term stimulation, suggesting that levels of GDNF protein being produced and/or secreted depend on physiological demands on the skeletal muscle cells.

3.5. ACKNOWLEDGEMENTS

Special thanks to Dr. Damon A. Miller (Electrical and Computer Engineering) and Benjamin VanDyken (Mechanical Engineering student) at Western Michigan University for designing and assembling the stimulation apparatus, based primarily on Marotta et al., 2004

This work was supported by NIH Grant 1R15AG022908-01A2, MSU-KCMS, and Western Michigan University

3.6. REFERENCES

An, M.C., Lin,W., Yang,J., Dominguez, B., Padgett, D., Sugiura,Y., Aryal, P., Gould,T.W., Oppenheim, R.W., Hester, M.E., Kaspar, B. K., Ko, C.-P., Lee, K.-F., 2010. Acetylcholine negatively regulates development of the neuromuscular junction through distinct cellular mechanisms. Proc. Ntl. Acad. Sci. 143, 10702–10707.

Angka, H.E., Geddes, A.J., Kablar, B., 2008. Differential survival response of neurons to exogenous GDNF depends on the presence of skeletal muscle. Dev. Dynam. 237, 3169–3178.

Bayol, S., Brownson, C., Loughna, P.T., 2005. Electrical stimulation modulates IGF binding protein transcript levels in C2C12 myotubes. Cell Biochem. Funct. 23, 361–365.

Bohn, M.C., 2004. Motoneurons crave glial cell line-derived neurotrophic factor. Exp. Neurol. 190, 263–275.

Choi, R.C.Y., Siow, N.L., Cheng, A.W.M., Ling, K.K.Y., Tung, E.K.K., Simon, J., Barnard, E.A.,Tsim, K.W.K., 2003. ATP acts via P2Y1 Receptors to stimulate acetylcholinesterase and acetylcholine receptor expression: transduction and transcription control. J.Neurosci.23, 4445–4456.

Cote, M.P., Azzam, G.A., Lemay, M.A., Zhukareva, V., Houle´, J.D., 2011. Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury. J.Neurotraum. 28, 299–309.

Donnelly, K., Khodabukus, A., Philip, A., Deldicque, L., Dennis, R. G., Baar, K. A., 2010. A novel bioreactor for stimulating skeletal muscle in vitro. Tissue Eng.16, 711–718.

Dudanova, I., Gatto, G., Klein, R., 2010. GDNF acts as a chemoattractant to support ephrin A-induced repulsion of limb motor axons. Curr. Biol. 20, 2150–2156.

Dupont-Versteegden, E. E., Houle´ , J. D., Dennis, R. A., Zhang, J., Knox, M., Wagoner, G., Peterson, C. A., 2004. Exercise-induced gene expression in soleus muscle is dependent on time after spinal cord injury in rats. Muscle Nerve 29, 73–81.

Eftimie, R., Brenner, H. R., Buonanno, A., 1991. Myogenin and myo-D join a family of skeletal muscle genes regulated by electrical activity. Proc. Natl. Acad. Sci. 88, 1349–1353.

Hashimoto, M., Nitta, A., Fukumitsu, H., Nomoto, H., Shen, L., Furukawa, S., 2005. . Involvement of glial cell line-derived Neurotrophic factor inactivation processes of rodent macrophages. J. Neurosci. Res.79, 476–487.

Henderson, C.E., Phillips, H.S., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M., Simpson, L.C., Moffet, B., Vandlen, R.A., Koliatsos, V.E., Rosenthal, A., 1994. GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle. Science 66, 1062–1064.

Houenou, L .J., Oppenheim, R.W., Li, L., Lo, A.C., Prevette, D., 1996. Regulation of spinal motoneuron survival by GDNF during development and following injury. Cell Tissue Res. 286, 219–223.

Jankovic, S. M., Kouvelas, D., Mirtsou-Fidani, V., 1998. Time course of isolated rat fundus response to muscarinic agonists: a measure of intrinsic efficacy. Physiol. Res. 47, 463–470.

Kramer, E. R., Knott, L., Su, F., Dessaud, E., Krull, C.E., Helmbacher, F., Klein, R., 2006. Cooperation between GDNF/Ret and ephrinA/ EphA4 signals for motor-axon pathways election in the limb. Neuron 50, 35–47.

Lee, H. H. C., Choi, R. C. Y., Ting, A. K .L., Siow, N. L., Jiang, J. X. S., Massoulie, J., Tsim, K. W. K., 2004. Transcriptional regulation of acetylcholine-associated collagen CoIQ .J. Biol. Chem., 279, 27098–3178.

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

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

Ling, K. K. Y., Siow, N. L., Choi, R. C. Y., Tsim, K. W. K., 2005. ATP potentiates the formation of AChR aggregate in the co-culture of NG108-15 cells with C2C12 myotubes. Fed. Eur. Biochem. Soc. Lett. 579, 2469–2474.

Longmore, J., Jani, B., Bradshaw, C. M., Szabadi, E., 1986. Effects of locally administered anticholinesterase agents on the secretory response of human accrine sweat gland to acetylcholine and carbachol. Br. J. Clin. Pharmacol. 21, 131–135.

Marotta, M., Bragos, R., Gómez-Foix, M., 2004. Design and performance of an electrical stimulation for long-term contraction of culture muscle cells. Biotechniques 36, 68–73.

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

Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K., Rosenthal, A., 1996. Renal and neuronal abnormalities in mice lacking GDNF. Nature 382, 789–792.

Naveilhan, P., ElShamy, W. M., Ernfors, P., 1997. Differential regulation of mRNAs for GDNF and its receptors ret and GDNFRα after sciatic nerve lesion in the mouse. Eur. J. Neurosci. 9, 1450–1460.

Nedachi, T., Fujita, H., Kanzaki, M., 2008. Contractile C2C12 myotubes model for studying exercise-inducible responses in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 295, 1191–1204.

Nguyen, Q.T., Parsadanian, A. S., Snider, W. D., Lichtman, J. W., 1998. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science 279, 1725–1728.

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

Poo, M.-M., 2001. Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. 2, 24–32.

Protas, L., Shen, J. B., Pappano, A.J., 1998. Carbachol increases contractions and intracellular Ca transients in guinea pig ventricular myocytes. J. Pharm. Exp. Ther. 284, 66–74.

Siow, N .L., Choi, R. C. Y., Cheng, A. W. M., Jiang, J. X. S., Wan, D.C.C., Zhu, S.Q.,

Tsim, K. W. K., 2002. Acyclic AMP-dependent Pathway regulate expression of acetylcholinesterase during myogenic differentiation of C2C12 cells. J.Biol. Chem. 277, 36129–36136.

Springer, J. E., Seeburger, J. L., He, J., Gabrea, A., Blanken horn, E .P.,1995. cDNA sequence and differential mRNA regulation of two forms of Glial and rat skeletal muscle. Exp. Neurol. 131, 47–52.

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

Suzuki, H., Hase, A., Kim, B.Y., Miyata, Y., Ikuya, N., Arahata, K., Akazawa, C., 1998b. Up-regulation of glial line-derived neurotrophic factor (GDNF) expression in regeneration muscle fibers in neuromuscular diseases. Neurosci. Lett. 257,165–167.

Taylor, P., Brown, J.H., 1999. Basic neurochemistry: Molecular, cellular and medical aspects. Sixth ed, Society for Neurochemistry, LaJolla, CA.

Thelen, M. H. M., Simonides, W. S., van Hardeveld, C., 1997. Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca 2+-ATPase gene.

Biochem. J. 321, 845–848.

Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Arenas, E., Ibanez, C., 1995. Peripheral expression and Biological activities gdnf, a new neurotrophic factor for avian and mammalian peripheral neurons. J.Cell Biol. 130, 137–148.

Tung, E. K. K., Choi, R. C. Y., Siow, N. L., Jiang, J. X. S., Ling, K. K. Y., Simon, J., Barnard, E. A., Tsim, K. W. K., 2004. P2Y2 receptor Activation regulates the expression of acetylcholinesterase and acetylcholine receptor genes at vertebrate neuromuscular junction. Mol. Pharmarcol. 66, 794–806.

Vianney, J.-M., Spitsbergen, J. M., 2011.Cholinergic neurons regulate secretion of glial cell line-derived neurotrophic factor by skeletal muscle cells in culture. BrainRes.1390, 1–9.

Wang, C. -Y., Yang, F., He, X.-P., Chow, A., Du, J., Russel, J.T., Lu, B., 2001. Ca 2+ binding protein frequenin mediates GDNF-induced potentiation of Ca 2+ channels and transmitter release. Neuron 32, 99–112.

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

Williams, R. S., Neufer, P. D., 2011. Regulation of gene expression in skeletal muscle by

contractile activity. Comprehensive physiology supplement 29: Handbook of physiology, Exercise: Regulation and Integration of Multiple Systems, pp. 1124–1150.

Xie, K., Wang, T., Alafsson, P., Mizuno, K., Lu, B., 1997. Activity-dependent expression of NT-3 in muscle cells in culture: implications in the development of neuromuscular junctions. J.Neurosci.17, 2947–2958.

Yafel, D., Saxel, O., 1977. Serial passaging and differentiation of Myogenic cells isolated from dystrophic mouse muscle. Nature 270, 725–727.

Yang, L .-X., Nelson, P. G., 2004. Glia cell line-derived neurotrophic factor regulates the distribution of acetylcholine receptors in mouse primary skeletal muscle cells. Neurosci. 128, 497–509.

Zebedin, E., Sandtner, W., Galler, S., Szendroedi, J., Just, H., Todt, H., Hilber, K., 2004. Fiber type convertion alters in activation of voltage-dependent sodium currents in murine C2C12 skeletal muscle cells. Am. J. Physiol. Cell Physiol. 287, 270–280.

Zhang, L., Ma, Z., Smith, G. M., Wen, X., Pressman, Y., Wood, P.M., Xu, X. M., 2009. GDNF-enhanced axonal regeneration and myelination following spinal cord injury is mediated by primary effects on neurons. Glia 57, 1178–1191.

Zwick, M.,Teng, L., Mu, X., Springer, J. E., Davis, B. M., 2001. Overexpression of GDNF induces and maintains hyperinnervation of muscle fibers and multiple end-plate formation. Exp.Neurol.171, 342–350.

CHAPTER 4

DIFFERENTIAL REGULATION OF GDNF AND NGF IN ATRIAL CARDIOMYOCYTES BY NEUROTRANSMITTER AND ELECTRICAL STIMULATION

4.1. INTRODUCTION

Neurotrophic factors have been shown to play a key role in neuronal growth, survival, and maintenance. In the peripheral nervous system, one major source of neurotrophic factors are the target tissues found in the periphery (Trupp et al., 1995). It has been shown that motor neurons in the periphery depend in part on the neurotrophic factors supplied by their targets (Bohn, 2004; Zwick et al., 2001). The heart is a target tissue supplied by both sympathetic and parasympathetic branches of the autonomic nervous system, and it is the most innervated target tissue in the periphery (Mitchell, 1952). The heart was also shown to express neurotrophic factors including nerve growth factor (NGF: Crowley et al., 1994; Furukuwa et al., 1984; Govoni et al., 2011; Levi-Montalcini, 1953 & 1987; Rana et al., 2011).

The expression of NGF in the heart and its support of the autonomic nervous system have been extensively examined. NGF was found to be critical for survival, nerve patterning, and development of the sympathetic neurons innervating the heart (Levi-Montalcini, 1987; Vo and Tomlinson, 1999). However, regulation of NGF expression in relation to heart function is not fully understood (Reviewed by Govoni et al., 2011; Ieda and Fukuda, 2009). Recent studies have also reported the expression of other neurotrophic factors including GDNF family ligands in the mammalian heart (Hiltunen et al., 2000; Martinelli et al., 2002; Rana et al., 2011). Results from other investigators have started

shedding light on the preferences of autonomic neurons for different neurotrophic factors. While neurturin (NRTN) a second member of the GDNF family was reported to be exclusively associated with cholinergic (parasympathetic) neurons in the heart (Hiltunen et al., 2000; Mabe et al., 2006), GDNF was shown to affect both noradrenergic (sympathetic) and cholinergic (parasympathetic) neurons (Martinelli et al., 2002; Rana et al., 2011).

GDNF levels have been shown to be elevated in chemically sympathectomized rats, possibly to support sympathetic nerve regeneration (Martinelli et al., 2002). GDNF was also shown to have neurotrophic effect on parasympathetic neurons in culture (Buj-Bello et al., 1995) and was found to be essential for parasympathetic nerve development *in vivo* and *in vitro* (Enomoto et al., 2000; Hasan, 2013). These observations suggest that GDNF may play a significant role in supporting these two antagonizing branches of autonomic systems. Although two GDNF family ligands are expressed in the heart and have been shown to exert neurotrophic activity (Hiltunen et al., 2000; Hoover et al., 2004; Martinelli et al., 2002), factors that regulate their synthesis and release are yet to be understood. This current study examines the regulation of expression of GDNF and NGF in cardiac muscle. The question being asked is whether the regulation of GDNF and NGF production by cardiac muscle is regulated in a similar manner.

In the current study, we utilized HL-1 cells, a murine atrial-derived cell line that continuously contracts in culture: 1) to explore the expression of GDNF and NGF, 2) to examine the roles of acetylcholine and norepinephrine on GDNF and NGF expression, and 3) to study the effect of electrical activity on levels of GDNF and NGF expression.

Results suggest that GDNF and NGF may be regulated differently by neurotransmitters released by sympathetic or parasympathetic neurons. The effect of direct electrical stimulation on GDNF and NGF production depends on the frequency and duration of stimulation. The results also suggest that HL-1 atrial cells can serve as useful model that can be used to study the cellular regulation of neurotrophic factor expression in the heart.

4.2. EXPERIMENTAL PROCEDURE

The experimental procedures used in this study have been explained in detail in chapter two. However, the following are brief explanations, including modifications for specific sets of studies under this section. All drugs were purchased from Sigma unless otherwise noted.

4.2.1. Cell culture procedure

HL-1 cells were used in this study because they display *in vitro* phenotypic characteristics of adult cardiomyocytes, including the ability to contract continuously in culture (Claycomb et al., 1998). The cells were obtained as a gift from Dr. W. Claycomb (Lousiana State University Medical Center, New Orleans, LA) and were grown according to Dr. Claycomb's Lab protocol (personal communication) with minor modifications. Briefly, culture medium consists of Claycomb medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY), 1% norepinephrine and 1% L-glutamine. All culture plates were obtained from USA Scientific, Inc. Ocala, FL, unless stated otherwise. Before seeding cells, plates were coated with a mixture of 0.02% gelatin (Fisher Scientific, Fair Lawn, NJ) and 0.5% fibronectin.

HL-1 cells were initially seeded on 100-mm plates for 4–5-days. This time in culture allowed the cells to reach confluence and differentiate to become contractile. To remove the cells from 100-mm plates, 3 ml of 0.05% trypsin/EDTA was added twice. The first addition of trypsin/EDTA was followed by 2 minutes incubation in standard incubator, followed by a 3 minute incubation after the second addition of trypsin/EDTA. Equal amount of soybean trypsin inhibitor was added to stop the activity of trypsin. The plate then was rinsed using 6 ml of wash medium (consists of Claycomb medium supplemented with 5% FBS and 1% antibiotic-antimycotic). Cells were centrifuged at 500 x g for 5 minutes. Supernatant was removed by aspiration and the cell pellets were gently suspended in 3 ml of supplemented Claycomb medium. The cells then were transferred to 6- or 12-well plates for experimental procedures. All treatment procedures were performed on differentiated, contracting cells. All cultures were maintained at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. The medium was renewed daily.

4.2.2. Sample collection and cell harvesting

All treatments were performed on differentiated, contracting cells. To determine GDNF or NGF protein concentration in culture medium, a 1ml sample of culture medium was collected from each culture dish between 0, 1, 2, 4, and 24 hours following treatment and kept at -20°C. To harvest cells, culture medium was removed, cells were washed with Dulbecco's phosphate buffered saline (DPBS). The cells were scraped from the dish using a cell lifter (USA Scientific). To examine intracellular GDNF or NGF, cells were spun in a cold centrifuge at 13.5 x g and supernatant was removed and stored at -20°C. GDNF or NGF protein content were measured by an enzyme-linked immunosorbent assay (ELISA). GDNF or NGF protein molecular sizes were determined by western blot analysis.

4.2.3. GDNF protein detection by ELISA

The GDNF ELISA procedure was performed as previously described in Chapter 3 and in Vianney and Spitsbergen, (2011). To determine NGF concentration in cells, NGF primary antibody (0.4µg/ml: R&D Systems, Minneapolis, MN, USA) and anti-NGF secondary antibody conjugated to biotin (100ng/ml: R&D Systems, Minneapolis, MN, USA) were used. Plate readings were taken every 30minutes until the standard curve was developed. For each assay a standard curve was calculated from known NGF concentration.

4.2.4. Protein localization by Immunocytochemistry

Procedures were adopted as previously described in Vianney and Spitsbergen, (2011) with minor modification. Briefly, cells were grown on cover slips pre-coated with gelatin/fibronectin. Cells were maintained at 37°C in a standard incubator and allowed to differentiate. Differentiated cells were washed with Dulbecco's phosphate buffer saline (DPBS) and fixed with 2.5% paraformaldehyde for 30 minutes at room temperature. Cells were then washed three times for 5 minutes each with DPBS. Cover slips were blocked with PBS containing 4% bovine serum albumin and 0.2% triton X-100 for 10 minutes. Cells were then incubated at 4° C overnight with a 1:100 dilution of goat anti-NGF (Abcam, Cambridge, MA), rabbit anti-GDNF (Santa Cruz Biotechnology, CA), or monoclonal antibody to sarcomeric myosin heavy chain (MF 20, Developmental Studies Hybridoma Bank, Iowa). Cells bound with primary antibodies were washed in PBS, followed by binding to secondary antibodies consisting of donkey anti-rabbit IgG conjugated to AlexaFluor® 488 (1:100), or donkey anti-mouse IgG conjugated to AlexFluor® 568 (1:100) and donkey anti-rabbit conjugated to AlexaFluor 647® (1:50)

(Life Technologies). Negative controls consisted of cover slips containing cell to which no primary antibodies were added. Slides were incubated for 2 hours at room temperature. Coverslips with cells were washed and mounted on a glass slide with 50%s glycerol/50% PBS and sealed. Images were captured using a Zeiss LSM 510 laser scanning confocal microscope.

4.2.5. Detection of GDNF protein size by Western blotting analysis

The size of the GDNF protein was determined by Western blotting, as previous described in Vianney and Spitsbergen (2011), but with some modifications. Culture medium and cell samples were loaded with Laemmli 2X loading buffer to make a final volume of 20µl. Controls consisted of a protein ladder (New England BioLabs, Ipswich, MA, USA) and GDNF protein (positive control, R&D Systems). The samples were boiled for 5 min and then loaded into a 15% polyacrylamide gel. The gel was run in separating buffer at two different voltages, 100V and 150V, respectively. The transfer of protein from the gel to the polyvinylidene difluoride (PVDF; Life Technologies) membrane was performed at 15V for 2h. The PVDF membrane was blocked with I-Block (Applied Biosystems, Foster City, CA, USA) for 1h at room temperature on a shaking platform. The membrane was incubated with a primary antibody against GDNF or NGF (Santa Cruz Biotechnologies) in I-Blocking buffer overnight at 4 °C on a shaking platform. The membrane was washed 3 times, for 5, 10, and 20 min, while shaking. The membrane was then incubated with an anti-rabbit HRP-conjugate secondary antibody (ECL; GE Healthcare) in I-Blocking buffer for 2h at room temperature, while shaking. GDNF or NGF protein bands were detected with Gel Logic 2200 Pro (Carestream, Molecular imaging)

with exposure time of 3–15 minutes, or with chemiluminsescence visualized on BioMax XAR film (Sigma) with exposure times of 1-3 minutes.

4.2.6. Treatment with neurotransmitters

Cells were grown on 12-well plates as previously described. On the day of each experiment, fresh culture medium containing norepinephrine (NE: 0.1mM and 1mM) or acetylcholine (ACh: 0.1µM, 1µM, 100µM) was added to differentiated HL-1 cells. Samples of conditioned culture medium and harvested cells were taken after 30 min, 1h, 2h, 4h and 24h. Control plates were processed in the same manner except that no NE or ACh was added. Both harvested cells and culture medium samples were stored at -20°C. GDNF or NGF protein content was measured by ELISA.

4.2.7. Blockade of NE and ACh effects

HL-1 cells were grown on 12-well plates and allowed to differentiate and start contracting. Cells were treated with fresh medium containing 100µM atropine or 100µM propranolol for 25 min. Following incubation with antagonists, the cells were washed twice with fresh culture medium and fresh medium pretreated with NE or ACh was added. Controls consisted of plates without treatments and plates that were treated with NE or ACh without antagonists. Samples were collected at intervals of 30min, 1h, 2h, 4h, and 24h. GDNF or NGF protein content was measured by ELISA.

4.2.8. Electrical stimulation

Electrical stimulation apparatus used in this work based on Marotta et al., (2004) included the use of switched semicircular electrodes as part of a culture dish lid, a series coupling capacitor (0.47µF) and current sampling resistors (approximately 100Ω).

Following Donnelly et al. (2010) stainless steel electrodes were used instead of platinum electrodes used by Marotta et. al. (2004) and Thelen et al. (1997). A Grass Technologies S88 stimulator provided voltage pulses to a custom interface box. Electrodes were secured to the culture dish lid with bolts, which also provided convenient wire connection points using wires with spade lugs. Within the interface box a unity gain voltage buffer provided current to up to six pair of electrodes connected to six switches, which enabled each pair of electrodes to be individually selected. Cells were stimulated at 1Hz or 5Hz for 30min, 90min, 12h, 24h, and 48h. Typical pulse amplitudes were in the range of 20-25V, with approximate 30 ms widths at the voltage buffer output. Using this apparatus, three wells were stimulated simultaneously and three wells served as controls. All cultures were maintained at 37°C in water-saturated incubator of 95% air and 5% CO_2 during stimulation periods. GDNF or NGF protein content was determined by ELISA.

4.2.9. Statistical analysis

Statistical analysis was performed using a student's t-test or analysis of variance (ANOVA) followed by Tukey's test. P value ≤ 0.05 were considered statistically significant. All data values are reported as the mean ± standard error of the mean (SEM).

4. 3. RESULTS

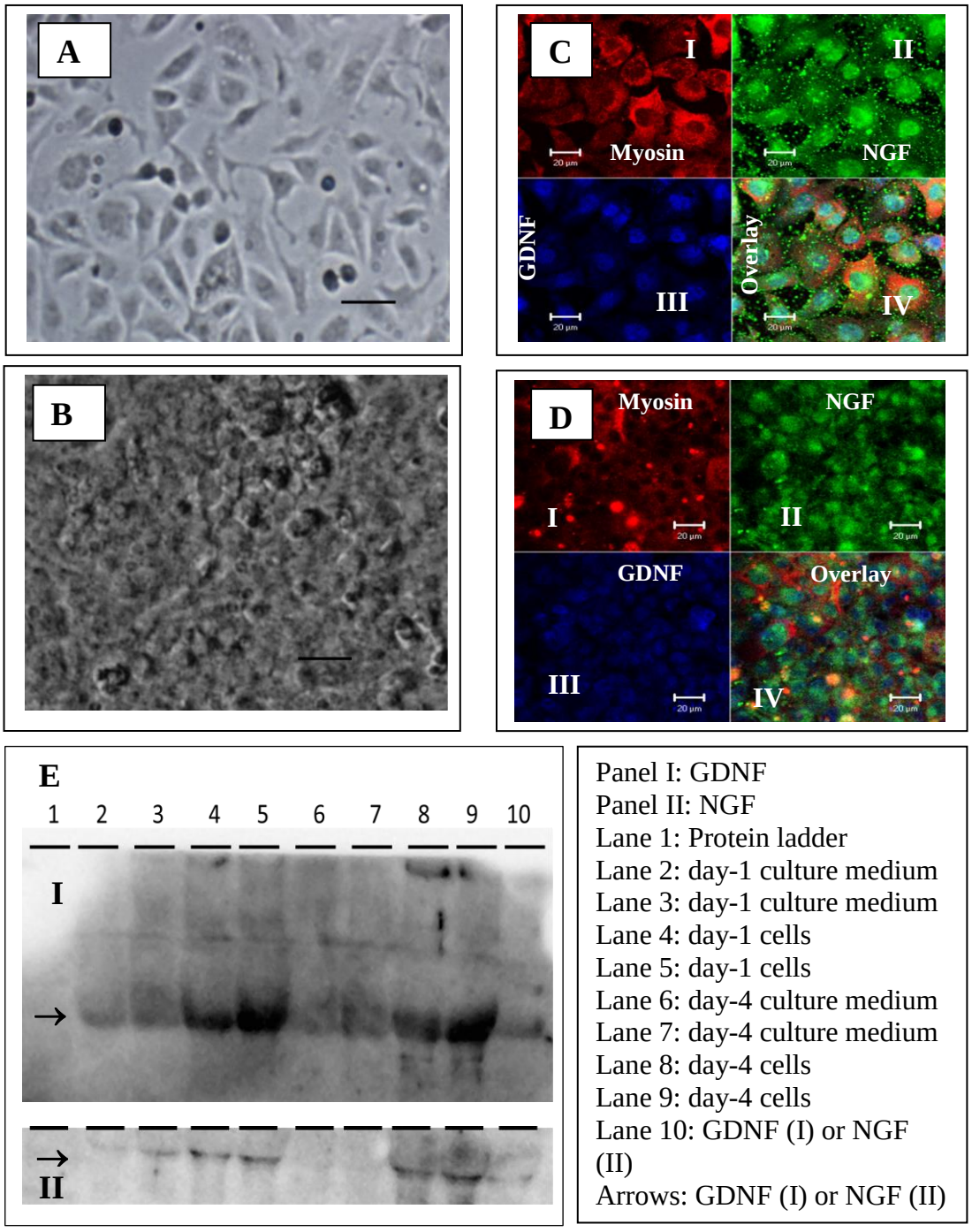
4. 3. 1. Characterization of GDNF and NGF

We first observed the growth of atrial cells and found that contraction starts on day 2 to 3, when cells start fusing to form a single layer. Figure 4. 1 panel (A) shows the seeded HL-1 cells 24h after sub-culturing (1-day-old); no contraction is observed at this stage of

growth. Figure 4.1 panel (B) shows 4-day-old cells, at this stage cells have fused to make a single layer and contract continuously.

The results confirmed that HL-1 cells express GDNF and NGF and that expression starts early after seeding; that is, both non-contractile and contractile cells were shown to synthesize the neurotrophic factors. Figure 4.1 panel (C) and panel (D) show 1-day-old and 4-day-old HL-1 cells, respectively, immunolabeled for GDNF and NGF. Both GDNF and NGF seem to colocalize with sarcomeric myosin but the proteins do not colocalize with each other (Fig. 4.1C, panel II-IV & Fig. 4.1D, panels II-IV). In addition, GDNF in 1-day-old cell seem to be mostly localized in the nucleus compare to the 4-day-old cells. GDNF and NGF produced by HL-1 cells were also released into culture medium. Western blotting analysis was performed to determine the molecular size(s) of both GDNF and NGF that are produced by HL-1 atrial cells. Results showed that the cell express GDNF and NGF of the molecular sizes of about 45kDa and 27kDa, respectively, panel (E).

Characterization of GDNF and NGF in HL-1 cells



Panel I: GDNF
Panel II: NGF
Lane 1: Protein ladder
Lane 2: day-1 culture medium
Lane 3: day-1 culture medium
Lane 4: day-1 cells
Lane 5: day-1 cells
Lane 6: day-4 culture medium
Lane 7: day-4 culture medium
Lane 8: day-4 cells
Lane 9: day-4 cells
Lane 10: GDNF (I) or NGF (II)
Arrows: GDNF (I) or NGF (II)

Figure 4.1. HL-1 cells express GDNF and NGF. Cells grown to full confluence started to contract continuously in culture. **Panel A**, shows non-contractile (1-day-old) and **panel B**, shows contractile (4-day-old) HL-1 cells as viewed at 20x magnification, bar = 30µm. For immunocytochemical processing, cells were grown on cover slips pre-treated with gelatin/fibronectin. Cells were fixed and bound with antibody against sarcomeric myosin heavy chain, goat anti-NGF, and rabbit anti-GDNF, followed by incubation in secondary antibodies conjugated to Alex Fluor® 568 (donkey anti-mouse IgG), AlexaFluor® 488 (donkey anti-goat), or Alex Fluor® 647 (donkey anti-rabbit IgG). Slides were viewed using a laser scanning confocal microscope (Zeiss LSM 510). **Panel C**, shows non-contractile (1-day-old) and **panel D**, contractile (4-day-old) cells. For both C and D: panels I, sarcomeric myosin (red); II, NGF (green); III, GDNF (blue); IV, overlay. **Panel E**, GDNF and NGF molecular sizes, as detected by Western blot analysis. GDNF band is ~ 45kDa, and NGF band is ~27kDa.

GDNF and NGF protein released into culture medium was quantified by ELISA. Neurotrophic factor levels retained in cells and that secreted into culture medium were shown to differ. The cells retained higher concentrations of intracellular GDNF or NGF than was released into culture medium (Fig. 4.2A panels I & II). It was also observed that the levels of GDNF and NGF synthesized or secreted by the cells significantly differ. The levels of GDNF secreted were about 200-300pg/ml compared to NGF which ranged from 20-30pg/ml (Fig. 4.2B). Similarly, intracellular GDNF was about 300-500pg/ml, compared to NGF content of 30-40pg/ml (Fig. 4.2C).

Intracellular and secreted GDNF and NGF content in HL-1 cells

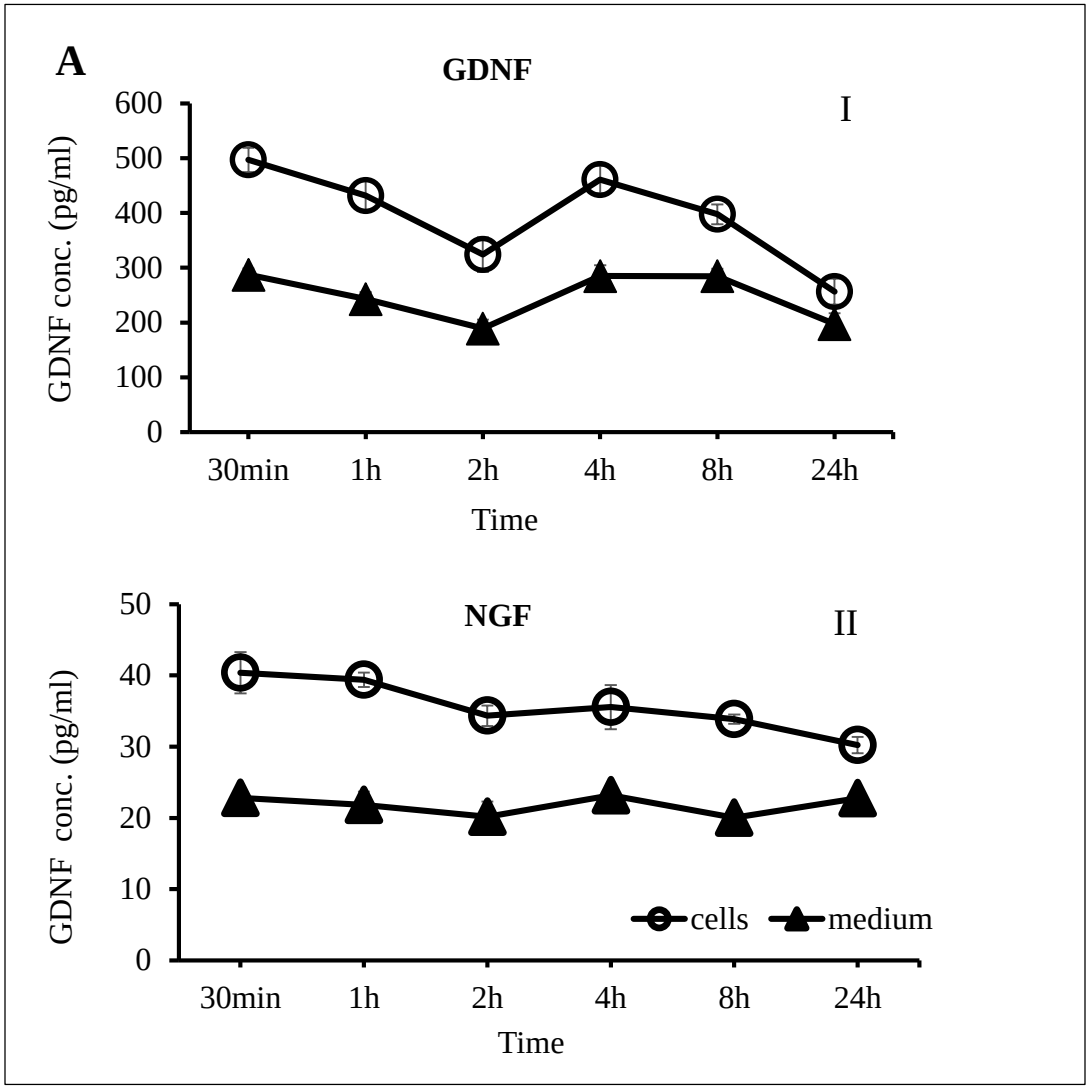


Figure 4.2A. Levels of GDNF and NGF produced in HL-1 cells in culture. Cells were grown in 12-well plates and allowed to differentiate into contractile cells. GDNF and NGF protein content was determined by ELISA. HL-1 cells retain more intracellular GDNF and NGF than they release into culture medium. **Panel I**, GDNF and **panel II**, NGF. Values are presented as mean ± S.E.M. $P \leq 0.05$, N=8.

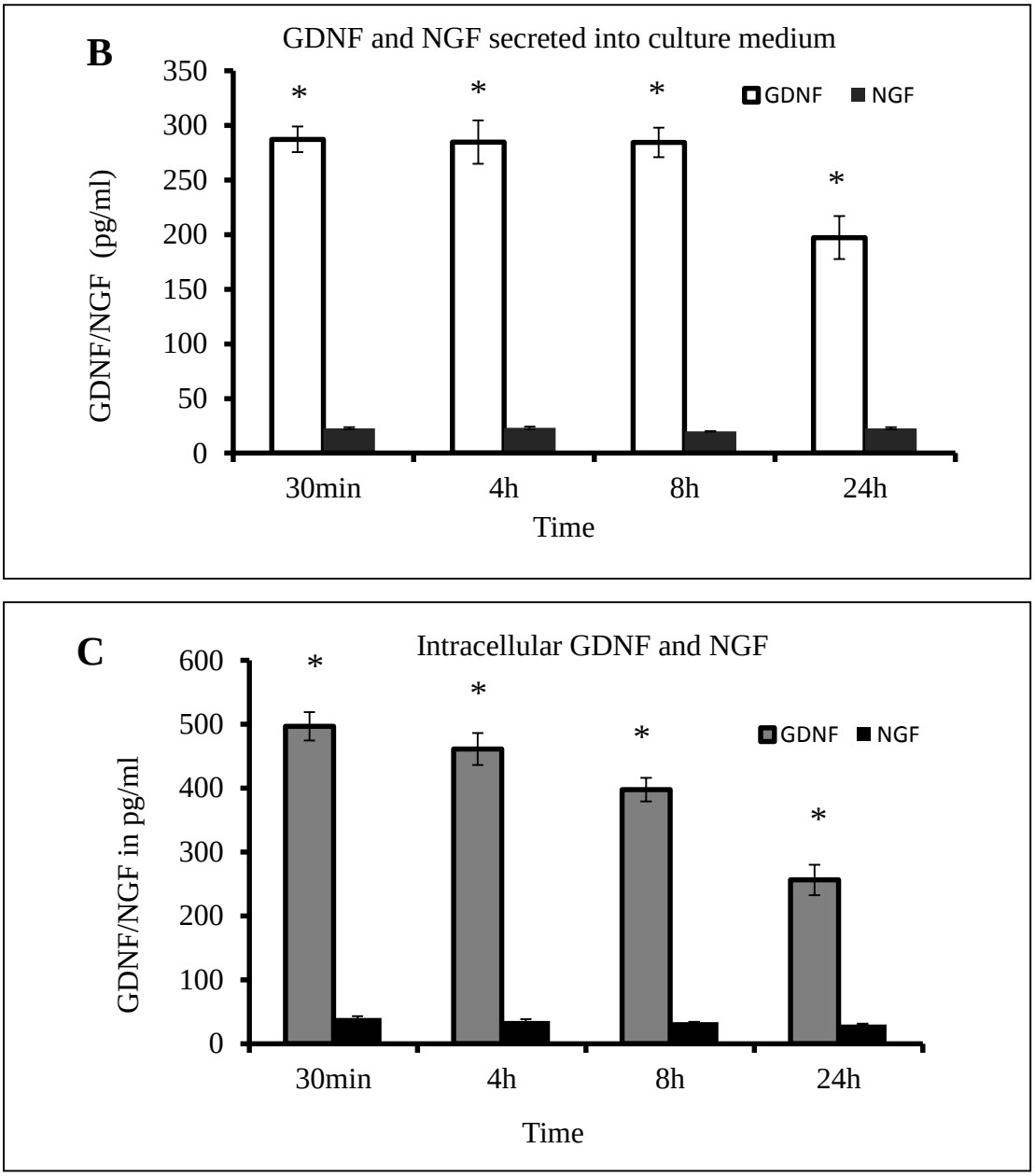


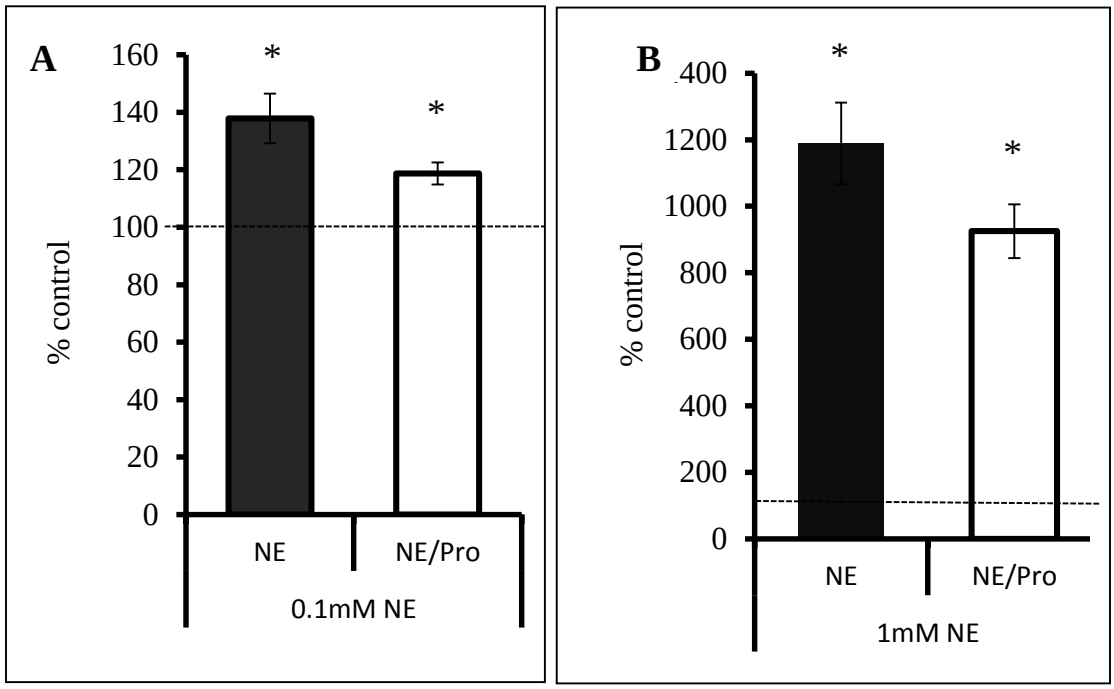
Figure 4.2B-C. Levels of GDNF and NGF produced by HL-1 cells in culture. Cells were allowed to differentiate into contractile cells as in **A** above. GDNF and NGF protein content was determined by ELISA. HL-1 cells express higher levels of GDNF than NGF.

B, GDNF and NGF secreted into culture medium and **C**, GDNF and NGF retained in cells. Values are presented as mean ± S.E.M. $P \leq 0.05$, $N = 8$.

4.3.2. Effects of norepinephrine on GDNF and NGF production in HL-1 cells

To test whether sympathetic neurons are involved in regulating GDNF and NGF production in HL-1 atrial cells, the cells were treated with different concentrations of norepinephrine (NE), (0.1mM, or 1mM). The results showed that the neurotransmitters appeared to have opposite effects on GDNF protein expression. GDNF protein content significantly increased in culture medium in samples collected after 2 hours following NE treatment (Fig. 4.3A & 4.3B). Also, it was noted that although both 0.1mM and 1mM increased GDNF, the higher concentration (1mM) significantly increased levels of GDNF about 8-fold (Fig. 4.3B). Contrasted with 2h samples, there was a significant decrease on GDNF protein levels in culture medium samples collected 24 hours after NE treatments (Fig.4.3C). For both time points, propranolol, a β-adrenergic receptor antagonist, did not reverse the action of NE on GDNF production (Fig. 4.3A-C). Similar results were observed for intracellular GDNF (data not shown).

Effects of NE on GDNF secretion by HL-1 cells at 2h



Effects of NE on GDNF secretion by HL-1 at 24h

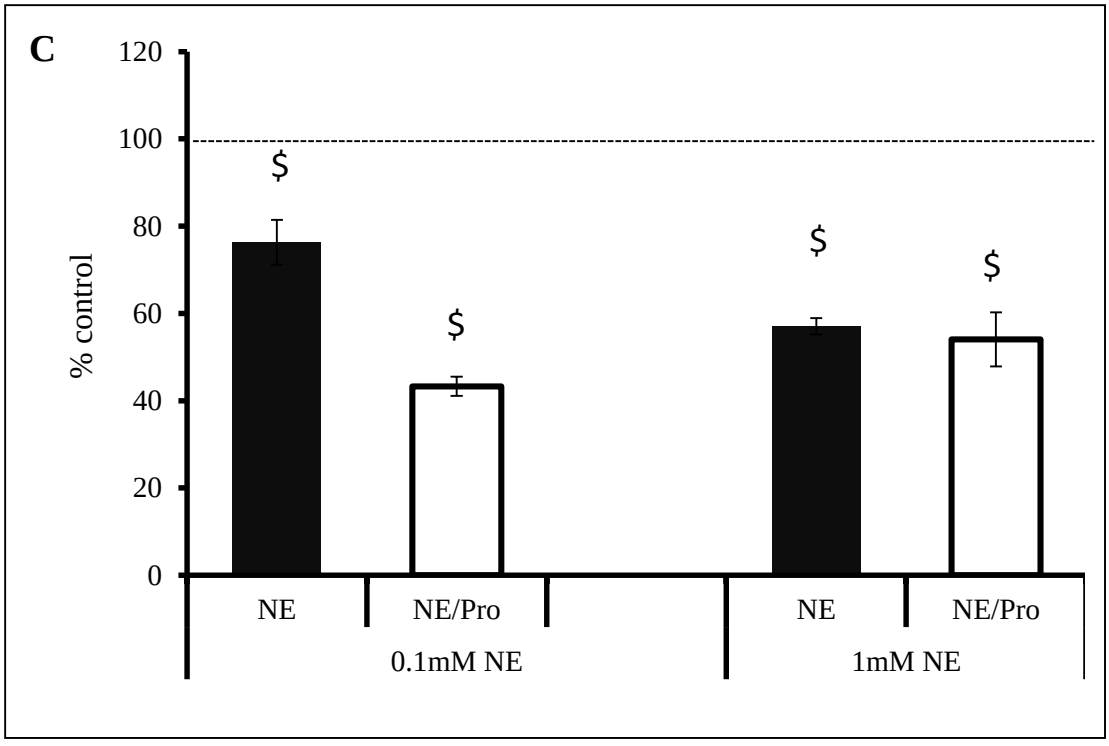
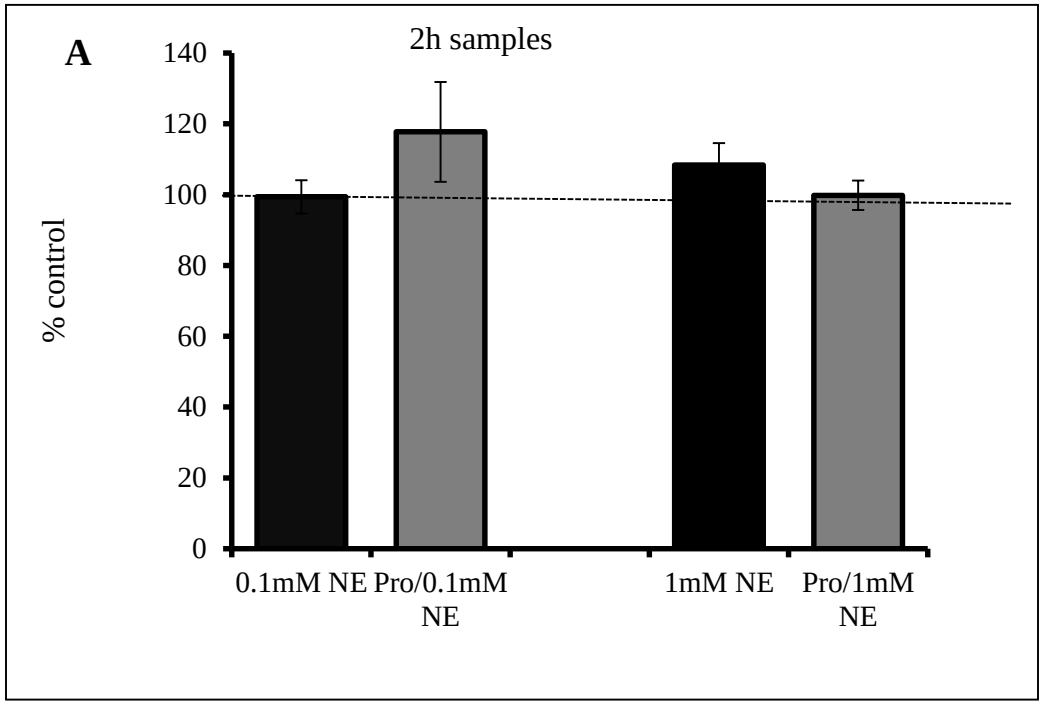


Figure 4. 3. Effect of norepinephrine on GDNF production. Cells were grown on 12-well plates and allowed to differentiate as in previous sections. Cells were treated with NE alone or NE with propranolol. GDNF protein content was measured by ELISA. **A & B**, NE increases GDNF secretion at 2h. **C,** NE decreases GDNF in 24h samples. Propranolol failed to reverse the action of NE, **A-C**. An asterisk (*) indicates a significant increase from control. A ($) sign indicates a significant decrease from control, P≤ 0.05. Values are presented as mean ± S.E.M, N= 6.

Results also showed that NE had no effect on NGF protein synthesized and released in culture medium 2h following NE treatment (Fig. 4.4A). However, high concentration of NE (1mM) showed a significant increase in NGF protein in culture medium samples collected 24h after treatment (Fig. 4.4B). The effect of NE treatment on intracellular NGF was similar (data not shown).

Effect of Norepinephrine on NGF production



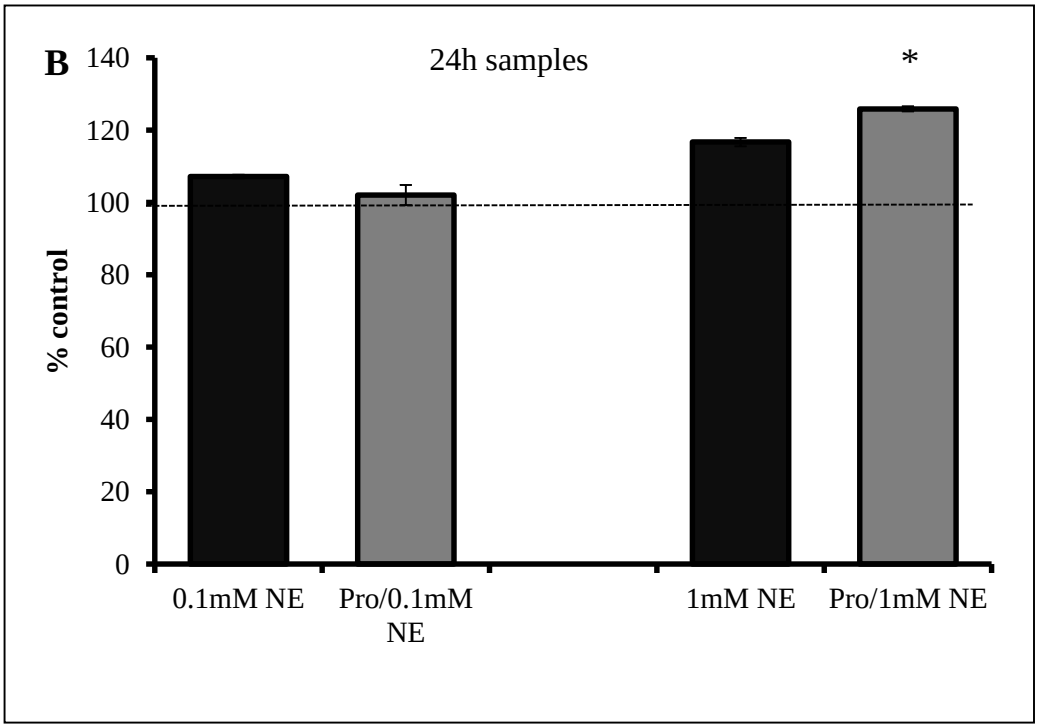
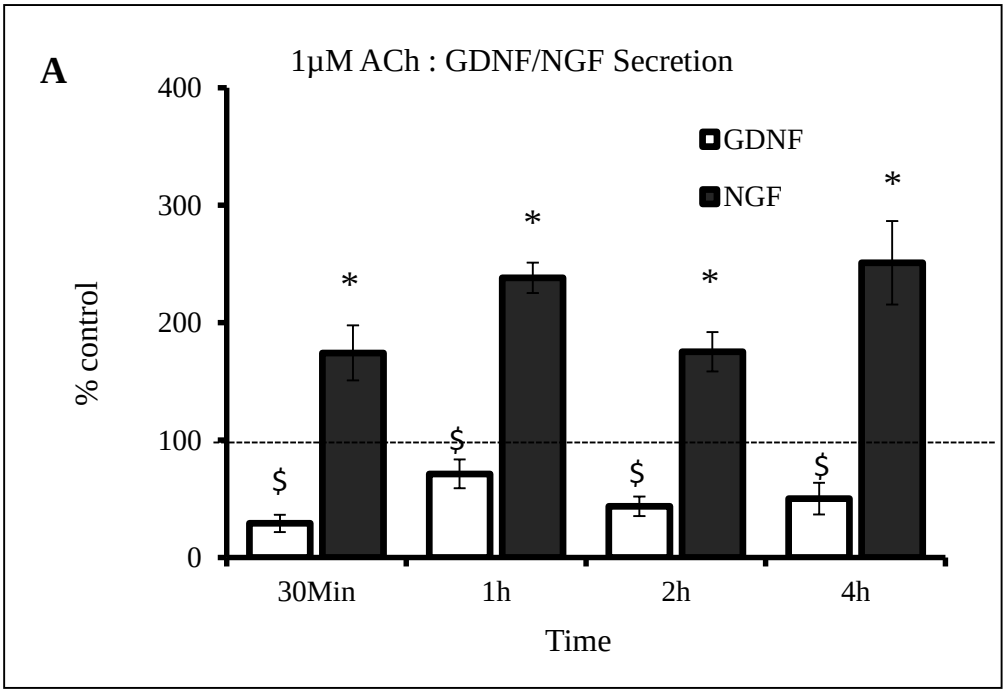


Figure 4.4. Effect of norepinephrine on NGF production. Differentiated cells were treated with NE alone or NE with propranolol (Pro). **A**. NE had no effect on NGF production at 2h. **B**. Treatment with 1mM NE increased NGF secretion at 24h. **A-B**. Propranolol had no effect on NGF production. NGF protein content was quantified by ELISA, $P \leq 0.05$. Values are presented as mean ± S.E.M, N= 6.

4.3.3. Effect of acetylcholine on GDNF and NGF in HL-1 cells

Next, we asked whether cholinergic neurons may regulate GDNF or NGF in the heart. In order to examine whether parasympathetic nerve fibers innervating the heart play a role in regulating GDNF or NGF expression, cells were treated with ACh at 1 or 100µM. Samples were collected between 30min to 4h as in previous studies (Chapter 3 and Vianney et al., 2014), ACh showed no effect after 24h. Treatment with ACh had opposite effects on GDNF and NGF expression in HL-1 cells. While application of 1µM ACh significantly decreased GDNF protein secretion (Fig. 4.5A) and content in cells (Fig. 4.5B), the same concentration of ACh increased levels of NGF protein in culture medium and cells (Fig. 4.5A and 4.5B).

Effects of acetylcholine on GDNF and NGF production by HL-1 cells



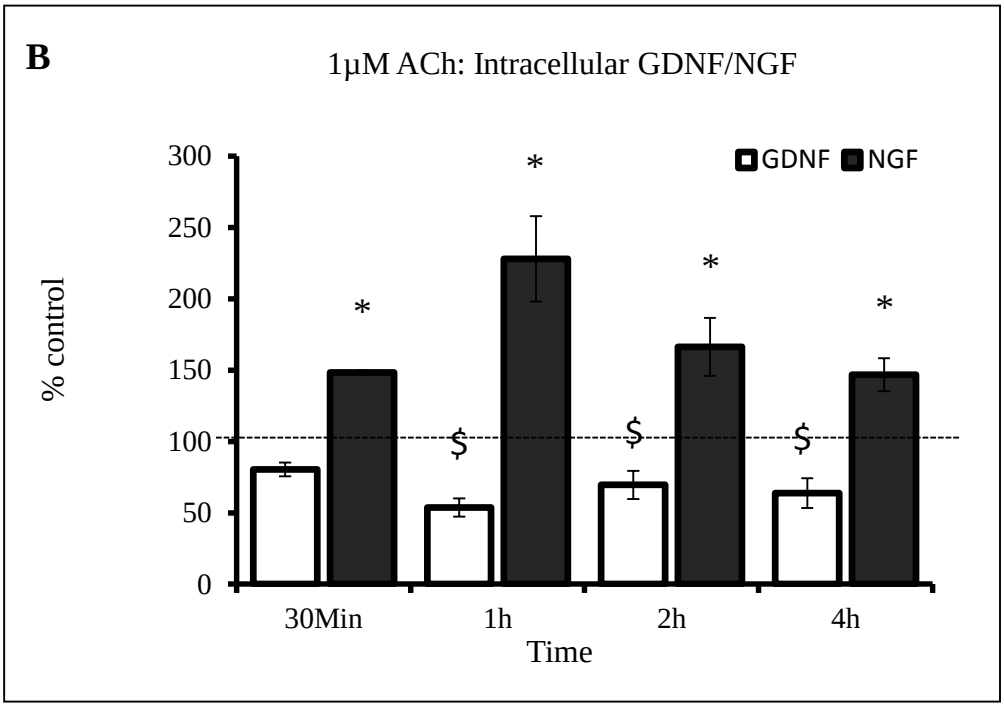
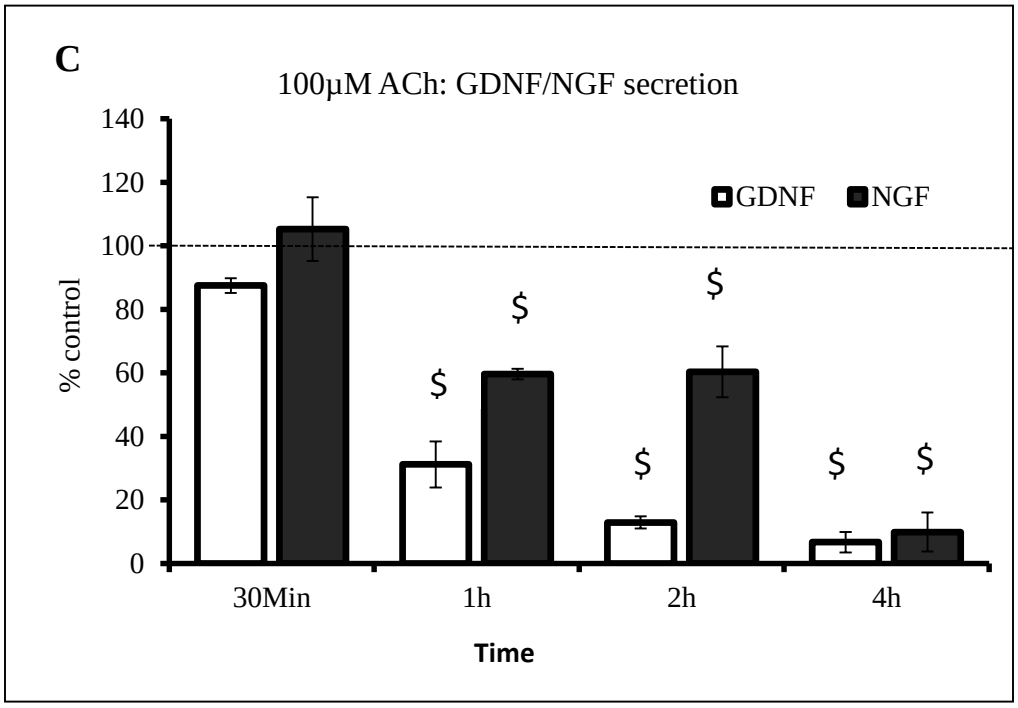
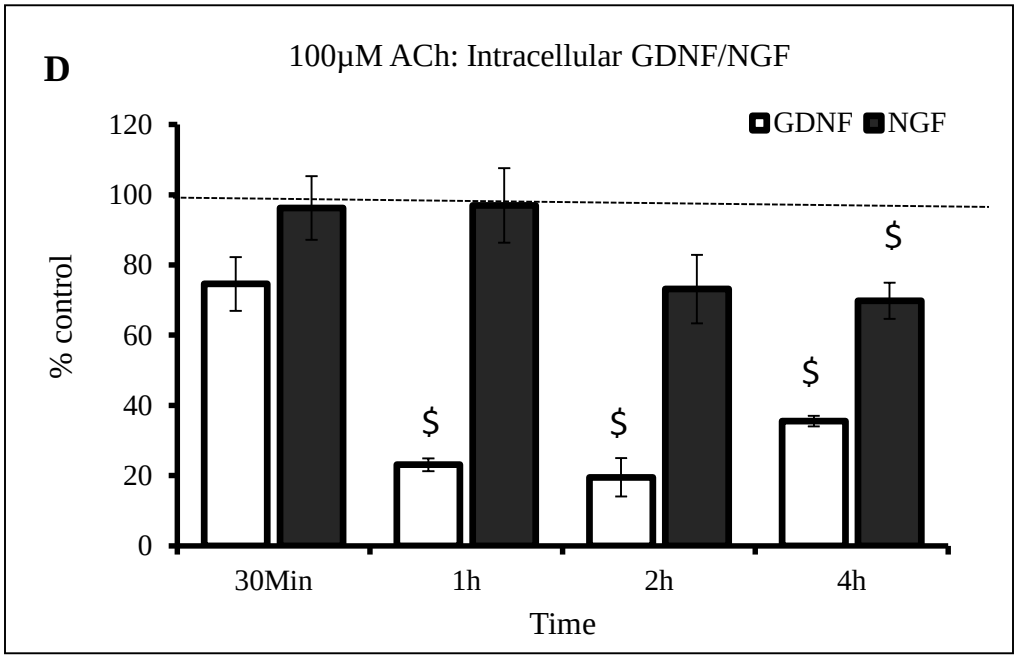


Figure 4.5 A-B. Effect of ACh on GDNF and NGF production. Cells were grown as in previous experiments. **A.** 1μM ACh decreases GDNF and increases NGF content in culture medium. **B**. Similar results were observed for intracellular GDNF and NGF. An asterisk (*) indicates a significant increase and ($) sign indicates a significant decrease. Values are presented as mean ± S.E.M, N= 6.

Application of 100μM ACh decreased both NGF and GDNF secretion (Fig. 4.5C). The effect of ACh on NGF release seemed to be time dependent as the reduction in secretion increased with time, from no effect at 30min to about 90% reduction at 4h (Fig. 4.5C). The reduction in intracellular NGF was only significant at 4h (Fig. 4.5D). Cells were also treated with atropine, a non-specific antagonist for mAChRs to block the effect of ACh. Results showed that atropine failed to reverse the action of ACh on NGF (data not shown) and GDNF production (Fig. 5E).

Effects of acetylcholine on GDNF and NGF production by HL-1 cells





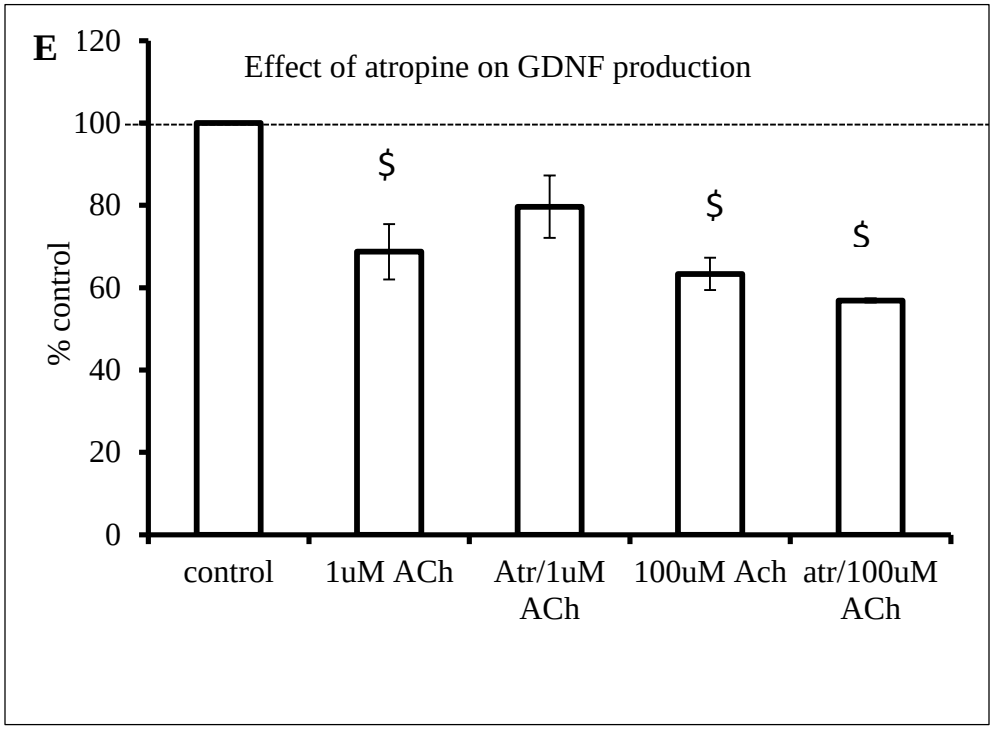


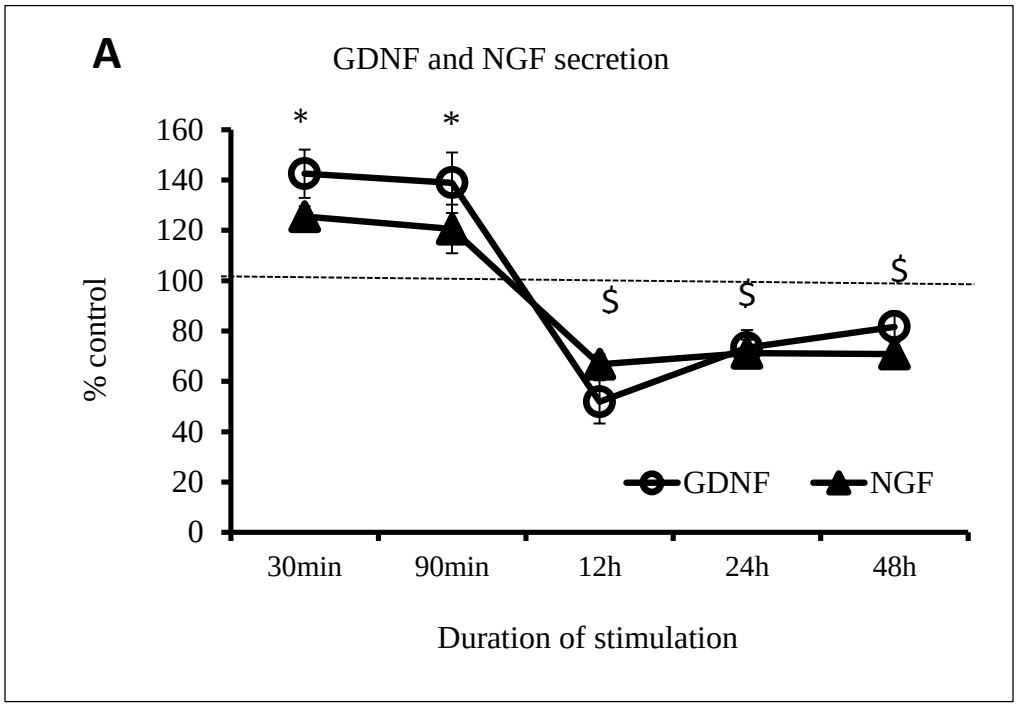
Figure. 4.5C-E. Effect of ACh on GDNF and NGF production. Cells were grown and allowed to differentiate as in previous experiments. **C**. ACh (100µM) decreases GDNF and NGF secretion into culture medium. **D**. The same concentration of ACh decreases intracellular NGF and GDNF. Atropine shows no effect on the production of GDNF (**E**) and NGF (data not shown). A dollar ($) sign indicates a significant decrease from control, $P \leq 0.05$. Values are presented as mean ± S.E.M. N = 6.

4.3.4. Effect of electrical stimulation

Short-term stimulation was defined as stimulation applied for 30 and 90 min whereas 12 to 48 hours of electrical stimulation was regarded as a long-term electrical stimulation. Cells remained healthy and intact during short- and long-term electrical

stimulation. Stimulation at 1Hz for 30 or 90 min resulted in a rapid increase in secretion of both GNDF (140% of control) and NGF (120% of control). The excitatory effect shifted to an inhibitory effect when the cells were stimulated for 12 – 48 hours (Fig. 4.6A). Intracellular GDNF and NGF were elevated when cells were stimulated for 30 minutes and significantly declined when cells were stimulated for 12, 24, and 48 hours (Fig. 4.6B).

Effects of 1Hz electrical stimulation on GDNF and NGF production



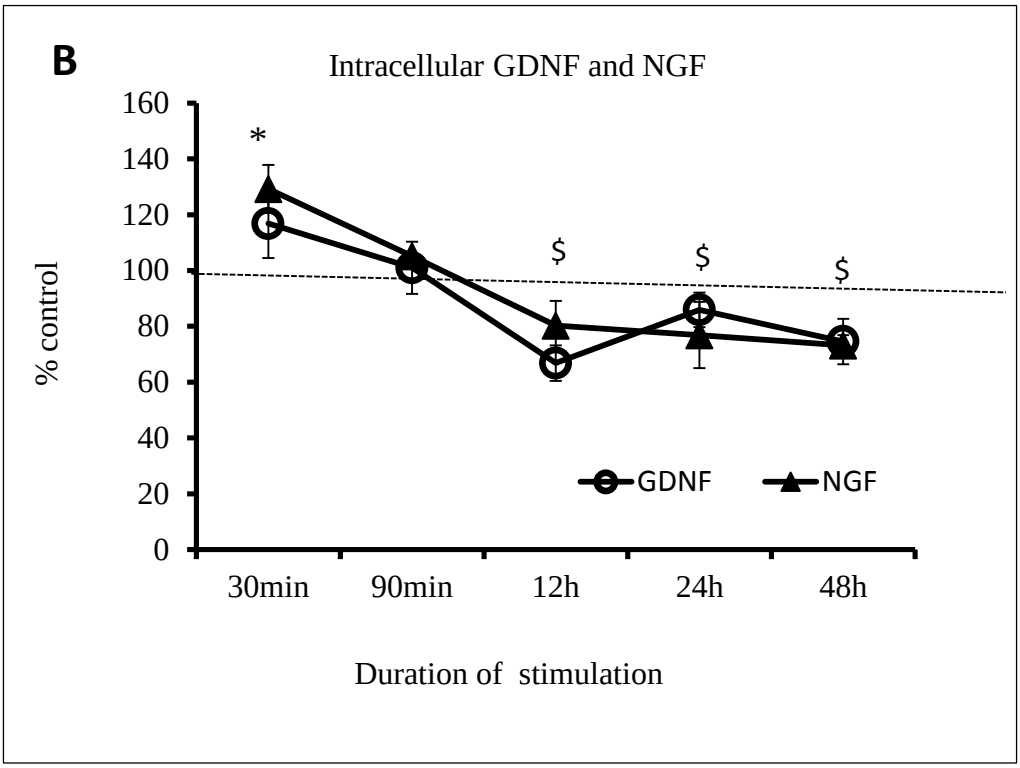
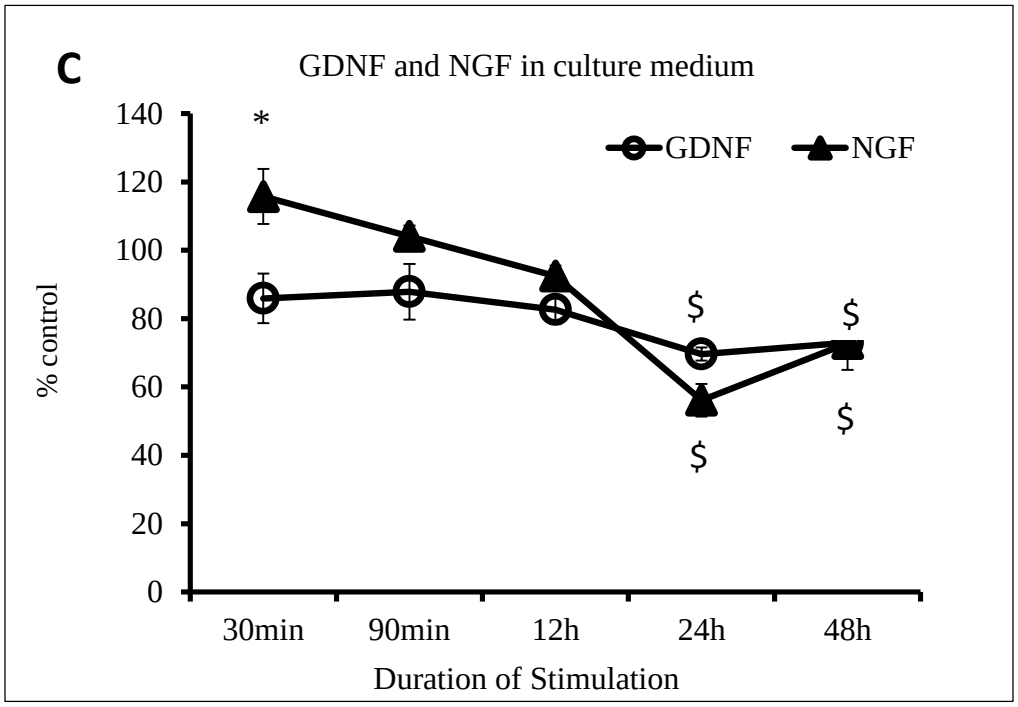


Figure. 4.6 A-B. Effect 1Hz electrical stimulation on GDNF and NGF production. Cells were grown in 6-well plates and allowed to differentiate. Cells were stimulated at 1Hz for 30min, 90min, 12h, 24h, and 48h. Short-term stimulation exerts an excitatory effect, while the long-term stimulation has an inhibitory effect on GDNF and NGF expression in HL-1 cells. GDNF and NGF secreted in culture medium, **A**, and intracellular GDNF and NGF, **B**. Protein content of GDNF or NGF was determined by ELISA. Asterisk (*) indicates a significant increase and a dollar sign ($) indicates a significant decrease for both GDNF and NGF. Values are presented as mean ± S.E.M. $P \leq 0.05$, N= 6.

The activity dependency of trophic factor production has been documented (Poo, 2001). To examine whether increased cardiomyocyte activity would alter GDNF and NGF

protein synthesis and release, the frequency of stimulation was increased to 5Hz. Results showed that a 5Hz frequency appeared to have different effects on GDNF and NGF during short-term electrical stimulation. GDNF secretion in HL-1 cells was inhibited about 20% at all time points, while a significant increase in NGF secretion was observed at 30min of electrical stimulation followed by a gradual decline (Fig. 4.6C). Intracellular GDNF was reduced about 20% in electrically stimulated cells (Fig. 4.6D). Also a brief rise to intracellular NGF was observed at 90min of stimulation, followed by a decline when cells were stimulated for 12 to 48hrs (Fig. 4.6D).

Effects of electrical stimulation on GDNF and NGF production in HL-1 cells at 5Hz



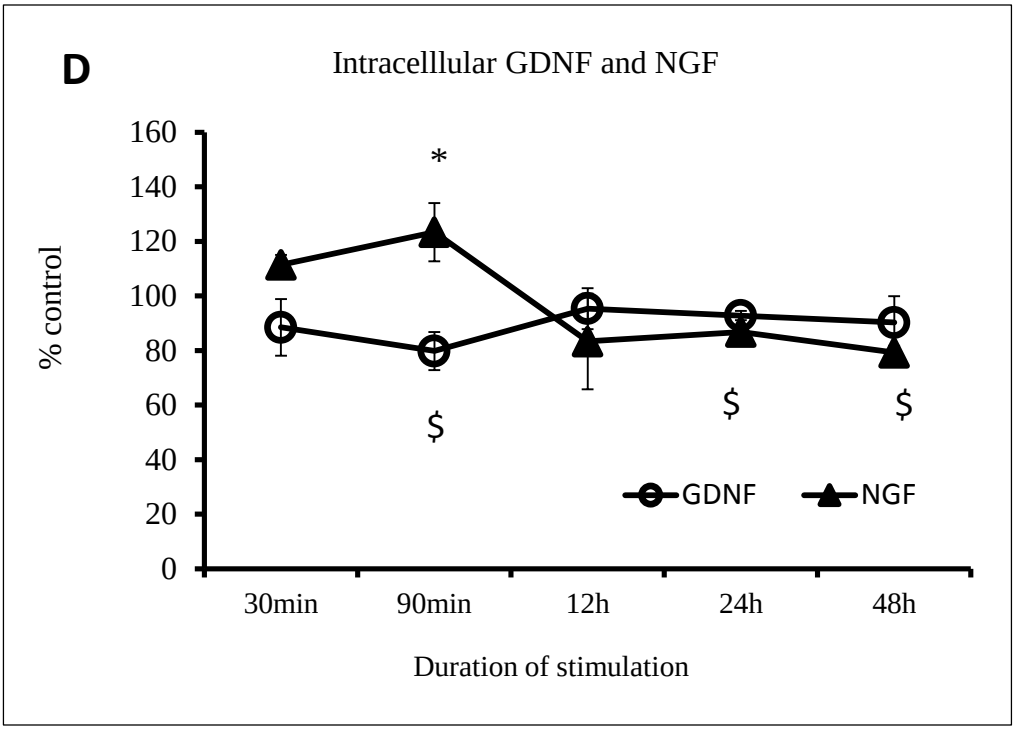


Figure 4.6 C-D. Effect of 5Hz electrical stimulation on GDNF and NGF production. Stimulation at 5Hz increased NGF secretion at 30min and decreased NGF and GDNF secretion at 24 and 48h. An asterisk (*) indicates a significant increase and a dollar sign ($) indicates a significant decrease for GDNF or NGF. Values are presented as mean ± S.E.M. $P \leq 0.05$, N=6.

4.4. DISCUSSION

In the current study we examined regulation of expression of the neurotrophic factors GDNF and NGF in HL-1 atrial cells. The levels of GDNF and NGF protein contents were measured following treatments with neurotransmitters or direct electrical stimulation. Results from this study suggest that acetylcholine, norepinephrine, and electrical

stimulation regulate GDNF and NGF production in HL-1 cells. The major observations include that 1) acetylcholine and norepinephrine have differential effects on GDNF and NGF production by HL-1 cells. 2) The direction of effect on GDNF or NGF production levels changes with the duration of exposure to and/or concentration of a neurotransmitter. 3) Low frequency electrical stimulation has similar effect on both GDNF and NGF production by the cells; whereas, high frequency electrical stimulation has opposite effects. 4) The excitatory or inhibitory effects on GDNF or NGF expression levels depends on duration and/or frequency of stimulation.

HL-1 cells were developed to serve as an *in vitro* model of adult atrial myocytes (Claycomb et al., 1998; White et al., 2004). The cells have been used in various studies including those examining cardiomyocyte growth (Brady et al., 2007; Nibbelink et al., 2007), cell signaling (Discoll et al., 2006), metabolism (Palanivel et al., 2006; Pineiro et al., 2005), electrophysiology (Yang et al., 2005), and response to pharmacological treatments (Chaudary et al., 2004). Here we report that HL-1 cells also express GDNF and NGF, neurotrophic factors that are utilized by sympathetic and parasympathetic neurons (Levi-Montalcini, 1987; Martinelli et al., 2002). Expression of GDNF and NGF proteins in HL-1 cardiomyocytes are observed in all times in cultures. However, the dynamics of GDNF and NGF protein production by HL-1 cells differ significantly. This suggests that each neurotrophic factor is regulated independently and possibly depends on demand for each neurotrophic factor.

We also examined whether neurotransmitters that are released by sympathetic and parasympathetic neurons regulate GDNF and NGF expression in HL-1 cells. Our results suggest that acetylcholine and norepinephrine have effects on production of GDNF and

NGF indicating that the neurotransmitters have a role in regulating the synthesis and secretion of the neurotrophic factors in cardiac muscle. Interestingly, GDNF and NGF are regulated in opposite manner by these neurotransmitters. We also found that the direction of the effects of each neurotransmitter depends on the duration of exposure and the concentration of neurotransmitter; with higher concentrations of norepinephrine causing a greater activation of GDNF secretion. Long-term exposure to norepinephrine results in inhibition of GDNF secretion, while increasing NGF production. One of the interpretations may be that changes in GDNF and NGF production with time may depend on changes in neural demand.

Treatments with norepinephrine appear to upregulate NGF in astroglial and fibroblast cells (Furukuwa et al., 1989). Other studies however, have showed that administration of norepinephrine resulted in decreasing NGF in iris (Hellweg et al., 1988), brown adipocytes (Nisoli et al., 1996), cardiomyocytes (Kaye et al., 2000), and heart (Kimura et al., 2010; Qin et al., 2002). Similar to the observation in the current study, in these studies, time and concentration of norepinephrine were shown to be important factors in determining the direction of changes in NGF in tissues.

Decreased production of GDNF following treatment with acetylcholine is a similar effect to what has been observed in skeletal muscle (McCullough, 2011; Vianney et al., 2014). This effect of acetylcholine on GDNF production may help explain the inhibitory effect of cholinergic neurons on GDNF production in skeletal muscle (Vianney and Spitsbergen, 2011).

We also observed that when high concentration of carbachol, an acetylcholine agonist, was applied to HL-1 cells, the rate of contraction of the cells decreased from two

beats per second (120bpm) to ~0.3 beats per seconds (18bpm). Slowing of heart rate has been shown to be mainly associated with activation of M_2 muscarinic acetylcholine receptors (mAChRs) (Stengel et al., 2000). In this study, treatment of HL-1 cells with acetylcholine causes GDNF production to decrease. It may be suggested that GDNF production in cardiac muscle is activity-dependent and the neurotrophic factor is regulated by acetylcholine via M_2 receptors. On the other hand, acetylcholine acts on upregulating NGF production, which is predominantly associated with sympathetic fibers that raise heart rate.

Adrenergic and muscarinic receptor blocking agents, propranolol and atropine used in this study, show small effects. One of the reasons may be the concentrations used. Another reason may be the specificity of the receptors as propranolol and atropine are non-specific blocking agents. Future studies will consider dose responsive curves to determine the minimum and maximum effect of these agents. Also, the studies will use specific blocking agents for adrenergic receptors as well as muscarinic receptors.

One of the aims of electrical stimulation studies was to bypass the action of neurotransmitters in the signaling cascades that may be regulating GDNF or NGF. Expression of GDNF and NGF is also regulated by electrical activity (Rana et al., 2011; Saygili et al., 2011). In this study, we found that the effect of electrical stimulation is not uniform, rather, the direction of the effects changes with frequency and duration of exposure. The rise and fall of neurotrophic factor production observed with electrical stimulation, can be correlated with a biphasic effect observed in heart. The physiological response to ACh was believed to be caused by action of both excitatory and inhibitory pathways utilizing M_1, M_2, or M_3 receptors, respectively (Kitazawa et al., 2009; Tanaka et

al., 2001). These receptors in turn, are coupled with different G-proteins that are involved in stimulatory or inhibitory pathways (Wang et al., 2004). Because electrical stimulation bypasses the mAChRs activation step, it will be of interest to examine whether the increase and decrease of GDNF production that are caused by a short- and long-term electrical stimulation, involve the G-proteins that are responsible for inhibitory or excitatory pathways.

In summary, the information presented here confirms that HL-1 atrial cells synthesize and secrete GDNF and NGF, and the production of these neurotrophic factors is regulated by acetylcholine, norepinephrine, and electrical stimulation. The results also show that the concentration of the neurotransmitters, frequency of electrical stimulation, and duration of treatments are the key factors in regulating the levels of GDNF and NGF production in HL-1 atrial cells. The results suggest that HL-1 cells can serve as useful model for the study of cellular regulation of neurotrophic factors in the heart. Finally, the results also suggest that GDNF and NGF production by cardiac muscle cells may be differentially regulated by sympathetic and parasympathetic nervous system via neurotransmitter release.

4.5. ACKNOWLEDGEMENTS

Special thanks to Dr. Damon A. Miller (Electrical and Computer Engineering) and Benjamin VanDyken (Mechanical Engineering student) at Western Michigan University for designing and assembling the stimulation apparatus, based primarily on Marotta et al., 2004.

Thanks to Jorge Ochoa, high school student at KCMS working in Dr. John Spitsbergen Laboratory for videotaping cardiac cell contraction in culture.

This work was supported by NIH Grant 1R15AG022908-01A2, MSU-KCMS, and Western Michigan University

4.6. REFERENCES

Bohn, M.C., 2004. Motoneurons crave glial cell line-derived neurotrophic factor. Exp. Neurol.190, 263–275.

Brady, N.R., Hamacher-Brady, A., Yuan, H., Gottlieb R.A., 2007. The autophagic response to nutrient deprivation in the hl-1 cardiac myocyte is modulated by Bcl-2 and sarco/endoplasmic reticulum calcium stores. FEBS. 274, 3184–3194.

Buj-Bello, A., Buchman, V.L., Horton, A., Rosenthal, A., Davies, A.M., 1995. GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron. 15, 821–828.

Chaudary, N., Naydenova, Z., Shuralyona, I., Coe, I.R., 2004. The adenosine transporter, mENT1is a target for adenosine receptor signaling and protein kinase Cϵ in Hypoxic and pharmacological preconditioning in the mouse cardiomyocyte cell line, HL-1. J. Pharmacol. Exp. Therapeutics 310, 1190–1199.

Claycomb, W.C., Lanson, N.A. Jr., Stallworth, B.S., Egeland, D.B., Delcarpio, J.B., Bahinski, A., Izzo, N.J. Jr., 1998. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc. Natl. Acad. Sci. 95, 2979–2984.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M,P., 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76, 1001–11.

Donnelly, K., Khodabukus, A., Philip, A., Deldicque, L., Dennis, R.G., Baar, K.A., 2010. A novel bioreactor for stimulating skeletal muscle in vitro. Tissue Eng.16, 711–718.

Driscoll, W.J., Hill, D., Smalstig, A., Mueller, G.P., 2006. Murine atrial HL-1 cells express highly active peptidylglycine α-amidating enzyme. Peptides 27, 1547-1553.

Enomoto, H., Heuckeroth, R.O., Golden, J.P., Johnson, E.M., Milbrandt, J., 2000. Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. Dev. 127, 4877–4889.

Furukawa, Y., Furukawa, S., Satoyoshi, E., Hayashi, K., 1984. Nerve growth factor secreted by mouse heart cells in culture. J. Biol. Chem. 259, 1259–1264.

Furukuwa, Y., Tomioka, N., Sato, W., Satoyoshi, E., Hayashi, K., Furukawa, S., 1989. Catecholamines increase nerve growth factor mRNA content in both mouse astroglial cells and fibrolast cells. FEBS Lett. 2, 463–467.

Goldberg, L.I., Bloodwell, R.D., Braunwald, E., Morrow, A.G., 1960. The direct effects of norepinephrine, epinephrine, and methoxamine on myocardial contractile force in man. Circulation 22, 1125–1132.

Govoni, G., Pascalea, A., Amadioa, M., Calvillo, L., D'Elia, E., Ceredad, C., Fantuccie, P., Ceronif, M., Vanolih, E., 2011. NGF and heart: Is there a role in heart disease? Pharmacological Res. 63, 266–277.

Hasan, W., 2013. Autonomic cardiac innervation. Development and adult plasticity. Organogenesis 9, 176-193.

Hellweg, R.I., Bandtlow, C.E., Heumann, R., Korsching, S., 1988. Nerve growth factor synthesis in cultured rat iris: modulation by endogenous transmitter substances. Exp. Cell Res. 179, 18–30.

Hiltunen, J.O., Laurikainen, A., Airaksinen, M.S., Saarma, M., 2000. GDNF family receptors in the embryonic and postnatal rat heart and reduced cholinergic innervation in mice hearts lacking ret or GFRα2. Dev. Dynam. 219, 28–39.

Hoover, D.B., Ganote, C.E., Ferguson, S.M., Blakely, R.D., Parsons, R.L., 2004. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc. Res. 62, 112–121.

Ieda, M., Fukuda, K., 2009. Cardiac innervation and sudden cardiac death. Curr. Cardiol. Rev. 5, 289–295.

Kaye, D.M., Viddadi, G., Gruskin, S.L., Du, X-J., Esler M.D., 2000. Reduced myocardial nerve growth factor expression in human and experimental heart failure. Circ. Res. 86, e80–e84.

Kimura, K., Kanazawa, H., Ieda, M., Kawaguchi-Manabe, H., Miyake, Y., Yagi, T., Arai, T., Sano, M., Fukuda, K., 2010. Norepinephrine-induced nerve growth factor depletion causes cardiac sympathetic denervation in severe heart failure. Autonomic Neurosci. Basic and Clinical, 156, 27–35.

Kitazawa, T., Asakawa, K., Nakamura, T., Teraoka, H., Unno, T.,Komori S-I., Yamada, M., Wess, J., 2009. M3 muscarinic receptors mediate position inotropic responses in mouse atria: a study with muscarinic receptor knockout mice. J. Pharmacol. and Exp. Therapeutics 330, 487–493.

Levi-Montalcini, R., 1987. The nerve growth: thirty-five years later. The EMBO J. 6, 1154–1154.

Levi-Montalcini, R., Hamburger, V., 1953. A diffusible agent of mouse sarcoma producing hyperplasia of sympathetic ganglia and hyperneurotization of the chick embryo. J Exp. Zool. 123, 233–288.

Mabe, A.M., Hoard, J. L., Doffourc, M.M., Hoover, D.B., 2006. Localization of cholinergic innervation and neurturin knockout mice. Cell Tissue Res. 326, 57–67.

Marotta, M., Bragos, R., Gómez-Foix, M. 2004. Design and performance of an electrical stimulation for long-term contraction of culture muscle cells. Bio Techniques 36, 68–73.

Martinelli, P.M., Camargos, E.R.S., Morel, G., Ravares, C.A.P., Nagib, P.R.A., Machado, C.R.S., 2002. Rat heart GDNF: effect of chemical sympathectomy. Histochem. Cell Biol. 118, 337–343.

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

Mitchell, G.A.G., 1953. The innervation of the heart. Br Heart J. 15, 159–171.

Miwa, K., Lee, J-K., Takagishi, Y., Opthof, T., Fu, X., Hirabayashi, M., Watabe, K., Jimbo, Y., Kodama, I., Komuro I., 2013. Axon guidance of sympathetic neurons to cardimyocytes by glial cell line-derived neurotrophic factor (GDNF). PLoS ONE 8, e65202.

Nibbelink, K.A., Tishkoff, D. X., Hershey, S. D., Rahman, A., Simpson, R. U., 2007. 1,

25 (OH)2-vitamin D3 actions on cell proliferation, size expression, and receptor localization, in the HL-1 cardiac myocyte. Steroid Biochem, Mol. Biol.103, 533–537.

Nisoli, E., Tonello, C., Benarese, M., Leberini, P., Carruba, M. O., Expression of nerve growth factor in brown adipose tissue: implications for thermogenesis and obesity. Endocrinol. 137, 495–503.

Palanivel, R., Eguchi, M., Shuralyova, I., Coe, I., Sweeney, G., 2006. Distinct effects of short-and long-term leptin treatment on glucose and fatty acid uptake and metabolism in HL-1 cardiomyocytes. Metabolism Clinical and Exp. 55, 1067–1075.

Pineiro, R., Iglesias, M.J., Gallego, R., Raghay, K., Eiras, S., Rubio, J., Dieguez, C., Gualilo, O., Gonzalez-Juanatey, J.R., Lago, F., 2005. Adiponectin is synthesized and secreted by human and murine cardiomyocytes. FEBS Lett. 579, 5163–5169.

Poo, M.-M., 2001. Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. 2, 24–32.

Qin, F., Vulapalli R.S., Stevens, S.Y., Ling, C-S., 2002. Loss of cardiac sympathetic neurotransmitters in heart failure and NE infusion is associated with reduced NGF Am. J. Physiol. Heart Circ. Physiol. 282, H363–H371.

Rana, O.R., Saygili, E., Gemein, C., Zink M. D. H., Buhr, A., Saygili, E., Mischke K.,

Nolte, K. W., Weis, J., Weber, C. Marx, N., Schauerte, P., 2011. Chronic electrical neuronal stimulation increases cardiac parasympathetic tone by eliciting neurotrophic effects. Circ. Res. 108, 1209–1219.

Saygili, E., Schauerte, P., Küppers, F., Heck, L., Weis, J., Weber, C., Robert H.G. Schwinger, R. H. G., Hoffmann, R., Schröder, J. W., Marx, N., Rana, O. R., 2010. Electrical stimulation of sympathetic neurons induces autocrine/paracrine effects of NGF Mmediated by TrkA. J. Mol. Cellular Cardiol. 49, 79–87.

Stengel, P.W., Gomeza, J., Wess, J., Cohen, M., L., 2000. M2 and M4 receptor knockout mice: muscarinic receptor function in cardiac and smooth muscles in vitro. J Pharmacol and Exp. Therapeutics 292, 877–885.

Tanaka, H., Nishimaru K., Kobayashi, M., Matsuda, T., Tanaka Y., Shigenobu, K., 2001. Acetylcholine-induced positive inotropy mediated by prostaglandin released from endocardial endothelium in mouse left. Naunyn Schmiedebergs Arch Pharmacol. 363, 577–82.

Taylor, P., Brown, J.H., 1999. Basic neurochemistry: Molecular, cellular and medical aspects. Sixth ed, Society of Society for Neurochemistry, La Jolla, CA.

Thelen, M.H.M., Simonides, W.S., van Hardeveld, C., 1997. Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent

transcription of the fast-typesarcoplasmic-reticulum Ca2+-ATPasegene. Biochem. J. 321, 845–848.

Trupp, M., Rydén, M., Jörnvall, H., Funakoshi, H., Timmusk, T., Arenas, E., Ibáñez, C. F., 1995. Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J. Cell Biol. 130, 137–148

Vianney, J.-M., Spitsbergen, J.M., 2011. Cholinergic neurons regulate secretion of glial cell line- derived neurotrophic factor by skeletal muscle cells in culture. Brain Res. 1390, 1–9.

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

Vo, A.A., Tomlinson, D.R., 1999. The regeneration of peripheral noradrenergic nerves after chemical sympathectomy in diabetic rats: Effects of nerve growth factor. Exp. Neurol. 157, 127–134.

Wang, Z., Shi, H., Wang, H., 2004. Functional M3 mucarinic acetylcholine receptors in mammalian hearts. Br. J. Pharmacol. 142, 395–408.

White, S.M., Constantin, P.E., Claycomb, W.C., 2004. Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. Am. J. Physiol. Heart Circ. Physiol. 286, H823–H829.

Yang, Z., Shen, W., Rottman, J.N., Wikswo, J.P., Murray, K.T., 2005. Rapid stimulation causes electrical remodeling in cultured atrial myocytes. J. Mol. Cell. Cardiol. 38, 299-308.

Zwick, M., Teng, L., Mu, X., Springer, J.E., Davis, B.M., 2001. Overexpression of GDNF induces and maintains hyperinnervation of muscle fibers and multiple end-plate formation. Exp. Neurol. 171, 342–350.

CHAPTER 5

A COMPARISON OF EFFECT OF NEUROTRANSMITTER AND ELECTRICAL STIMULATION ON GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR EXPRESSION IN VOLUNTARY AND INVOLUNTARY MUSCLE

5. 1. INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is regarded as a potent survival factor for sub-populations of neurons including somatic and autonomic motor neurons. These neurons have been shown to depend, in part, on GDNF synthesized and secreted by their target tissues (Henderson et al., 1994; Martinelli et al., 2002; Shneider et al., 2009). It has been shown that a number of different tissues in the periphery express GDNF (Moore et al., 1996; Sariola and Saarma, 2003). However, these target tissues differ in their composition, function, and in the case of different muscle cell types, their contractile characteristics. Whether the processes regulating GDNF production in these different tissues is similar or different is poorly understood.

Neuronal-muscle co-culture studies suggest that neurons may regulate the levels of GDNF synthesized and/or secreted by skeletal muscle (Vianney and Spitsbergen, 2011). Also, acetylcholine, a major neurotransmitter released by cholinergic neurons, has been shown to regulate GDNF production in skeletal muscle (Vianney et al., 2014). These two observations lead to the hypothesis that GDNF in target tissues may be in part, regulated by neurons via neurotransmitter release. Neuronal populations innervating muscle tissues differ in the type of neurotransmitters they release and the effects those transmitters exert on muscle contractile activity.

Preferential utilization of different neurotrophic factors by different branches of the nervous system has been suggested. In the somatic nervous system, cholinergic motor neurons have been shown to depend on GDNF secreted by skeletal muscle (Angka et al., 2008), while in the heart, GDNF supports both sympathetic and parasympathetic fibers (Enamoto, et al., 2000; Martinelli et al., 2002). Nerve growth factor (NGF) has been shown to be primarily associated with the sympathetic nerves (Heumann et al., 1984; Habecker et al., 2008). Neurturin (NTRN), another member of GDNF family ligands, was shown to be exclusively associated with cholinergic neurons in the autonomic nervous system (Hiltunen et al., 2000; Hiltunen and Airaksinen, 2004; Hoover et al., 2004; Mabe and Hoover, 2009). It is of interest to determine whether expression of these neurotrophic factors is regulated in a similar fashion in different target tissues. A better understanding of processes regulating GDNF expression in different target tissues may help to identify potential roles for altered GDNF expression in the development of nervous system dysfunction with disease and aging.

The current study examines GDNF expression in skeletal and cardiac muscle cells; these cells were used as study models for voluntary and involuntary muscles. The study examines and compares the effect of acetylcholine, a neurotransmitter released by both cholinergic neurons in the somatic and autonomic systems. Also, the effect of direct electrical stimulation (ES) on GDNF production in both muscle cell types is investigated. The similarities and differences between the effects of acetylcholine with that of electrical stimulation, on GDNF regulatory processes are discussed. The results suggest that electrical stimulation and exposure to acetylcholine cause different effects on GDNF expression in voluntary and involuntary muscles.

5. 2. EXPERIMENTAL PROCEDURE

This study was aimed at comparing GDNF production in voluntary and involuntary muscles at the cell level. To ensure controlled experimentation, experiments on voluntary and involuntary muscle cells were performed side-by-side to reduce variability that could occur if each cell type were examine alone. C2C12 skeletal muscle cells were selected as a model for voluntary muscle and HL-1 cardiac muscle cells were selected as a model for involuntary muscles.

5.2.1. HL-1 cell culturing

The HL-1 cells were obtained as a gift from Dr. W. Claycomb (Lousiana State University Medical Center, New Orleans, LA). The HL-1cells retain ultrastructural characteristics in vitro of adult atrial cardiac muscle cells, including the ability to spontaneously contract in culture (Claycomb, 1998). The cells were grown and maintained as previous described in Claycomb et al. (1998). Briefly, undifferentiated HL-1 cells were seeded onto 100-mm plates (USA Scientific, Inc.) pre-treated plates with fibronectin dissolved in 0.02% gelatin and maintained in Claycomb's Dulbecco's Modified Eagle's Medium (CDMEM), supplemented with 10% fetal bovine serum (Sigma), 1% antibiotic–antimycotic (Life Technologies, Grand Island, NY), 1% norepinephrine (Sigma) and 1% L-glutamine (Sigma). Cells were incubated at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2. Three-day-old cardiac cells were subcultured (transferred) and allowed to grow in 12-well plates. For all experiments, cells were grown in 12-well plates except for electrical stimulation studies where 6-well plates were used (USA Scientific, Inc.). The medium was renewed with fresh culture medium every 1 to 2 days until

treatment. All treatments were performed on confluent cultures which were spontaneously contracting.

5.2.1.1. Sample collection for HL-1 cardiac cell

To determine GDNF protein concentrations in culture media, conditioned samples were collected from each culture dish 30min to 24h following treatments. To harvest cells, culture medium was removed, cells were washed with Dulbecco's phosphate buffered saline (DPBS: Sigma, St. Louis, MO) and 1ml of sample buffer (a mixture of phosphate buffered saline, 0.005% Tween-20, 0.5% bovine serum albumin, 0.1mM benzethonium chloride, 2mM benzemidine, 0.4M NaCl, 2mM EDTA and 164µl/100ml aprotinin) was added. Cells were scraped from the dish using a cell lifter (USA Scientific, Ocala, FL). To examine intracellular GDNF, cells were spun in a cold centrifuge at 13.5 x g and supernatant was removed and stored at -20°C. GDNF protein content in each experiment was measured by an enzyme-linked immunosorbent assay (ELISA) as described in Vianney and Spitsbergen (2011).

5.2.2. C2C12 cell culturing

The C2C12 mouse skeletal muscle cell line was purchased from American Type culture collection (ATTC: Manassas, VA, USA). The C2C12 cell line was extracted from 2-month old mouse thigh muscle and have been used by researchers because of their in vivo skeletal muscle phenotype, including expression of contractile proteins (Ling et al., 2005; Yafel and Saxel, 1997). C2C12 culturing procedure were performed according to the ATTC protocols and as described by Vianney and Spitsbergen (2011), with some minor modifications. Briefly, C2C12 myoblasts were initially seeded on 100-mm plates (USA Scientific, Inc.) and maintained in Dulbecco's Modified Eagle's medium (DMEM: ATCC)

supplemented with 10% fetal bovine serum (Sigma) and 1% antibiotic-antimycotic (Life Technologyies, Carlsbad, CA, USA). Cells were incubated at 37°C in water-saturated atmosphere of 95% air and 5% CO_2. For all experiments cells were grown in 12-well plates except for electrical stimulation studies where 6-well plates were used (USA Scientific, Inc.). Cells were seeded as myoblasts and differentiation was induced by replacing the growth medium with DMEM supplemented with 10% horse serum and 1% antibiotic-antimyocotic. The medium was renewed every one to two days. Myotube studies were performed on myotubes that had been maintained in culture for 6 to 7 days.

5.2.2.1. Sample collection for C2C12 cells

To determine GDNF protein concentration in culture medium, conditioned samples were collected from each culture dish at 2, 4, and 24 h following treatment and kept at -20°C. To harvest cells, culture medium was removed, cells were washed with calcium/magnesium-free buffer and 1ml of sample buffer was added to each culture dish containing either myoblastas or myotubes. To harvest myoblasts, the cells were scraped from the dish using a cell lifter (USA Scientific, Ocala, FL). For myotubes, 1ml of sample buffer was added causing cells to detach from the culture dish. To examine intracellular GDNF, cells were spun in a cold centrifuge at 13.5 x g and supernatant was removed and stored at -20°C. GDNF protein content in each experiment was measured by ELISA.

5.2.3. Western blot analysis

To examine size of GDNF protein molecules, culture medium and cell samples were loaded with Laemmli 2X loading buffer to make a final volume of 20µl. Controls consisted of a protein ladder (New England BioLabs), GDNF protein (positive control) and NGF protein (negative control). The samples were boiled for 5 min then loaded into a 15%

polyacrylamide gel. The gel was submerged and run in separating buffer at two different voltages, starting at 100V then switched to 150V. The transfer of protein from the gel to the polyvinylidene difluoride (PVDF; Invitrogen) membrane was performed at 12V for 1h and 30min. The PVDF membrane was blocked with I-Block (Applied Biosystems, Foster City, CA, USA) for 1 h at 4 °C on a shaking platform. The membrane was then incubated with a primary antibody against GDNF (Santa Cruz Biotechnologies) in I-Blocking buffer solution overnight at 4 °C on a shaking platform. The membrane was washed for 5, 10, and 20 min with 1X phosphate buffered saline (PBS) while shaking. The membrane was then incubated with a HRP-conjugated secondary antibody (ECL; GE Healthcare) in I-Blocking buffer for 1 h at room temperature while shaking then washed with 1X PBS. GDNF protein was detected with chemiluminescence and was visualized on BioMax XAR film (Sigma) with exposure times of 1-3 min or with luminescence detection machine (Carestreem, GE Healthcare) with exposure time of 1-15min.

5.2.4. Treatment with acetylcholine

Acetylcholine (ACh) was used because it is the neurotransmitter that is released by both somatic motor neurons and parasympathetic neurons innervating the heart. For each experiment, old medium was removed and fresh medium containing 0.1µM, 1µM, or 100µM ACh was added to cells. Conditioned culture medium and harvested cell samples were taken at 2, 4, and 24 hours following treatments. Control plates were processed the same except that, no neurotransmitter was added. GDNF protein content was determined by ELISA.

5.2.5. Electrical stimulation of the cells

Cells were electrically stimulated as in previous studies (Vianney et al., 2014). Cells were stimulated at 1Hz or 5Hz with 24V 30ms pulse for 30min – 48hours. All cultures were maintained at 37°C in water-saturated incubator of 95% air and 5% CO_2 during stimulation periods.

5.2.6. Statistical analysis

Statistical analysis was performed using a Student's t-test. P values ≤ 0.05 were considered statistically significant. All data values are reported as the mean ± standard error of the mean (S.E.M).

5. 3. RESULTS

5.3.1. GDNF protein in skeletal and cardiac muscle cells

Results from western blot revealed that both skeletal and cardiac muscle cells expressed and secreted GDNF with a molecular size approximately 45kDa (Figure 5.1). Differentiated skeletal muscle cells (myotubes) contained an additional band with molecular size around 25kDa, and this band was only observed in culture medium samples (Figure 5.1A). Cardiac muscle cells express GDNF with a single molecular size of 45kDa (Figure 5.1B).

GDNF molecular sizes in skeletal and cardiac muscle cells

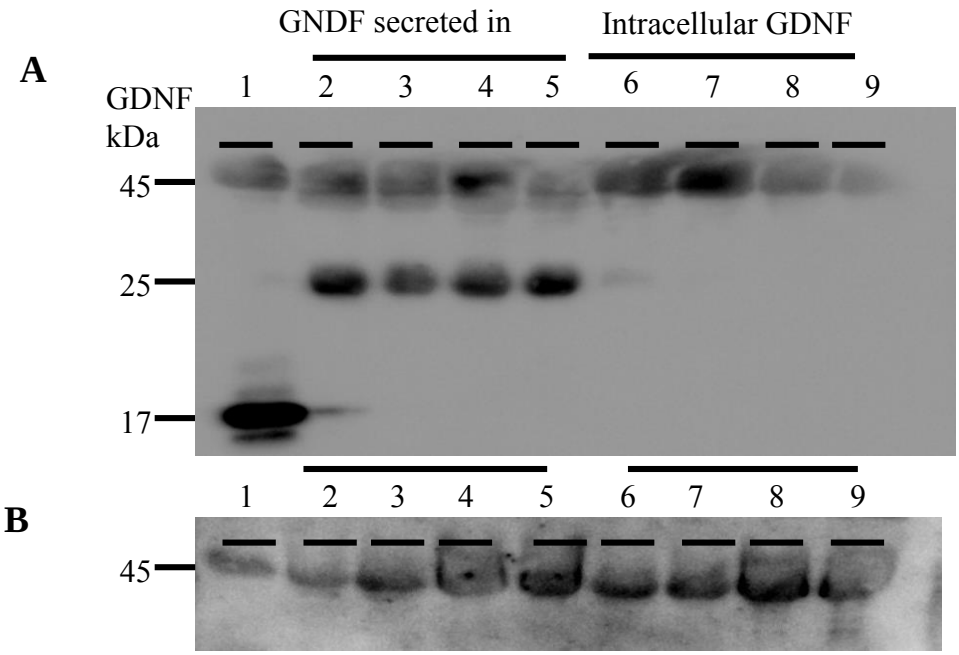
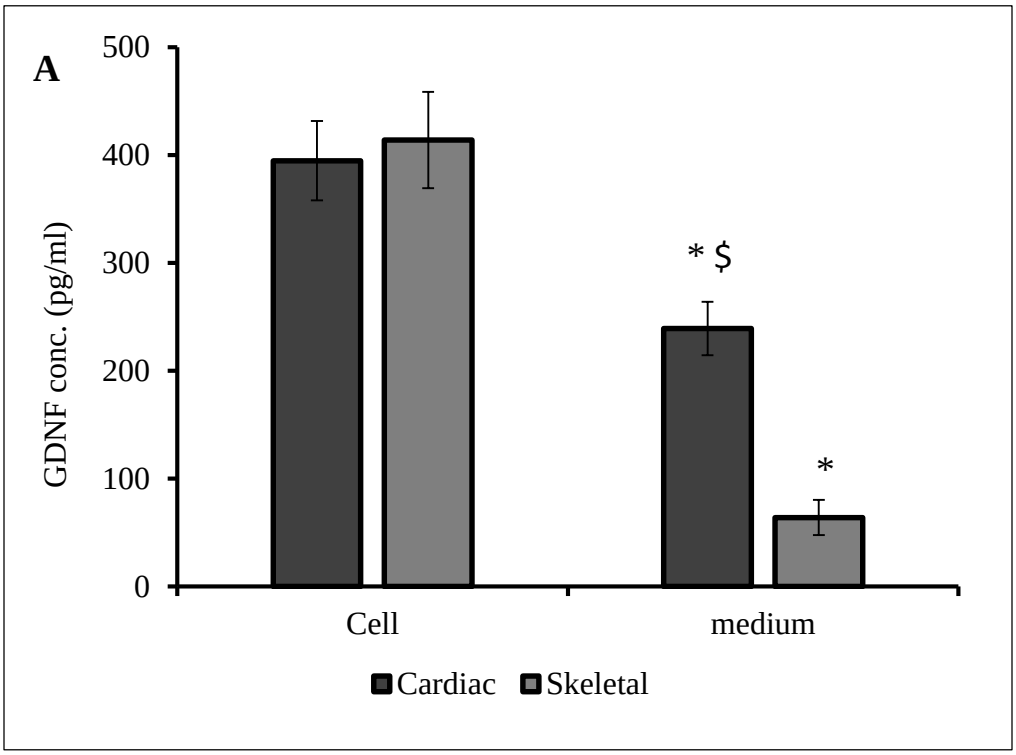


Figure 5.1. GDNF molecular sizes in skeletal and cardiac muscle cells. Cells were grown and allowed to differentiate according to the protocol for each cell type. Samples were collected from 7-day-old myotubes and 5-day-old cardiac muscle cells. A. Both cell types express GDNF of ~45kDa. Myotubes release an additional band of ~25kDa. Lane 1, GDNF positive control show ~45kDa and ~17kDa; Lanes 2-5, two bands, 45kDa and 25kDa of GDNF released by myotubes; Lanes 6-9, intracellular GDNF in myotubes. Lane 10, protein ladder. B. Cardiac muscle cells GDNF with size of ~45kDa; lanes are assigned as in (A) above.

Skeletal and cardiac muscle cells contained similar amounts of GDNF protein; however, cardiac cells secreted more GDNF protein (Table 1 and Figure 5.2A). Figure

5.2B shows percentage contribution to total GDNF by either GDNF secreted or GDNF retained in cells. Intracellular skeletal muscle GDNF contributed about 87% of total GDNF produced by myotubes whereas GDNF released into culture medium contributed only 13% to the total GDNF. In cardiac cells, intracellular GDNF and extracellular GDNF was about 60% and 40% respectively (Figure 5.2B).

GDNF production in skeletal and cardiac muscle cells



Percentage contribution of intracellular and secreted GDNF

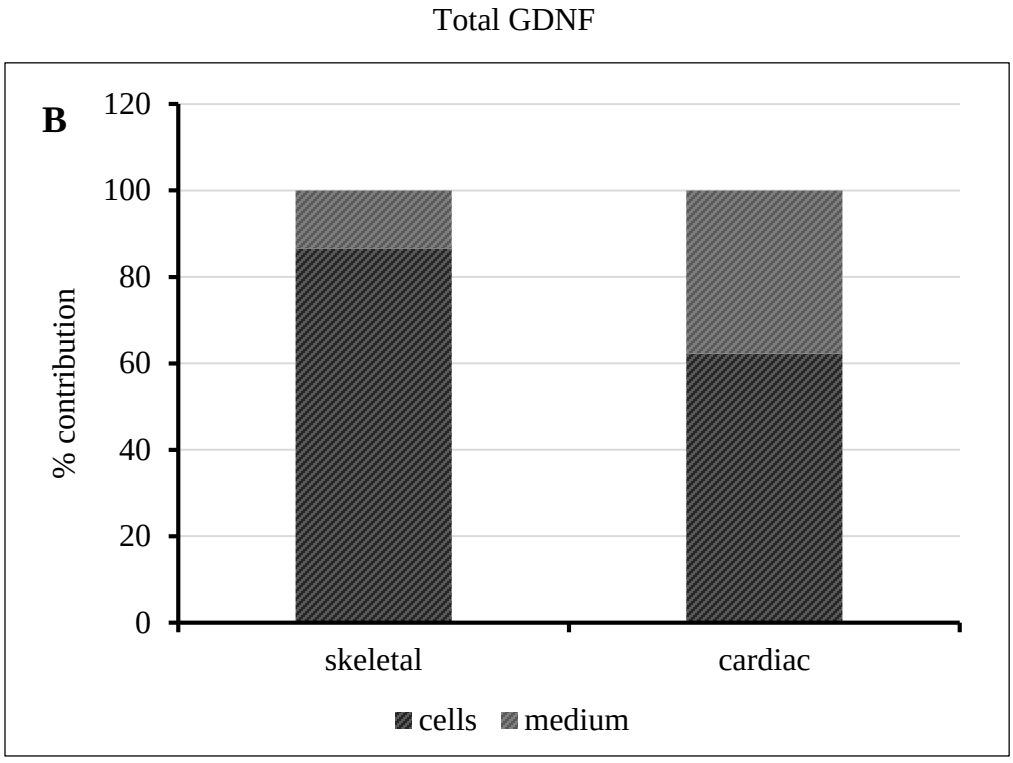


Figure 5.2A-B. GDNF production in skeletal and cardiac muscle cells. **A,** Both cell types synthesize GDNF protein and both cell types secrete less protein than they retain inside the cell. Cardiac muscle cells secrete higher levels of GDNF protein than skeletal muscle cells **B**. Intracellular GDNF contributes 87% or 62% in skeletal muscle or cardiac muscle, respectively. Values are presented as mean, S.E.M. Asterisk (*) indicates a significant difference ($P \leq 0.05$) between levels of GDNF that is retained in cells and GDNF that is secreted in culture medium in each cell type. A dollar sign ($) indicates a significant difference between cell lines, N=8.

Table 5.1: Ratios of synthesized to secreted GDNF

	Skeletal muscle cells			Cardiac muscle cells		
Time	synthesis	secretion	ratio	synthesis	secretion	ratio
30min				497	287*	1.7
1h	281	55*	5	432	243*	1.8
2h	376	29*	13	324	139*	2
4h	313	30*	10	461	285*	1.6
8h	474	67*	7	398	284*	1.4
12h	566	64*	9			
24h	473	137*	3	256	197*	1.3

GDNF synthesis and secretion concentration in pg/ml

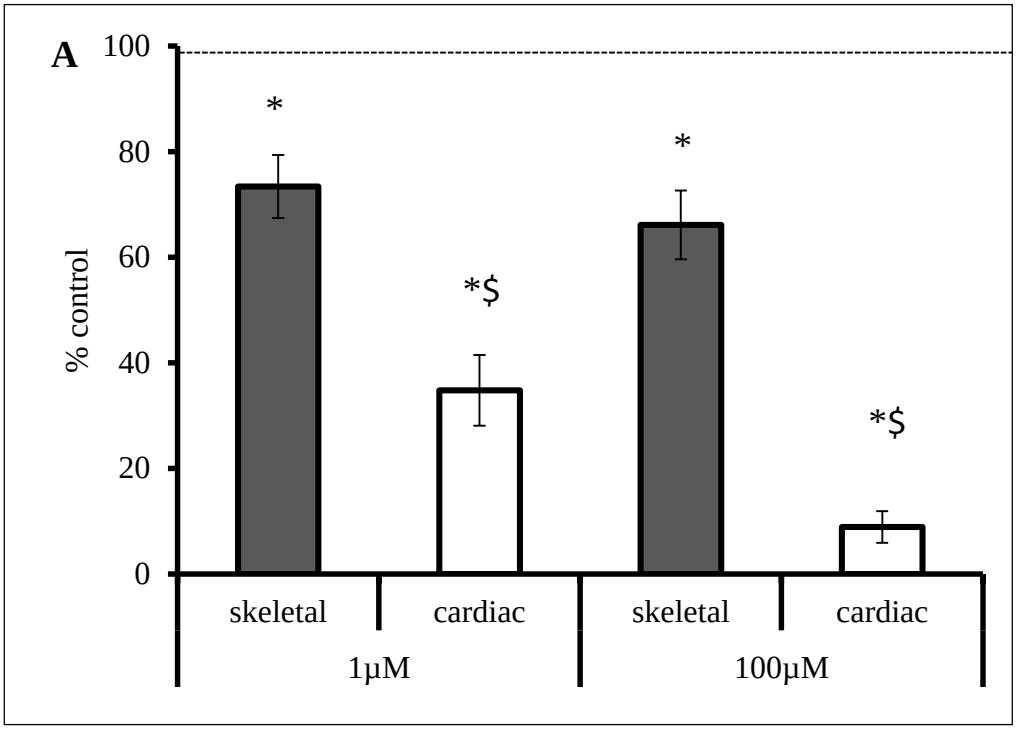
Table 5.1. GDNF synthesis and secretion in skeletal muscle and cardiac muscle control cells. The table shows the ratio between intracellular and secreted GDNF by skeletal and cardiac muscle cells. Levels of GDNF secreted by skeletal muscle cells varies with time. The lowest levels appeared to be at 2h and 4h as the ratio between intracellular GDNF and GDNF secreted is higher compared to other time points. Unlike skeletal muscle cells, the intracellular GDNF and GDNF that is secreted in cardiac cells is almost a 1:2 ratio. Asterisk (*) indicates significance difference between GDNF protein in cells and GDNF secreted into culture medium in both cell types, N=8.

5.3.2. Effect of ACh on GDNF expression by skeletal and cardiac muscle cells

Treatment with ACh had a similar effect on GDNF production in both cardiac and skeletal muscle cells. Following 2h treatment at both concentrations (1µM and 100µM) GDNF secretion was inhibited in both cell types, the effect was much greater in cardiac muscle cells than in skeletal muscle cells (Figure 5.3A). Treatment with ACh also decreased intracellular GDNF content in cardiac cells, whereas there was no effect on intracellular GDNF in skeletal muscle cells (Figure 5.3B).

Effect of acetylcholine on GDNF production in skeletal and cardiac muscle cells

GDNF content in culture medium



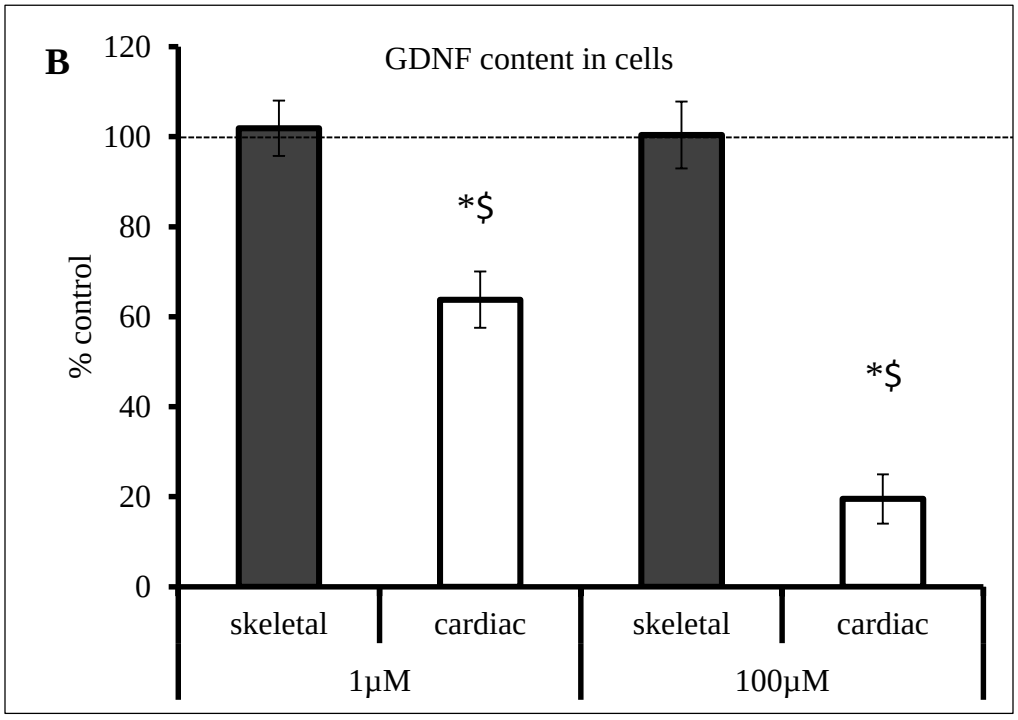


Figure 5.3. Effect of ACh on GDNF production by skeletal and cardiac muscle cells. Cells were plated and allowed to differentiate into myotubes (skeletal) or beating cells (cardiac). Culture medium containing 1µM or 100µM ACh was added to 7-day-old myotubes or 5-day-old cardiac cells. **A**. ACh inhibits GDNF secretion in culture medium in both cell types although GDNF levels are more reduced in cardiac cells compared to skeletal muscle cells. **B**. ACh affects intracellular GDNF levels in cardiac muscle cells but had no effect on intracellular GDNF in skeletal muscle cells. GDNF protein concentration was determined by ELISA. Values are presented as Mean ± S.E.M. An asterisk (*) indicates a significant deference from control and the dollar sign ($) indicates a significant difference between cell types ($P \leq 0.05$), $N = 6$.

5.3.3. Effect of electrical stimulation on GDNF expression in skeletal and cardiac muscle cells

We examined the effect of electrical impulses on GDNF expression in skeletal muscle and cardiac muscle cells. Thirty minutes of electrical stimulation at 1Hz caused an increase in GDNF secretion in cardiac muscle cells and a decrease in GDNF secretion in skeletal muscle cells (Figure 5.4). When the duration of electrical stimulation was increased from 30 minutes to 48 hours, levels of secretion of GDNF by cardiac cells are significantly inhibited compared to control, while GDNF secretion in skeletal muscle is significantly increased compared to control (Figure 5.5A). Intracellular GDNF content was altered in a similar manner to secreted GDNF in both cell types following low frequency stimulation (data not shown). Skeletal muscle cells showed a similar response to high frequency stimulation (5Hz) as low stimulation; however, cardiac muscle cells, displayed a decline in GDNF secretion (Figure 5.5B) and intracellular content (data not shown) at all times following stimulation at 5Hz.

Effect of short-term electrical stimulation on skeletal and cardiac muscle cells

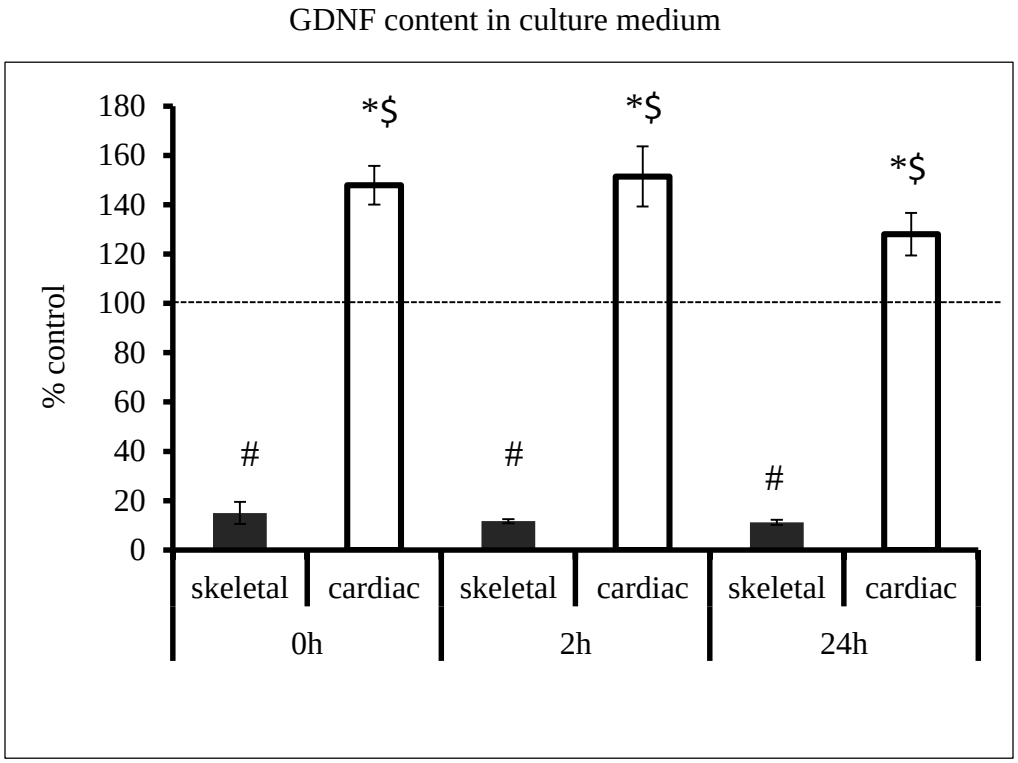


Figure 5.4. Opposite effect on GDNF secretion in skeletal and cardiac muscle cells following 1Hz electrical stimulation for 30minutes. Cells were plated on 6-well plates and allowed to differentiate. Electrical stimulation for 30min at low frequency (1Hz) have opposite effects on GDNF production by skeletal and cardiac muscle. GDNF protein concentration was determined by ELISA. Values are presented as Mean ± S.E.M. Asterisk (*) indicates a significant increase from control, the pound sign (#) a significant decrease from control, and the dollar sign ($) a significant difference between skeletal muscle and cardiac muscle, ($P \leq 0.05$), N=6.

Effect of 1Hz on GDNF production by muscle cells

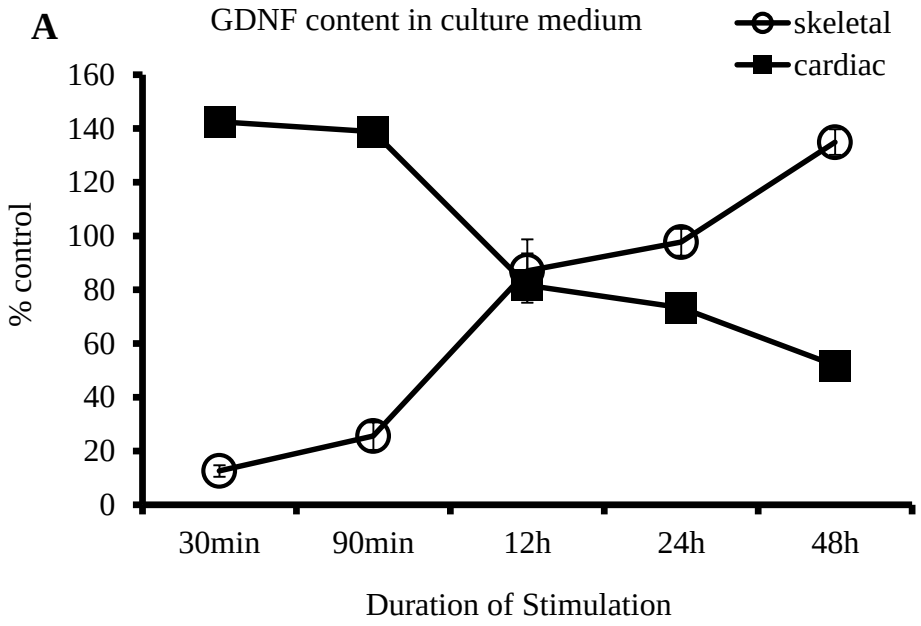


Figure 5. 5A. Effect of short- and long-term electrical stimulation on GDNF production in skeletal and cardiac muscle – effect of 1Hz. Cells were electrically stimulated at 1Hz for 30min, 90min, 12h, 24h, and 48h at 1Hz. With short-term ES, the excitatory effect in cardiac muscle cells or inhibitory effect in skeletal muscle cells are reduced as the duration of ES is increased. With long-term ES the inhibitory effect in skeletal muscle becomes a stimulatory effect and the early stimulatory effect in cardiac muscle becomes inhibitory. Electrical stimulation at 1Hz has a similar effect on intracellular GDNF of both skeletal and cardiac muscle cells (data not shown). GDNF protein content was determined by ELISA. Values are presented as mean ± S.E.M. ($P \leq 0.05$). N=6

Effect 5Hz frequency on GDNF secretion

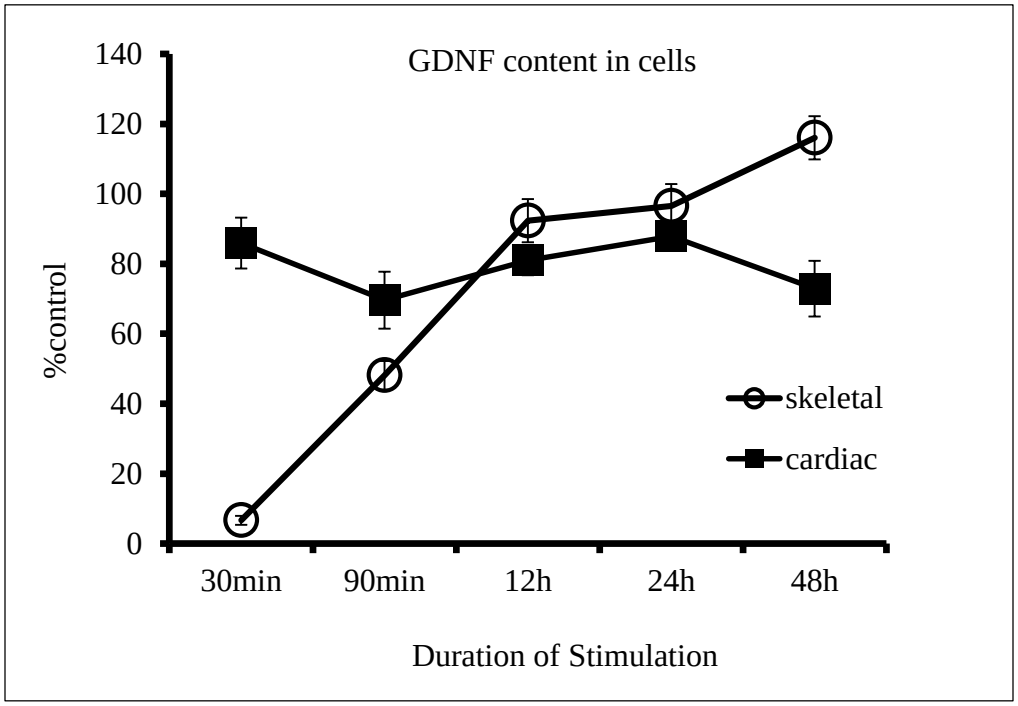


Figure 5.5B. Effect of short- and long-term electrical stimulation on GDNF production in skeletal and cardiac muscle - effect of 5Hz. Cells were electrically stimulated at 5Hz for 30min, 90min, 12h, 24h, and 48h at 5Hz. In skeletal muscle cells ES at 5Hz causes an early inhibition of GDNF production that transitions to a stimulatory effect with long-term ES. Stimulation of cardiac muscle cells at 5Hz leads to an inhibition of GDNF production at all times. The 5Hz frequency has similar effect on intracellular GDNF in both skeletal and cardiac muscle cells (data not shown). GDNF protein content was determined by ELISA. Values are presented as mean ± S.E.M. ($P \leq 0.05$), N=6.

5. 4. DISCUSSION

In the current study, we have examined GDNF production in voluntary and involuntary muscle cells. The pattern of GDNF protein expression in control muscle cell types was assessed, and changes in expression following chemical or electrical stimulation was quantified. The overall results show that 1) both skeletal muscle and cardiac muscle cells express approximately equal amounts of intracellular GDNF protein. However, the amount secreted into culture medium by both cell types differs. 2) Acetylcholine, the neurotransmitter released by somatic and parasympathetic neurons, inhibits GDNF production in skeletal and cardiac muscle cells. 3) Direct electrical stimulation (ES) has opposing effects of GDNF expression in skeletal muscle and cardiac muscle cells. The results suggest that GDNF production in skeletal muscle and cardiac muscle is dependent on ongoing activity. Also, the results demonstrate that the same stimulus may exert very different effects on GDNF production in different muscle types. These effects depend on the duration of exposure, intensity, or concentration of stimulus. Skeletal muscle and cardiac muscle cells express GDNF of the same molecular weight, although the cleavage and secretion processing may differ between the cell types.

Skeletal muscle and cardiac muscle cells express GDNF protein of molecular weight about 45kDa, which may represent a dimerized form of activated GDNF (Xu et al., 1998). Skeletal muscle cells also appear to release GDNF of molecular size about 25kDa, which may represent the monomer form of GDNF (Xu et al., 1998). This monomer form of GDNF was only observed in the skeletal muscle culture medium. On the other hand, cardiac muscle cells synthesize and secrete the dimerized form of GDNF. Both the dimer and

monomer forms may represent pro-GDNF, the precursor form of active GDNF (Xu et al., 1998). Other studies have shown that a number of other cell types secrete pro-GDNF (Lonka-Nevalaita et al., 2010) and the proteolytic cleavage of the mature pro-protein can occur either inside the cell or outside the cells, or both (Lonka-Nevalaita et al., 2010; Teng et al., 2005). Because of the GDNF molecular size observed in the current study, it appears as though both differentiated skeletal muscle cells and cardiac muscle cells may release pro-GDNF. Our western blot did not detect GDNF in the final mature form (15 to 20 kDa), a form in which both pre- and pro- amino acid portions have been cleaved. This may suggest that GDNF expressed in skeletal muscle and cardiac muscle are released in pro-forms and undergo cleavage after being released. The pro-forms of neurotrophins (both pro-BDNF and pro-NGF) have been shown to play a role in apoptotic pathway in cells (Teng et al., 2005). However, a study conducted by Kust et al. (2014) investigating the effect of mature GDNF and its precursors (pre-GDNF, pro-GDNF, pre-pro GDNF) on spinal ganglia cells, demonstrated that these GDNF forms exert trophic support on neuronal growth, although at different degrees. The mature GDNF was show to have the maximum trophic effect on neuronal growth with pro-GDNF having the least trophic effect (Kust et al., 2014). Moreover, as stated earlier, cardiac muscle cells have been shown to release only dimerized GDNF, whereas differentiated skeletal muscle release both the monomer and dimer GDNF. Since different biological activities of neurotrophin monomers and dimers have been proposed (Kolbeck et al., 1994), this suggests that there may be similarities and differences in function of GDNF in skeletal muscle and cardiac muscle.

The results also suggest that GDNF processing within skeletal and cardiac muscles may differ. Skeletal muscle cells show a higher ratio of intracellular to extracellular GDNF,

where cardiac cells secrete a much higher ratio of the GDNF being produced. The differences between cardiac muscle cell GDNF and skeletal muscle cell GDNF handling may suggest constitutive and regulated pathways (Nevalaita and Saarma, 2010), where in cardiac muscle transport vesicles containing GDNF fuse immediately in the cell membrane upon arrival, while GDNF in skeletal muscle may accumulate at the target membrane until the cells are triggered to release it. The secretion pattern of GDNF observed in skeletal and cardiac muscle cells in the current study, may explain why GDNF levels in skeletal muscle increase with increased activity (Gyorkos et al., 2014; Gyorkos and Spitsbergen, 2014; Vianney et al., 2014), whereas in cardiac cells the prolonged activity leads to a decline in GDNF. The proposed regulated and constitutive mechanisms of secretion for GDNF in skeletal muscle cells and cardiac muscle cells may also correlate with the contractile characteristics of skeletal (voluntary) muscle and cardiac (involuntary) muscle.

Our results support previous *in vitro* and *in vivo* studies where increased electrical stimulation of skeletal muscle or increased intensity of exercise in rats increased GDNF production in skeletal muscle cells or skeletal muscle fibers (Gyorkos and Spitsbergen, 2014; Vianney et al., 2014). On the other hand, in cardiac cells, GDNF declines as the duration or intensity of stimulation increases, suggesting that the cardiac cells have a mechanism that prevents an increase in GDNF expression with chronic activity or high intensity activity. This limit on trophic factor production may be due to the fact that overexpression of neurotrophic factors in the heart is not always desirable (Ieda and Fukuda, 2009). The effects of electrical stimulation in the current study also demonstrates that the same stimulus may induce very different effects on GDNF production in voluntary

and voluntary muscles and that, duration and/or intensity of exposure are critical determinants of the effect on GDNF production.

ACh negatively regulates GDNF secretion in both muscle cell types. However, in cardiac muscle cells both intracellular and secreted GDNF protein levels are decreased following ACh treatment. The mechanism underlying the inhibition of GDNF in both skeletal muscle and cardiac muscle cells has yet to be determined. However, the same observations have been observed with other ACh studies. In developing NMJ, ACh regulates NMJ in a negative fashion by inhibiting extra synaptic growth (An et al., 2010); or, by causing a decrease in acetylcholine receptors that are spontaneously formed at the NMJ (Lin et al., 2005; Xiong and Lin, 2005). Similarly, in vitro studies suggest thatACh inhibits GDNF in skeletal muscle (McCullough et al., 2011; Vianney et al., 2014). Therefore, it can be postulated that the presence of ACh functions by preventing extra production of the NMJ components including some of the neurotrophic factors produced by a muscle target. Figure 7 attempts to illustrate an event or a relationship of the synaptic components that have been reported to be associated with GDNF at the NMJ. Both GDNF and its receptors are expressed in skeletal muscle, this suggests an autocrine mode of action of GDNF, whereby GDNF enhances the insertion of AChRs in the absence of innervation (Young and Nelson, 2004) When ACh is present it acts to inhibit AChR insertion, which indirectly inhibits the action of GDNF on enhancing the insertion of AChRs. The same mechanism of action may also be true in cardiac muscle.

We believe that the current study is the first to examine the regulation of GDNF production in voluntary and involuntary muscles. Our results show that ACh alters GDNF production in cardiac and skeletal muscles, suggesting that somatic and autonomic nervous

system may be involved in regulation of GDNF production in the heart and skeletal muscle. The effects of electrical stimulation, which bypasses the neurotransmitter signaling steps at the neuromuscular junction events following muscle depolarization, are also important regulators of GDNF expression in the target tissues.

Because GDNF is a naturally occurring neurotrophic factor and provides trophic support for the nervous system, understanding endogenous factors that control GDNF production may help to explain why diseases associated with neural-target dysfunction occur. Furthermore, determining the similarities and differences on how GDNF expression is regulated in target tissues may also provide a better understanding of how these processes may be modulated therapeutically. However, further studies are needed to determine the potential mechanism(s) responsible for differential regulation of GDNF in voluntary and involuntary muscle cells.

5.5. SUMMARY

- The study compares the regulation of production of GDNF in voluntary (skeletal) muscle and involuntary (cardiac) muscle following electrical and chemical stimulation.

- Results suggest that electrical stimulation has opposing effects on GDNF production in cardiac muscle and skeletal muscle. GDNF levels increase with long-term electrical stimulation in skeletal muscle and decrease with the long term electrical stimulation in cardiac muscle.

- Treatment with acetylcholine inhibits GDNF production in both cardiac and skeletal muscle cells.

- The current results suggest that GDNF expression may be differentially regulated in the heart and skeletal muscle. Therefore:

- Understanding the regulation of GDNF production in these target tissues will provide a better understanding of how these processes may be modulated therapeutically.

5.6. ACKNOWLEDGEMENTS

Special thanks to Dr. Damon A. Miller (Electrical and Computer Engineering) and Benjamin VanDyken (Mechanical Engineering student) at Western Michigan University for designing and assembling the stimulation apparatus, based primarily on Marotta et al., 2004.

This work was supported by NIH Grant 1R15AG022908-01A2, MSU-KCMS, and Western Michigan University

5.7. REFERENCES

An, M.C., Lin, W.,Yang, J., Dominguez, B., Padgett, D., Sugiura, Y., Aryal, P., Gould, T.W., Oppenheim, R.W., Hester, M.E., Kaspar, B.K., Ko, C.-P., Lee, K.-F., 2010. Acetylcholine negatively regulates development of the neuromuscular junction through distinct cellular mechanisms. Proc. Ntl. Acad. Sci.143, 10702–10707.

Angka, H.E., Geddes, A.J., Kablar, B., 2008. Differential survival response of neurons to exogenous GDNF depends on the presence of skeletal muscle. Dev. Dynam. 237, 3169–3178.

Claycomb, W.C, Lanson, N.A Jr., Stallworth, B.S., Egeland, D.B., Delcarpio, J.B., Bahinski, A., Izzo, N.J Jr., 1998. HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc. Natl. Acad. Sci. 95, 2979–2984.

Enomoto, H., Heuckeroth, R.O., Golden, J.P., Johnson, E.M., Milbrandt, J., 2000. Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. Dev. 127, 4877– 4889.

Gyorkos, A. M., Spitsbergen, J.M., 2014. GDNF content and NMJ morphology are altered in recruited muscles following high-speed and resistance wheel training. Physiol. Rep. 2, 1–11.

Gyorkos, A.M., McCullough, M.J., Spitsbergen, J.M. (2014), Glia cell line-derived neurotrophic factor (GDNF) expression and NMJ plasticity in skeletal muscle following endurance exercise. Neurosci. 257, 111–118.

Habecker, B.A., Bilimoria, P., Linick, C., Gritman, K., Lorentz, C.U., Woodward, W., Birren, S.J., 2008. Regulation of cardiac innervation and function via the p75 neurotrophin receptor. Auton. Neurosci. 140, 40–48.

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

Heumann, R., Korsching, S., Scott, J., Thoenen, H., 1994. Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. Embo Journal 3, 3183–3189.

Hiltunen, P.H., Airaksinen, M.S., 2004. Sympathetic cholinergic target innervation requires GDNF family receptor GFRα2. Mol. Cell Neurosci. 26, 450–457.

Hiltunen, J.O., Laurikainen, A., Airaksinen, M.S., Saarma, M., 2000. GDNF family receptors in the embryonic and postnatal rat heart and reduced cholinergic innervation in mice hearts lacking ret or GFRα2. Dev. Dyn. 219, 28–39.

Hoover, D.B., Ganote, C.E., Ferguson, S.M., Blakely, R.D., Parsons, R.L., (2004) Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc. Res. 62, 112–121.

Ieda, M., Fukuda, K., 2009. Cardiac innervation and sudden cardiac death. Curr. Cardiol. Rev. 5, 289–295.

Kolbeck, R., Jungbluth, S., Barde, Y-A., 1994. Characterisation of neurotrophin dimers and monomers. Eur. J. Biochem. 225, 995–1003.

Kust, N., Panteleev, D., Mertsalov, I., Savchenko, E., Rybalkina, E., Revishchin, A., Pavlova, G., 2014. Availability of pre- and pro-regions of transgenic GDNF affects the ability to induce axonal sprout growth. Mol. Neurobiol. DOI 10.1007/s12035-014-8792-8.

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

Ling, K.K.Y., Siow, N.L., Choi, R.C.Y., Tsim, K.W.K., 2005. ATP potentiates the formation of AChR aggregate in the co-culture of NG108-15 cells with C2C12 myotubes. Fed. Eur. Biochem. Soc. Lett. 579, 2469–2474.

Lonka-Nevalaita, L., Lume, M., Leppanen, S., Jokitalo, E., Peranen, J., Saarma, M., 2010. Characterization of the intracellular localization, processing, and secretion of two glial cell line-derived neurotrophic factor splice isoforms. J. Neurosci. 30, 11403–11413.

Mabe, A.M., Hoover, D.B., 2009. Structural and functional cardiac cholinergic deficits in adult neurturin knockout mice. Cardiovasc. Res. 82, 93–99.

Martinelli, P.M., Camargos, E.R.S., Morel, G., Ravares, C.A.P., Nagib, P.R.A., Machado, C.R.S., 2002. Rat heart GDNF: effect of chemical sympathectomy. Histochem. Cell Biol. 118, 337–343.

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

Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L.F., Ryan, A.M., Carver-Moore, K., Rosenthal, A., 1996. Renal and neuronal abnormalities in mice lacking GDNF. Nature 382, 789–792.

Sariola, H., Saarma, H., 2003. Novel functions and signaling pathways for GDNF. J. Cell Science 116, 3855–3862.

Shneider, N.A., Brown, M.N., Smith, C.A., Pickel, J., Alvarez, F.J., 2009. Gamma motor neurons express distinct genetic markers at birth and require muscle spindle-derived GDNF for postnatal survival. Neural Dev. 4, 42. doi: 10.1186/1749-8104-4-42.

Teng, H.K., Teng, K.K., Lee, R., Wright, S., Tevar, S., Almeida, R.A., Kermani, P., Torkin, R., Chen, Z-Y., Lee, F.L., Kraemer, R.T., Nykjaer, A., Hempstead, B.L. 2005. Pro BDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J. Neurosci. 25, 5455–5463.

Vianney, J.-M., Spitsbergen, J.M., 2011. Cholinergic neurons regulate secretion of glial cell line-derived neurotrophic factor by skeletal muscle cells in culture. Brain Res. 1390, 1–9.

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

Xiong, W.C., Mei, L., 2005. An unconventional role of neurotransmission in synapse formation. Neuron 46, 521–528.

Xu, R.Y., Pong, K., Yu, Y., Chang, D., Liu, S., Lile, J.D., Treanor, J., Beck, K.D., Louis, J.-C., 1998. Characterization of two distinct monoclonal antibodies specific for glial cell line-derived neurotrophic factor. J. Neurochem. 70, 1383–1393.

Yafel, D., Saxel, O., 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270, 725–727.

Yang, L.-X., Nelson, P.G., 2004. Glia cell line-derived neurotrophic factor regulates the distribution of acetylcholine receptors in mouse primary skeletal muscle cells. Neurosci. 128, 497–509.

CHAPTER 6

SUMMARY, CONCLUSION, AND FUTURE STUDIES

6.1. REGULATION OF GDNF EXPRESSION IN A NERVE-MUSCLE CO-CULTURE SYSTEM

The overall goal of this study was to investigate whether neural cells control their own supply of muscle-derived GDNF. Preliminary studies showed that GDNF production was inhibited in co-cultures of skeletal muscle myotubes and cholinergic neurons as GDNF content in co-cultured cells declined compared to controls. Also, immunocytochemical staining using anti-GDNF showed that cholinergic neurons grown with skeletal muscle myotubes were stained positive for GDNF, whereas, these neurons did not contain GDNF when they were grown alone. Why cholinergic neurons appear to contain GDNF when in contact with skeletal myotubes is not clear. Further studies are needed to investigate whether:

1. Cholinergic neural cells express GDNF when in contact with skeletal myotubes. In situ hybridization is one of the useful experiments that allows one to examine gene expression at the mRNA level. Thus, studies aimed at examining which cell type, (neural cells, skeletal myotubes, or both) are expressing GDNF mRNA will give more insight into how target-derived GDNF production is regulated.

2. GDNF protein secreted by skeletal myotubes is taken up by neural cells. One of the experiments to examine this hypothesis may involve growing neural cells and

muscle in compartment chambers (Klusch et al., 2013) and use retrograde tracing methods (Choi et al., 2002) to detect retrograde transport of GDNF.

6.2. GDNF SYNTHESIS/SECRETION PATTERNS IN SKELETAL AND CARDIAC MUSCLE CELLS

We have examined GDNF production in voluntary and involuntary muscles using skeletal and cardiac muscle cells, respectively. Overall findings show that both cell types synthesize equal amounts of GDNF but the cells differ in secretion of GDNF. While skeletal muscle myotubes release less than a quarter of the total GDNF they produce, cardiac muscle cells release almost a half of the total GDNF produced. This suggests that GDNF secretion pathways in voluntary and involuntary muscles may differ. One possibility is that skeletal muscle GDNF secretion may be regulated, whereas in cardiac muscle, GDNF may be constitutively released. However, it is not clear whether skeletal muscle normally secretes low levels of GDNF, as it was observed in the current study; or, the low levels of secreted GDNF may be caused by a defect in GDNF protein variants (the two forms of GDNF) processing that has been observed in other cell models (Lonka-Nevalaita et al., 2010; Oh-Hash et al., 2009). Therefore, further studies may be needed to examine whether:

3. a) The two forms of GDNF mRNA and corresponding GDNF protein are expressed in skeletal and cardiac muscles in vivo and in vitro using PCR/RT-PCR and western blot analysis.

b) Both regulated and constitutive pathways of GDNF exist in skeletal and cardiac muscles. Immunocytochemical techniques may help detect which form of GDNF is localized in regulated and/or constitutive vesicles (Geng et al., 2011; Lonka-Nevalaita et al., 2010)

6.3. SOMATIC AND AUTONOMIC NEURONS REGULATE NEUROTROPHIC FACTOR PRODUCTION VIA NEUROTRANSMITTER EFFECTS

Table 6. 1: Effect of neurotransmitters and electrical stimulation on GDNF and NGF production in cardiac muscle cells.

	SHORT-TERM EFFECT			**LONG-TERM EFFECT**		
		GDNF	**NGF**		**GDNF**	**NGF**
ACh	1µM	↓	↑			
	100µM	↓	↓			
NE	0.1mM	↑	no effect		↓	no effect
	1mM	↑	no effect		↓	↑
ES	1Hz	↑	↑		↓	↓
	5Hz	↓	↑		↓	↓

ACh: acetylcholine; NE: Norepinephrine; ES: Electrical Stimulation. An upward (↑) represents an increase in neurotrophic factors production and a downward arrow (↓) represents a decrease in neurotrophic factors.

Table 6.1. Summary of results of short- and long-term treatment with neurotransmitter or electrical stimulation on GDNF and NGF production by HL-1 cells.

Acetylcholine (ACh): Both low and high concentrations of ACh inhibit GDNF production, while a low concentration of ACh stimulates NGF production and a high concentration of ACh inhibits it. Norepinephrine (NE): Both low and high concentrations of NE stimulate GDNF production. Short-term and long-term exposure to NE had no effect on NGF production. Electrical stimulation (ES): Short-term ES at low frequency (1Hz) increases both GDNF and NGF production and long-term stimulation decreases production of the neurotrophic factors. High frequency (5Hz) exerts similar effect on NGF production as that of low frequency stimulation. GDNF production is inhibited following both short- and long-term electrical stimulation when cells are stimulated at 5Hz.

6.3.1. Acetylcholine regulates GDNF production in skeletal and cardiac muscles

Cholinergic neurons inhibit GDNF expression in skeletal muscle (Vianney and Spitsbergen, 2011). Similarly, ACh inhibits GDNF production in both, skeletal muscle and cardiac muscle, suggesting that cholinergic neurons regulate GDNF in the muscle via ACh release. However, the mechanism underlying ACh inhibition of GDNF expression remains unclear. Lie and Weis (1998) observed that the amount of GDNF mRNA levels increased in denervated muscle compared to innervated muscle. Moreover, GDNF mRNA and GDNF protein levels were shown to increase after nerve injury and during nerve regeneration process, but the levels returns to normal with reinnervation (Naveilhan et al., 1997; Suzuki et al., 1998; Michalski et al., 2008; Yamada et al., 2004). All of the above may suggest that the reduction of GDNF protein production observed with an intact neural connection may be due to an inhibitory effect of ACh on GDNF expression in skeletal and cardiac muscle. However, this is one of possible potential mechanisms of action of GDNF.

Thus, further studies aimed at understanding normal regulatory process and GDNF expression levels may help understand and draw better conclusion with changes of GDNF observed with experimental treatments. Therefore:

4. Cross sectional and/or longitudinal studies could be performed to investigate the following broad questions:

a) What is the ongoing need for GDNF protein in tissue from healthy humans and model organisms?

b) Do different types of tissues normally differ in their GDNF protein content?

c) When are changes in GDNF protein content or mRNA observed in tissues following aging or with development of disease?

d) In (a) – (c) above, are there differences between different GDNF isoforms?

The possibility that GDNF may be acting in autocrine mode is another potential mechanism that may lead to decreased production of GDNF in these studies. GDNF autoregulation has been reported in neuronal cells *in vivo* and *in vitro*, where GDNF acts to increase its own expression (Barak et al., 2011; He and Ron, 2006). It may be interesting to examine:

5. Whether GDNF autoregulatory system exists in skeletal muscle and cardiac muscle. To do this, GDNF receptors (GFRα1) on the muscles may be blocked with anti- GFRα1 antibodies Ab1531 and/or AbG90 (Messer et al., 2000). Short-term electrical stimulation or ACh treatments may be performed on skeletal muscle cells (myotubes) in the presence of anti-GFRα1 antibodies. The hypothesis being tested is that the inhibitory effect that is observed with a short-term electrical stimulation or ACh is abolished when GDNF receptors are blocked.

6.3.2. ACh and norepinephrine have different effects on GDNF and NGF in cardiac muscle

Overall, the concentration and/or duration of exposure to a neurotransmitter are important in determining the direction of changes in GDNF and NGF expression in cardiac muscle. Moreover, levels of expression of GNDF and NGF are regulated differently by ACh and NE in cardiac muscle. While GDNF protein levels decrease with exposure to ACh, the levels increase with exposure to NE.

6. Studies aimed at determining the relationship between the rate of cardiac muscle cell contraction and levels of GDNF or NGF production may be valuable. In these studies changes in cardiac cell contraction may be modulated by addition of neurotransmitters, electrical stimulation, or other depolarizing agents such as potassium chloride (KCl).

6.4. ELECTRICAL STIMULATION REGULATES NEUROTROPHIC FACTORS PRODUCTION IN SKELETAL AND CARDIAC MUSCLES

6.4.1. Electrical stimulation has different effects on GDNF production in skeletal and cardiac muscles.

As shown in Chapter 5, Figure 5.5A, the response to electrical stimulation at 1Hz in skeletal and cardiac muscle is almost a mirror image, where short-term electrical stimulation inhibits GDNF production in skeletal muscle and increases GDNF production in cardiac muscle, while long-term stimulation increases GDNF production in skeletal muscle and decreases it in cardiac muscle. The mechanisms underlying the effect on GDNF production following short- and long-term electrical stimulation remain unclear. A further investigation examining these mechanisms may be based on the following assumptions:

7. a) It is well known that electrical stimulation produces changes in contractile proteins such as myosin heavy chain in vivo and in vitro (MHC: Bayol et al., 2005). Mature C2C12 myotubes are known to express predominantly fast MHC (Brown et al., 2012; Zebedin et al., 2004) and a switch from fast to slow MHC in C2C12 skeletal myotubes has been shown to be induced by chemical or electrical stimulation (Zebedin et., 2004). It may be of interest to examine whether changes in GDNF protein levels observed with electrical stimulation are due to changes in muscle characteristics, such as a switch from fast to slow MHC.

   b) Results from (a) above, may help explain whether or not the regulation of GDNF expression depends only on the duration and intensity of the stimulus or whether GDNF expression is linked to the muscle fiber composition.

8. Another observation was that electrical stimulation did not induce contraction in 9- to 14-day-old myotubes, yet there was a significant change in GDNF protein following stimulation. Similarly, treatment with acetylcholine or carbachol did not cause myotube contraction, however, there was a significant change in GDNF protein levels with treatment. Also treatment with a high concentration of carbachol decreases contraction in cardiac muscle cells. Thus, it may be hypothesized that changes in GDNF protein production in skeletal and cardiac muscles does not only depend on contractile activity, but may also depend on changes in other intracellular signals that occur with stimulation. Therefore, it may be reasonable to examine:

a) The role that ion channels play in the processes regulating GDNF production in skeletal and cardiac muscles.

9. Many cells express GDNF mRNA splice variants and their corresponding proteins (α-GDNF and β-GDNF) (Lie and Weis, 1998; Lonka-Nevalaita et al., 2010; Oh-Hash et al., 2009; Springer et al., 1995). Secretion of these GDNF isoforms has been shown to differ in cell models. Studies by Lonka-Nevalaita et al., (2010) observed that potassium chloride increases secretion of β-GDNF but not α-GDNF in PC-6.3 cells. Whether the different in secretion of GDNF forms is observed in skeletal and cardiac muscle is not known, therefore a study aimed at examining:

a) Whether treatments with neurotransmitter, electrical stimulation, or other depolarizing agents favor secretion of one form of GDNF and if,
b) Rise or fall of intracellular calcium may be involved. Since a number of cellular processes, including gene expression and muscle contraction, are regulated calcium, it is possible that GDNF release may be triggered by a rise or fall in calcium. Changes in GDNF mRNA and/or protein content can be examined along with intracellular Ca^{2+} concentration. Changes in intracellular Ca^{2+} can be triggered by neurotransmitter and by electrical stimulation. Changes in intracellular calcium may be blocked by thapsigargin (Engedal et al., 2013). The concentration of intracellular Ca^{2+} may be measured by various techniques either fluorescence or bioluminescence calcium indicators depending on study

goals and models to be used (Cobbold and Rink, 1987; Engedal et al., 2013; Grienberger and Konnerth, 2012; Russell, 2011; Tahakashi et al., 1999).

Overall, this study demonstrates that there are some similarities and differences in regulation of GDNF production in voluntary and involuntary muscles. These differences appear to depend on duration and intensity of exposure. Understanding the similarities and differences in the regulation of GDNF production in different tissues may provide a better understanding of how GDNF production may be modulated therapeutically.

6.5. REFERENCES

Barak, S., Ahmadiantehrani, S., Kharazia, V., Ron, D., 2011. Positive autoregulation of GDNF levels in the ventral tegmental area mediates long-lasting inhibition of excessive alcohol consumption. Transl. Psychiatry 1, 1- 9.

Bayol, S., Brownson, C., Loughna, P.T., 2005. Electrical stimulation modulates IGF binding protein transcript levels in C2C12 myotubes. Cell Biochem. Funct. 23, 361–365.

Brown, D.M., Parr, T., Brameld, J.M., 2012. Myosin heavy chain mRNA isoforms are expressed in two distinct chohorts during C2C12 myogenesis. J. Muscle Res. Cell Motil. 32, 383 – 390.

Choi, D., Li, D., Raisman, G., 2002. Fluorescent retrograde neuronal tracers that label the rat facial nucleus: a comparison of fast blue, fluoro-ruby, fluoro-emeral, fluoro-gold and diI. J. Neurosci. Methods 117, 167–172.

Cobbold, P.H., Rink T.J., 1987. Fluorescence and bioluminescence measurement of cytoplasmic free calcium. Biochem. 248, 313–328.

Engedal, N., Torgersen, M.L., Guldvik, I.J., Barfeld, S.J., Bakula, D., Sætre, F., Hagen, L.K., Patterson, J.B., Proikas-Cezanne, T., Seglen, P.O., Simonsen, A., Mills I.G.,

2013. Modulation of intracellular calcium homeostasis blocks autophagosome formation. Autophagy 9, 1475-1490.

Geng, Z., Xu F.-Y., Huang, S-H.,Chen, Z-Y., 2011. Sorting protein-related receptor SorLA controls regulated secretion of glial cell line-derived neurotrophic factor. J Biol Chem 286, 4187– 41882.

Grienberger, C., Konnerth, A., 2012. Imaging calcium in neurons. Neuron 73, 862–885.

He, D.-Y., Ron, D., 2006. Autoregulaion of glial cell line-derived neurotrophic factor expression: implications for the long-lasting actions of the anti-addiction drug, Ibogaine. FASEB 20, E1820–E1827.

Kluch, A., Ponce, L., Gorxelanny, C., Schäfer, I., Schneider S.W., Ringkamp, M., Holloschi, A., Schmelz, M., Hafner, M., Petersen, M., (2013). Coculture model of sensory neurites and keratinocytes to investigate functional interaction: Chemical stimulation and atomic force microscope-transmitted mechanical stimulation combined with live-cell imaging. J. Investigative Dermatol. 133, 1387–1390.

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

Lonka- Nevalaita, L., Lume, M., Leppänen, S., Jokitalo, E., Peränen, J., Saarma, M., (2010). Characterization of the intracellular localization, processing, and secretion of two glial cell line-derived neurotrophic factor splice isoforms. J. Neurosci. 30, 11403–11413.

Messer, C.J., Eisch, A.J., Carlezon, W.A., Whisler, K., Shen, L., Wold, D.H., Westphal, H., Collins, F., Russell, D.S., Nestler, E.J., 2000. Role for GDNF in biochemical and behavioral adaptations to drugs of abuse. Neuron 26, 247–257.

Michalski, B., Bain,. J. R., Fahnestock., M., 2008. Long-term changes in neurotrophic factor expression in distal nerve stump following denervation and reinnervation with motor or sensory nerve. J. Neurochem, 105, 1244–1252.

Naveilhan, P., ElShamy, W.M., Ernfors, P., 1997. Differential regulation of mRNAs for GDNF and its receptors ret and GDNFRα after sciatic nerve lesion in the mouse. Eur. J. Neurosci. 9, 1450–1460.

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

Russell, J.T., 2011. Imaging calcium signals in vivo: a powerful too in physiology and pharmacology. British J. Pharmacol. 163, 1605–1625.

Springer, J.E., Seeburger, J.L., He, J., Gabrea, A., Blankenhorn, E.P., 1995. cDNA sequence and differential mRNA regulation of two forms of glial and rat skeletal muscle. Exp. Neurol. 131, 47–52.

Suzuki, H., Hase, A., Kim, B.Y., Miyata, Y., Nonaka I, Arahata, K., Akazawa, C., 1998a. Up-regulation of glial line-derived neurotrophic factor (GDNF) expression in regeneration muscle fibers in neuromuscular diseases. Neurosci. Lett. 257, 165–167.

Takahashi, A., Camacho, P., Lechleiter, J.D., Herman, B., 1999. Measurement of intracellular calcium. Physiol. Rev. 79, 1089–1125.

Vianney, J.-M., Spitsbergen, J.M., 2011. Cholinergic neurons regulate secretion of glial cell line- derived neurotrophic factor by skeletal muscle cells in culture. Brain Res. 1390, 1–9.

Yamada, Y., Shimizu, K., Nitta, A., Soumiya, H., Fukumitsu, H., Furukuwa, S., 2004. Axonal regrowth downregulate the sysntesis of glial cell line-derived neurotrophic factor in the lesioned rat sciatic nerve. Neurosci. Lett. 264, 11-15.

Zebedin, E., Sandtner, W., Galler, S., Szendroedi, J., Just, H., Todt, H., Hilber, K., 2004. Fiber type convertion alters inactivation of voltage-dependent sodium currents in murine C2C12 skeletal muscle cells. Am. J. Physiol. Cell Physiol. 287, 270–280.