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The Role of Calcium in Regulation of Glial Cell Line Derived Neurotrophic Factor by Skeletal Muscle Cells

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THE ROLE OF CALCIUM IN REGULATION OF GLIAL CELL LINE DERIVED NEUROTROPHIC FACTOR BY SKELETAL MUSCLE CELLS

by

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Glial cell line-derived neurotrophic factor (GDNF) is an important signaling molecule for the somatic motor nervous system. GDNF protein is produced and secreted by skeletal muscle cells and helps maintain motor neuron innervation at the neuromuscular junction. Treatment with exogenous GDNF prevents denervation which is characteristic of aging and neurodegenerative disease. The therapeutic potential of GDNF cannot be fully explored without understanding the mechanisms by which GDNF protein production is regulated. The primary objective of this study was to determine the role of calcium in regulating GDNF protein expression by skeletal muscle cells. Skeletal muscle cells (C2C12) were grown in culture and allowed to differentiate into myotubes. The following treatments were given: Bay K8644 (100µM), an agonist for L-type calcium channels, nifedipine (100µM), a known antagonist for voltage-gated L-type calcium channels, and dantolene (100µM), an antagonist for ryanodine channels. Results suggest that expression of GDNF in skeletal muscle is regulated in a calcium-dependent manner. Acquiring a greater understanding of the role that calcium plays in regulating GDNF production may help to identify potential sites for therapeutic intervention to increase or decrease GDNF production.

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INTRODUCTION

The overarching goal of our laboratory is to gain a more complete understanding of the communication between neurons and target tissues. Neurotrophic factors have been discovered as playing a major role in the regulation of innervation of skeletal muscles, during both development and maintenance of the nervous system. The goal of the current study is to gain a greater understanding of glial cell line-derived neurotrophic factor (GDNF) protein expression by skeletal muscle in *vitro*. We begin with a brief review of the mammalian neuromuscular system and the neuroprotective effects of GDNF.

Structure and function of the mammalian nervous system

The nervous system allows animals to receive, interpret, and respond to our environment. There are two main types of neurons: sensory and motor neurons. Sensory neurons relay signals from the periphery to the central nervous system (CNS). The coordinated effort of sensory neurons allows for information sensed from our environment to travel to places where interpretation of the electrical signals occurs. Motor neurons relay information from the CNS out to effector organs such as skeletal muscle. The movement of impulses from the brain through the motor neurons allows for contraction of muscles for movement.

The motor neurons within the peripheral nervous system are classified as either somatic or autonomic. The autonomic nervous system consists of both the sympathetic and parasympathetic nervous systems. The sympathetic nervous system synapses with smooth muscle and glands and helps stimulate the flight-or-fight response. On the

other hand the parasympathetic nervous system acts in a contradictory manner and helps to regulate homeostasis in the body. The somatic branch of the peripheral nervous system is used for voluntary movement in skeletal muscles. The majority of motor neurons that innervate skeletal muscle are α motor neurons, which project from the CNS to the skeletal muscle fibers and are the largest neurons in the mammalian nervous system (Huxley, 1974).

Skeletal muscle and neuron interaction

Communication between myelinated motor neurons of the peripheral nervous system and skeletal muscle occurs at the neuromuscular junction. At the synaptic knob of the neuromuscular junction, neurotransmitters will mediate depolarization of the muscle sarcolemma. The action potential will propagate along the sarcolemma and throughout the transverse tubules, which stimulates a conformational shift in voltage-gated L-type calcium channels (v-g L-type Ca^{2+} channel in figure 1). The L-type Ca^{2+} channels are in direct contact with foot processes of the ryanodine receptors. Through depolarization of L-type channels, and their direct interaction with foot processes, ryanodine receptors along the sarcoplasmic reticulum are opened allowing for calcium release from internal stores (Catterall, 1991; Mattson, 2007).

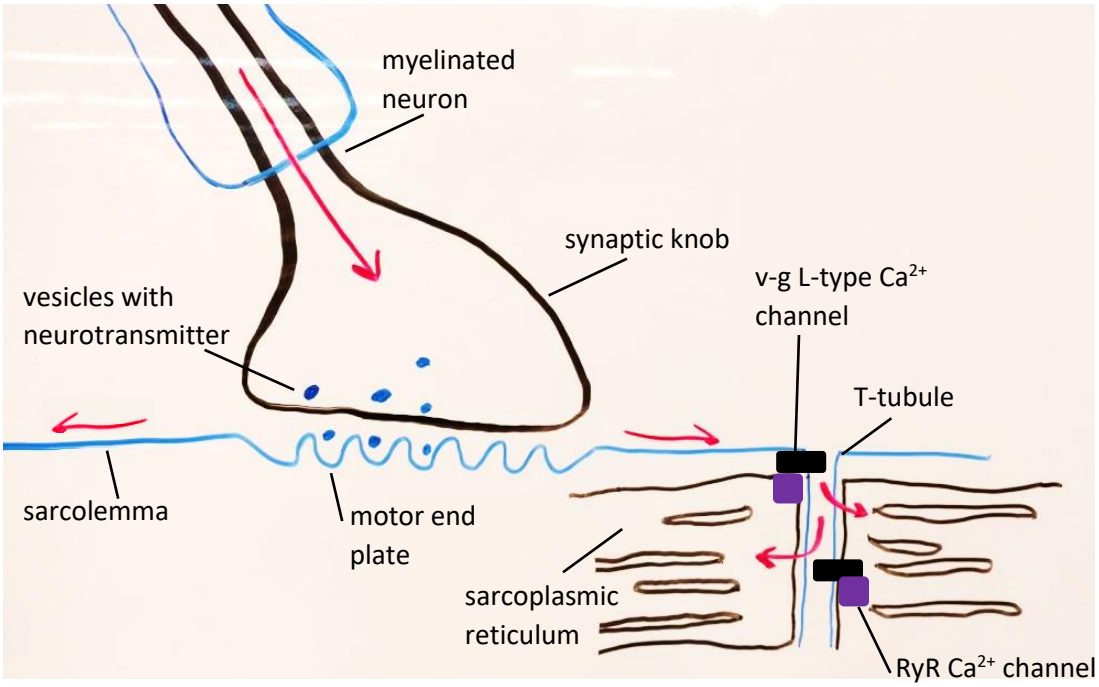

Figure 1: An illustration of the neuromuscular junction. The red arrows indicate the direction of action potential from the motor neuron to the muscle fiber.

Depolarization of voltage-gated L-type Ca^{2+} channels within the T-tubule stimulates the opening of ryanodine channels (RyR Ca^{2+} channels in figure 1) in the sarcoplasmic reticulum which act to release internal stores of calcium, further increasing intracellular calcium levels (Gonzalez- Freire et al., 2014). Due to its direct interaction with troponin, calcium is an important regulator of skeletal muscle contraction (Berchtold et al., 2000). In the presence of intracellular calcium, troponin binds to calcium and moves tropomyosin off the active sites, allowing for myosin heads to bind to actin. Thin actin filaments and the thick myosin filaments within a sarcomere will then form a sliding cross-bridge to cause muscle contraction (Huxley, 1974). The presence of calcium at the neuromuscular junction is necessary for the action potential from the motor neuron to cause a contractile response in the muscle fiber.

Neurotrophic factors

Within the nervous system small proteins called neurotrophic factors help regulate the development, survival, and functionality of motor neurons (Yan et al., 1993; Henderson et al., 1996). Neurotrophic factors within the peripheral nervous system can be made by target tissues such as skeletal muscle, by motor neurons, and by nearby glial cells. When neurotrophic factors are secreted by muscle tissues they have the ability to undergo retrograde axonal transportation up to neuronal cell bodies (Yamamoto et al., 1996; Lie and Weiss, 1998). At motor neurons the neurotrophic factor exhibits neuroprotective effects and can prevent neuronal cell death.

Neurotrophic factors are proteins that are necessary for the growth, development, and survival of the mammalian nervous system. Some neurotrophic factors include nerve growth factor (NGF), neurocytokines, neurotrophin 3, neurotrophin 4/5, brain derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF). The main function of neurotrophic factors is to act as neurocytokines and, upon synthesis and secretion, to facilitate communication between neurons and their target tissues (Morcuende et al., 2013). The GDNF family consists of neurturin (NTRN), artemin (ARTN), persephin (PSPN) and of course GDNF (Lin et al., 1993; Frostick et al., 1998). Due to the potent effects of GDNF on the peripheral nervous system the research described herein focuses on this molecule.

GDNF

GDNF was originally discovered in midbrain dopaminergic neurons of Sprague-Dawley rats (Lin et al., 1993). Shortly after the discovery of GDNF multiple researchers

began identifying which cell types in the mammalian body contained and produced GDNF (Springer et al., 1995). GDNF protein is widely distributed throughout both the central and peripheral nervous systems. Production and secretion of GDNF occurs in many cell types such as motor neurons, Schwann cells, skeletal muscle, and within the kidneys (Henderson et al., 1994; Yan et al., 1995; Yamamoto et al., 1996). Glial cell line-derived neurotrophic factor is the most potent neurotrophic factor for motor neurons in the peripheral nervous system and is crucial for the regulation of motor neuron survival (Henderson et al., 1994).

GDNF is part of the transforming growth factor β (TGF- β) superfamily (Lin et al., 1993). The members of this family have low amino acid sequence homology but all function as homodimers for receptor tyrosine kinase activation. All members of the GDNF family are produced as precursor proteins (preproGDNF) and upon secretion they are cleaved to the mature protein form (Airaksinen and Saarma, 2002). When first synthesized there are 211 amino acids in what is called pro-GDNF. This form then undergoes alternative splicing into the mature form of GDNF protein containing 134 amino acids (Wang et al., 2008). GDNF ligand will bind to GDNFα1 receptor on cell membranes and activate RET (receptor tyrosine kinase). The downstream signaling of RET causes activation of many intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK), which has been shown to contribute to both neurogenesis and neuronal survival (Wang et al., 2008).

Experiments using genetic knockout mice lacking GDNF, RET, or GFRα1 result in fatalities shortly after birth (Airaksinen and Saarma, 2002). This could be due to many phenotypic abnormalities including decreased amounts of motor neurons as well as

declines in myelination of neurons. When GDNF is absent in the developing embryo of rodents the kidneys fail to develop properly and in some cases may be completely absent (Trupp et al., 1995). Another cause of fatality in these knockout mice is suffocation due to inadequate innervation of the diaphragm. To ensure proper neuromuscular development the amount and location of GDNF is highly regulated (Keller-Peck et al., 2001; Oppenheim et al., 1995).

During embryonic development, Schwann cells are the primary producers of GDNF in the nervous system. In postnatal motor neurons, GDNF production at the neuromuscular junction supports terminal axon branching and synapse formation (Airaksinen and Saarma, 2002). Muscle cells express GDNF during development to promote motor neuron survival. GDNF has been found to play a crucial role during nervous system development and regeneration by acting as a chemoattractant (Tang et al., 1998). When target tissues are lacking in innervation they tend to secrete a greater amount of GDNF into their surrounding environment to attract nearby axons (Nagano and Suzuki, 2003).

The mammalian nervous system constantly exhibits plasticity beginning prenatally and continuing during maturation and into aging. During embryonic development there is typically an overproduction in the amount of neurons needed in the adult nervous system (Henderson et al., 1994). Motor neuron plasticity allows for this unnecessary hyper-innervation to undergo modification. As aging continues, degeneration will occur allowing for each muscle fiber to reach a favorable density of innervation (Oppenheim et al., 2000; Tintignac et al., 2015). Motor neuron degeneration is a key stage in development. This process of neuronal retraction is dependent on the

presence and absence of target tissues and the corresponding demand for innervation. One of the methods by which target tissues and neurons can communicate with each other is through the use of target-derived neurotrophic factors (Oppenheim et al., 1995; Nguyen et al., 1998).

Neurotrophic factors such as GDNF play a role in maintenance of the neuromuscular junction. When insufficient levels of GDNF are present, neuronal cell death can occur, resulting in compromised structure and function of neuromuscular junctions. Decreased levels of GDNF have been found in association with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Duchenne type muscular dystrophy (DMD) (Suzuki et al., 2008; Yamamoto et al. 1996). Similarly, when axonal dysfunction or nerve injury occurs GDNF plays a prominent role in the repair and regeneration of the nervous system (Magill et al., 2010).

GDNF may ameliorate disease and injury

Duchenne type muscular dystrophy and ALS are characterized by progressive loss of skeletal muscle strength and reduction of muscle fiber innervation. Patients with DMD displayed a decreased density of neuromuscular junctions in regenerating muscle fibers and a subsequent up-regulation of GDNF (Suzuki et al., 1998). GDNF remained elevated until neuromuscular junctions were regenerated. This result suggests that GDNF may act as a signal between muscle and neurons for inducing and maintaining innervation following degeneration. Additional research regarding the synthesis of GDNF may provide improved therapeutic methods, given the ability of GDNF to ameliorate neuronal degeneration (Deschenes et al., 1993; Suzuki et al., 1998).

Similarly, an increased expression of GDNF mRNA was found in motor neurons following denervation of skeletal muscle (Springer et al., 1995). Many studies suggest that neuroplasticity is a cycle characterized by denervation and retraction of motor neurons, and subsequent regeneration of the neuromuscular junction. When rates of denervation outpace rates of reinnervation, a negative phenotype will develop over time causing decreased functionality of the neuromuscular system. When levels of GDNF were increased by the addition of exogenous GDNF, neurodegeneration was prevented in mature neurons (Henderson et al., 1994; Oppenheim et al., 1995). In GDNF-overexpressing transgenic mice, motor neurons displayed greater neuroprotection when compared to age-matched control animals (Lee et al., 2000).

Researchers used human mesenchymal stem cells to deliver GDNF directly into skeletal muscle of familial ALS model rats. The rats showed increased muscular levels of GDNF and retrograde transport of GDNF protein into motor neurons (Suzuki et al., 2008). Exogenous addition of GDNF demonstrated neuroprotective effects on neuron survival and function at the neuromuscular junction (Suzuki et al., 2008). A similar neuroprotective effect was observed in transgenic ALS mice following intramuscular injection of an adeno-associated virus carrying the gene for GDNF (Wang et al., 2002). Taken together these studies demonstrate that exogenous administration of GDNF delays the denervation characteristic of ALS, improves motor performance, and helps maintain axonal projections to skeletal muscles.

These promising results have prompted investigation to see if GDNF, along with other neurotrophic factors such as brain-derived neurotrophic factor (BDNF), are able to prevent the degeneration of dopaminergic neurons in the substantia nigra of

Parkinson's disease (PD) models. A recent study delivered GDNF into the brains of PD mouse models using GDNF-transfected macrophages (Zhao et al., 2014). Results revealed that GDNF helped provide protection against neuroinflammation and neurodegeneration and ameliorated disease pathology (Zhao et al., 2014). Considering the beneficial effects GDNF has on neurodegenerative disorders it may come as no surprise that GDNF has also been found to aid in the recovery of motor neurons following injury.

GDNF was found to be the most potent neurotrophic factor to promote functional recovery of motor neurons following sciatic nerve injury in rats (Chen et al., 2010; Yan et al., 1995). Researchers noted differing rates of early regeneration depending on which cell populations contained GDNF (Magill et al., 2010). Following nerve injury mice given an injection of GDNF in the target muscle displayed an increased number of nerve fibers, fiber density, and nerve percentage after two weeks than control mice and those given injection of GDNF in the neuron (Magill et al., 2010). After three weeks there was no difference in neuromuscular function between the mice given GDNF intramuscularly and those given GDNF in the neuron. This information reveals that the presence of GDNF in skeletal muscle has a greater effect on early regeneration than GDNF administered to motor neurons. Exogenous administration of GDNF in either muscles or neurons prompted a more robust recovery in structure and function of the neuromuscular system (Magill et al., 2010).

In neonatal rats, axotomy of motor neurons innervating extraocular eye muscles showed varied regeneration depending on the type of neurotrophic factor that was exogenously added at the site of injury (Morcuende et al., 2013). The results showed

that NGF, BDNF, NT-3, and GDNF all significantly rescued motor neurons from cell death with GDNF and NGF as the most potent survival factors for motor neuron regeneration. Simultaneous administration of GDNF with BDNF resulted in no additive effect from the positive effect GDNF demonstrated when administered on its own (Morcuende et al., 2013). Research regarding the high affinity for retrograde axonal transport by GDNF from muscle to motor neurons compared to other neurotrophic factors helps support the potent effects of GDNF on motor neuron regeneration (Zhao et al., 2014). In mature neuromuscular systems the presence of GDNF mRNA and protein is greatest earlier in development and decreases in an age dependent manner (Keller-Peck et al., 2001; Nagano et al., 2003).

Sarcopenia

As mammals age beyond adulthood and into old age there is a notable increase in sarcopenia. Sarcopenia is the loss of muscle mass and function associated with the aging process (Frontera et al., 2000). There are many postulated causes of sarcopenia, ranging from oxidative damage from mitochondrial dysfunction, to age-related denervation (Edstrom et al., 2007). Recent evidence shows that denervation of motor neurons precedes sarcopenia and if neuroprotective measures are taken there is a delay in the age-related changes in skeletal muscle (Deschenes et al., 2010). It is well documented that with age the plasticity of the neuromuscular system contributes to muscle fiber type switching. Fast motor neurons typically innervate large type II muscle fibers and slow motor neurons innervate smaller type I muscle fibers.

In the mature nervous system fast motor neurons are particularly susceptible to denervation and retraction. In order to reestablish muscle fiber innervation a nearby slow type motor neuron may branch out to form a new neuromuscular junction with the myotube (Edstrom et al., 2007). This change in innervation pattern results in a muscle fiber switch from a large fast fiber to a slower, smaller, less fatigable muscle fiber. If the muscle fiber is not reinnervated by either type of motor neuron then the fiber is not functional and will degrade with time. The loss of neuronal signaling and subsequent muscle loss contribute to a decline in muscle mass, strength, and overall coordination (Tintignac et al., 2015).

The onset and extent of sarcopenia with age may be dependent on the amount of regular muscle activity. In rats aged 21 months the soleus muscle, which is recruited for daily posture, showed no significant denervation when compared to rats aged 10 months (Deschenes et al., 2010). The plantaris is a skeletal muscle recruited for locomotion and in this sedentary study there was significant denervation of the elderly plantaris, without fiber type switching, compared to the young rats. This suggests that denervation is accelerated with age in muscles that are not recruited but that perhaps exercise can delay the onset of age-related denervation and subsequent sarcopenia. There is evidence that the introduction of physical activity may have a neuroprotective role in preservation of the integrity of the neuromuscular junction (Cheng et al., 2013; Deschenes et al., 1993). Our lab has found that levels of GDNF protein increase with exercise, suggesting that physical activity may be used as a method to alter levels of GDNF regulation and prevent degeneration (McCullough et al., 2011).

Activity-based neurophysiology

Previous studies from our laboratory demonstrated that the levels of production of neurotrophic factors may be regulated in an activity-dependent manner. Levels of GDNF protein extracted from skeletal muscle were greater in rodents subjected to both voluntary and involuntary physical exercise when compared to sedentary controls (McCullough et al., 2011; Wehrwein et al., 2002). Similarly, within exercised rats, GDNF protein levels varied depending on how heavily physical exercise recruited those muscle groups (Gyorkos et al., 2014). The results revealed that increased muscle activation resulted in greater intramuscular GDNF protein levels, which suggests that muscle activity may play a role in determining the expression of GDNF. Similar increases in GDNF protein can be found in cultured skeletal muscle cells subjected to electrical stimulation (Vianney et al., 2014).

Physical exercise in mammals has a well-documented positive impact on neurodegenerative diseases. Dorsal root ganglia extending from the spinal cord displayed neurotrophic factor-dependent neurite outgrowth following exercise, which may help ameliorate Parkinson's disease (Molteni et al., 2004). Interestingly, the importance of exercise with respect to regeneration of the peripheral nervous system has received much less attention.

A similar study used nerve transection to examine the role of exercise in nerve regeneration and protein levels of neurotrophic factors (Park and Hoke, 2014). The experiment was set up by transecting the median nerve followed by repair. Similarly, transection of the ulnar nerve was also performed but regeneration was inhibited by deflecting the ulnar nerve to the biceps muscle (Park and Hoke, 2014). Regeneration

was measured by forelimb grip function, motor action potentials, number of axons, and myofiber size in target muscles (Park and Hoke, 2014). After daily treadmill exercise there was significantly greater functionality and repair in the peripheral nerves beginning in as little as 1 week after surgery when compared to the sedentary sham control recovering mice. Immunocytochemistry showed that the axon number, axon diameter, and myelin thickness were all significantly greater in the exercise group. After 6 weeks the mice were sacrificed and GDNF protein levels in the serum, distal nerve, and target muscles were significantly greater than protein levels in the sedentary mice. This discovery suggests that exercise may increase generation of neurotrophic factors such as GDNF to aid in functional and structural regeneration following nerve injury (Park and Hoke, 2014). The protein levels of GDNF were elevated well after regeneration was underway which suggests a role for GDNF regarding long term maintenance of neuromuscular junctions in the peripheral nervous system.

Calcium

As mentioned previously, calcium plays a major role at the neuromuscular junction and during skeletal muscle contraction. With age, the neuromuscular system undergoes many notable changes, one of which includes significant alterations in calcium homeostasis. While the mechanisms underlying changes in the signaling pathways are currently unknown, there appears to be an age-related development of abnormal neuronal calcium signaling through calcium channels (Foster, 2007). These neuronal abnormalities have been observed in the neurodegenerative disorders

Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (Bezprozvanny. 2010).

Alterations in calcium homeostasis contribute to the demise of motor neurons in ALS (Berchtold et al., 2000). Calcium is a key player in many intracellular pathways so it is critical that calcium homeostasis is tightly regulated in all cells, particularly in neurons. The neurons resistant to degeneration in ALS are those which express a greater amount of Ca^{2+} binding proteins to prevent excitotoxicity (Mattson, 2007). Calcium ions play a large role in the release of neurotransmitters at the neuromuscular junction promoting signaling, neuron excitation, as well as intracellular processes such as gene expression and programmed cell death (Nikoletopoulou and Tavernarakis, 2012). Deregulation of neuronal Ca^{2+} homeostasis can lead to declines in both structure and functionality of neurons in the central and peripheral nervous systems. The current evidence of altered Ca^{2+} homeostasis in neurons suggests that calcium deregulation in target tissues may also contribute to degeneration of the neuromuscular junction.

Age may affect Ca^{2+} ion mobility in skeletal muscle cells. Calcium release from the sarcoplasmic reticulum can occur through RyR1 channels and is necessary for muscle contraction to occur. In aged rodents RyR1 channels were found to be oxidized and damaged to the point where they were described as "leaky" with a decline in functionality when compared to younger adult rodents (Andersson et al., 2011). The open state of RyR1 channels will act to raise intracellular Ca^{2+} concentrations and potentially alter calcium homeostasis. Calcium is critical for the excitation-contraction

coupling in skeletal muscles and alterations in the RyR1 receptor can lead to a decline in specific force production and increase age-dependent muscle weakness (Andersson et al., 2011). The mechanism responsible for the leak in RyR1 channels as well as the pathway between Ca^{2+} alterations and overall muscle weakness has yet to be determined. It is possible that the link between age-related muscle weakness and calcium is the neurotrophic factor, GDNF. Because of this, this study is designed to investigate the role of calcium in regulation of GDNF expression.

While there is a gap in current research examining how calcium ions can regulate GDNF production in skeletal muscle, there have been studies investigating the role of calcium in neuronal expression of BDNF. Calcium is a critical component of many activity dependent signaling pathways in both the CNS and the PNS. The transcription of GDNF, similar to that of BDNF, appears to be activity-dependent as seen with changes in physical exercise and electrical stimulation of cell populations (West et al., 2001). BDNF mRNA expression increases following electrical activity in the brain and displays a neuroprotective role in adulthood (Allen et al., 2013). After Ca^{2+} influx from electrical activity there are a large number of intracellular pathways which can respond by activating a cascade of intracellular signaling molecules thus amplifying the Ca^{2+} signal to the nucleus. Within DNA containing the BDNF gene there is an inactive CREB (cAMP response element binding protein) bound to the BDNF promoter (West et al., 2001). Upon CREB phosphorylation, transcription proteins are recruited to the BDNF promoter and BDNF mRNA is synthesized. In experiments where BDNF CRE or CREB function was inhibited, there was a dramatic loss of BDNF transcription and BDNF protein (Tao et al., 1998).

While there are a variety of pathways that are able to phosphorylate CREB, they are all dependent on intracellular calcium influx. Within neurons there are two main routes for calcium entry. The voltage sensitive calcium channels (L-VSCC) and the ligand gated ion channel N-methyl-D-aspartate-type glutamate receptor (NMDA-R) are responsible for a substantial cellular influx of calcium (Tao et al., 1998; West et al., 2001). Depending on the method of calcium entry there is either a sustained CREB Ser-133 phosphorylation or a transient CREB Ser-133 induced by L-VSCC and NMDA-R respectively. Additionally, this CREB phosphorylation is a developmentally regulated event during early neuronal development NMDA-R induced expression of early-intermediate genes occurs following calcium influx. This pathways allows for a transient increase in BDNF mRNA expression whereas activation of signaling pathways via L-VSCC creates a more sustained increase in BDNF expression (Tao et al., 1998; West et al., 2001). It is possible that a similar calcium-dependent pathway is activated for GDNF transcription.

Taken together, the previous studies suggest that GDNF expression may be activity dependent. The overall aim of the current study was to investigate the role of calcium in regulation of GDNF expression in skeletal muscle.

AIMS OF THE CURRENT STUDY

There are two hypotheses that are explored in the current study. The first hypothesis is that the use of a calcium channel agonist will increase GDNF expression by skeletal muscle. The second hypothesis is that the use of calcium channel

antagonists will decrease GDNF expression by skeletal muscle. By using both a calcium channel agonist and antagonists we can promote calcium flux and inhibit calcium flux respectively, to examine the effects of calcium on GDNF mRNA and protein levels in skeletal muscle.

MATERIALS AND METHODS

C2C12 mouse myoblast cell line

The C2C12 cell line is a prominently used *in vitro* model of skeletal muscle (Richler and Yaffee, 1970). C2C12 cells are myoblasts capable of differentiating into myotubes. Phenotypically, myotubes exhibit contractile abilities and are capable of expressing various neurotrophic factors (Vianney and Spitsbergen, 2014). The experiments described herein utilized the C2C12 cell line at an early passage number (passage 5-7) and its ability to create and secrete GDNF as a model for mammalian skeletal muscle.

Cell culture procedures

C2C12 mouse skeletal muscle cells were cultured on 100-mm plates (Falcon) in 89% Dulbecco's Modified Eagle's Medium (DMEM; ATTC). In addition to the DMEM, cell medium was supplemented with 10% fetal bovine serum (Mediatech, Manassas, VA) and 1% antibiotic-antimycotic (Invitrogen-GIBCO). C2C12 myoblasts were placed in 37°C incubator with an atmosphere containing 95% atmospheric air supplemented

with 5% CO_2. Cells, cell medium, incubator, and culture hood were kept under sterile conditions during all experimentation to prevent contamination of cells.

After subculture into 6-well plates (Falcon) C2C12 cells were allowed to reach 100% confluence. At this point in confluency the cell medium was switched to DMEM, 10% horse serum (Sigma), and 1% antibiotic-antimycotic (Invitrogen- GIBCO). Cell medium was removed by vacuum every 1-2 days. The myoblasts were allowed to mature into myotubes and at day 5-6 myotubes were subjected to treatment. Myotubes were treated with cell medium containing Bay K8644 (Bay K), nifedipine, or dantrolene (100μM) for each treatment for 24 hours prior to sample collection. Samples of myotubes were taken to examine intracellular GDNF and samples of cell medium were taken to examine extracellular GDNF levels.

GDNF protein quantification via ELISA

Levels of GDNF protein were quantified by enzyme linked immunosorbent assay (ELISA). The 96-well ELISA plate (Thermo Scientific) was first coated with GDNF primary antibody (1μg/mL; R&D Systems) and incubated overnight in a humidification chamber at room temperature. Each plate was rinsed with wash buffer containing phosphate buffered saline (PBS, pH 7.4) (deionized water supplemented with 0.9% NaCl, 0.24% monobasic NaH_2PO_4, 1.14% dibasic NaH_2PO_4,) and 0.05% Tween-20. During all rinses the plate was on low powered plate stirrer. Then a blocking solution of PBS containing 5% sucrose (MP Biomedicals, LLC) and 1% bovine serum albumin (BSA: Fisher Scientific) was added to the plate and incubated for exactly one hour.

Again the plate was rinsed after which the samples were added in the amount of 100µL/well. Samples were added in triplicate and a standard curve was performed. A standard curve using GDNF protein standard (R&D Systems) was performed during every ELISA containing samples. The ELISA plate with samples and standard curve was allowed to incubate for 2 hours. Following incubation, the plate was rinsed and a secondary antibody solution consisting of Tris buffered saline (TBS pH 7.3), 0.1% BSA, 0.05% Tween-20, and 0.02% anti-GDNF secondary antibody conjugated to biotin (R&D Systems) was added to the plate. ELISA plates were then incubated overnight and rinsed. Pierce High Sensitivity Streptavidin HRP (horseradish peroxidase; Thermo Scientific) was added as 0.5% to a solution containing PBS (pH 7.4) and 1% BSA. This solution was added to the ELISA plate in the amount of 100µL/well and allowed to incubate in the humidification chamber for 20 minutes. After this incubation the plate was rinsed and the color reagent (1-Step Turbo TMB- ELISA; Thermo Scientific) was added in the amount of 100µL/well. The plate was then placed in the humidification chamber and after 35 minutes 1M HCl was added to each well in the amount of 100µL/well. All ELISA plates were read by the plate reader at a wavelength of 450nm immediately following addition of HCl to the color reagent.

Total mRNA extraction from C2C12 cells

ELISA was used for protein quantification and mRNA levels were measured via real time polymerase chain reaction (qPCR). Cell medium (1mL) was removed from each well of the 6-well cell culture plate (Falcon) for protein analysis via ELISA. The

remaining cell medium was removed by vacuum suction in the sterile hood and the cells were rinsed with 3mL Tyrode's saline solution per well. The Tyrode's saline was then removed and a Trypsin- EDTA (Sigma) solution (90% Tyrode's saline, 10% EDTA) was added in the amount of 1mL per well. This solution was sufficient to remove all cells from the plate, and an equal amount of cell medium (1mL per well) was added to dilute and inhibit the Trypsin activity. The cells suspended in solution from each well were transferred to sterile 15mL centrifuge tubes and centrifuged for 5 minutes. TRI Reagent (1mL; Sigma) was added to each tube containing cells and mixed until cells were completely homogenized with the solution. An equal portion (1mL) of 95% ethanol was mixed into the TRI Reagent and cell solution after which 700µL of solution was placed into an RNase-Free spin column inside an RNase-Free collection tube (Zymo Research).

The procedure for mRNA extraction was provided by Zymo Research for Direct-zol RNA MiniPrep Plus as a method for using columns for RNA extraction directly from TRI Reagent samples. In order to prevent contamination of DNases and RNases, the work area and all tools were wiped with DNAase Away (Thermo Fisher) and RNase-Free micropipette tips were used (Ambion). All centrifugation steps were performed at 14,000 x g for 30 seconds. RNA Wash Buffer (400µL) was added to each column and centrifuged again. A DNA digestion buffer solution (6.25% DNase, 93.75% DNA Digestion Buffer) was added in the amount of 80µL directly to each column and allowed to incubate at room temperature for 15 minutes. Following incubation, 400µL of Direct-zol RNA PreWash was added to each column and centrifuged again. The flow-through was discarded and the collection tube was placed back under the spin column. The

addition of Direct-zol RNA PreWash (400µL) was added and centrifuged again. After repeating the Pre-Wash step 700µL of RNA Wash Buffer was added to each column and centrifuged again but this time for a complete 2 minutes so ensure complete removal of wash buffer from the samples. Every column was transferred to an RNase-free tube and 100µL of DNase/RNase-Free Water was added and centrifuged for 30 seconds. At this point the extracted RNA was frozen in -80°C until RNA quantification could occur.

Real time- PCR for mRNA quantification

Immediately prior to qPCR, all samples of RNA were measured with a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific) to ensure proper levels of RNA were present after the RNA extraction procedure. DNase/RNase free tips were used to transfer 1µL of extracted RNA onto the NanoDrop. DNase/RNase free water was used for calibration. An absorbency ratio of 2.0 for each sample at the 260nm/280nm wavelengths is generally accepted as “pure” for RNA. Additionally, the NanoDrop was used to measure the concentration of total RNA extracted and a level greater than 60 ng/uL was deemed appropriate for qPCR.

The procedure for qPCR was provided by Qiagen, as was the QuantiNova SYBR Green RT-PCR kit (Hilden, Germany). QuiantiTect Primmer Assays (Qiagen) were used for mouse GDNF (GDNF; Mm_Gdnf_1_SG), beta-actin (Actin; Mm_Actb_1_SG), and Glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Mm_Gapdh_3-SG). A 96-well PCR plate (Applied Biosystems) was used and a solution consisting of 10µL of 2x

QuantiNova SYBR Green RT-PCR Master Mix, 1µL QN ROX Reference Dye, 0.2µL of QN SYBR Green RT-Mix, 2µL primer, and 17µL RNase-Free Water was added to each well in the PCR plate. After the solution was added to the appropriated wells and with the appropriate primer, 2µL of sample RNA was added to the wells in triplicate. The PCR plate was covered with optical adhesive covers (Fisher Scientific) the entire plate was centrifuged at 14,000 x g for 30 seconds to ensure the solution was properly mixed before adding the plate to the real-time cycler. The cycler (StepOnePlus Real-Time PCR System by Applied Biosystems) was set for Ct quantification of RNA using SYBR Green One-Step set up. The cycler conditions were set to 40 cycles at the following conditions followed by the melt curve analysis. For the reverse transcription step the temperature was set to 50°C for 10 minutes. The PCR initial activation step was set to 2 minutes at 95°C, followed by a 5 second denaturation step also at 95°C. The combined annealing extension step was set for 10 seconds at 60°C.

For comparison of the GDNF mRNA between samples the Ct value was averaged between the triplicate samples. The delta-delta Ct value was calculated for every sample as outlined by Livak and Schmittgen (Livak and Schmittgen, 2001). This measurement compares the gene of interest to the housekeeping gene expression to give delta Ct. The delta Ct is then compared with the delta Ct for the untreated control samples to give delta-delta Ct. The delta-delta Ct is converted to fold change and the results were graphed.

Statistics

Comparisons between multiple groups were made using a one-way ANOVA. A Tukey's (Honest Significant Difference) test was used following the ANOVA to determine any significant difference between two means for GDNF protein data. For direct comparisons of mRNA levels a student's T-test was used to determine significance from vehicle control. A p-value of less than 0.05 was considered statistically significant for both the ELISA results and the qPCR data. The error bars on the graphs indicate the standard error of the mean.

RESULTS

Role of L-type Ca^{2+} channels on myotube GDNF protein levels

Myotubes express voltage-gated L-type calcium channels on the sarcolemma. In order to examine the role of L-type Ca^{2+} channels on GDNF protein levels, the agonist, Bay K8644 (Bay K) was used (figure 2). Bay K acts to increase calcium influx through voltage-gated L-type Ca^{2+} channels.

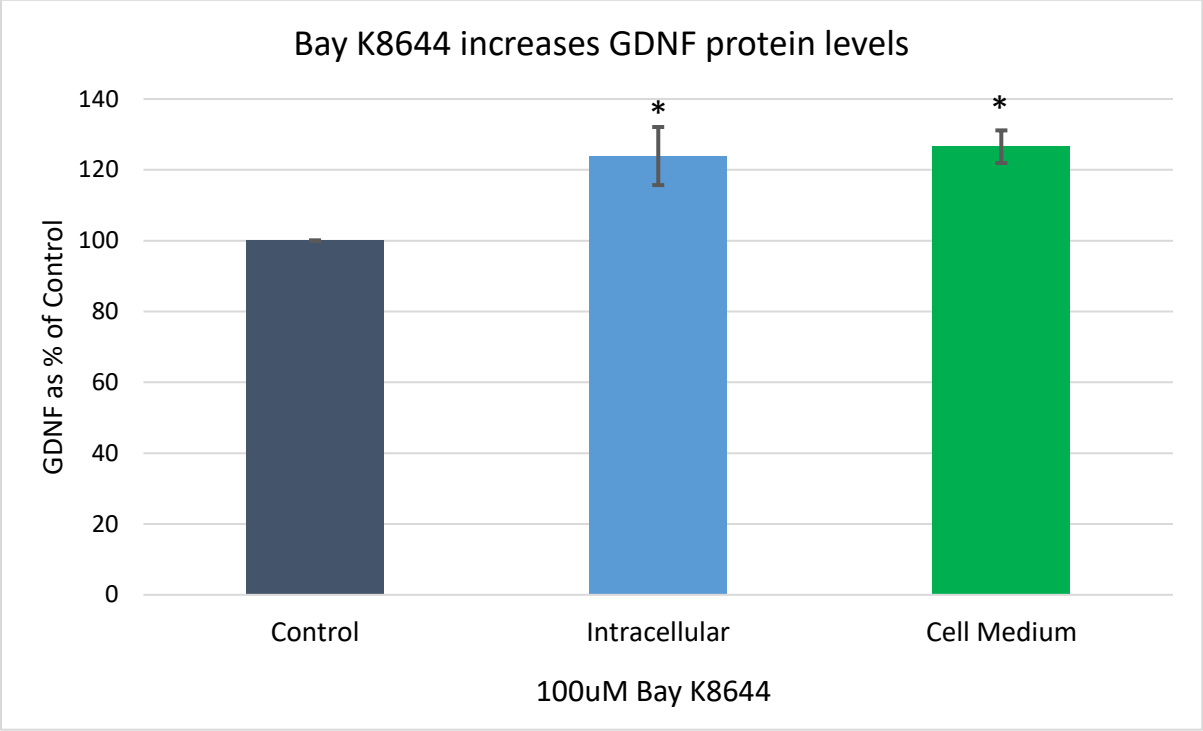

Figure 2: Effect of Bay K8644 on GDNF protein levels. C2C12 myotubes were treated with Bay K8644 (100μM) and GDNF levels were measured with ELISA. Results show that the intracellular GDNF protein levels were increased to 123.9% of control myotubes (n=8). Extracellular GDNF protein levels were increased to 126.5% of control (n=4). The values shown here indicate the sample mean ± SEM. The ***** indicates significance of $p<0.05$.

Following 24 hour treatment with Bay K, intracellular levels of GDNF protein were significantly increased to 124% of control ($p<0.05$). Extracellular levels of GDNF protein were increased to 127% of control myotubes ($p<0.01$). To demonstrate further that calcium influx through L-type channels affects GDNF levels we chose to examine the effects of an antagonist for L-type calcium channels on GDNF protein (figure 3).

Treatment with nifedipine (100μM) decreased intracellular GDNF to 65% of control ($p<0.01$). Additionally, the protein levels of GDNF found in the surrounding cell medium was also significantly decreased to 64% of GDNF present in vehicle control

treated myotubes ($p<0.05$) (figure 3). There was no significant difference between intracellular GDNF protein and secreted GDNF.

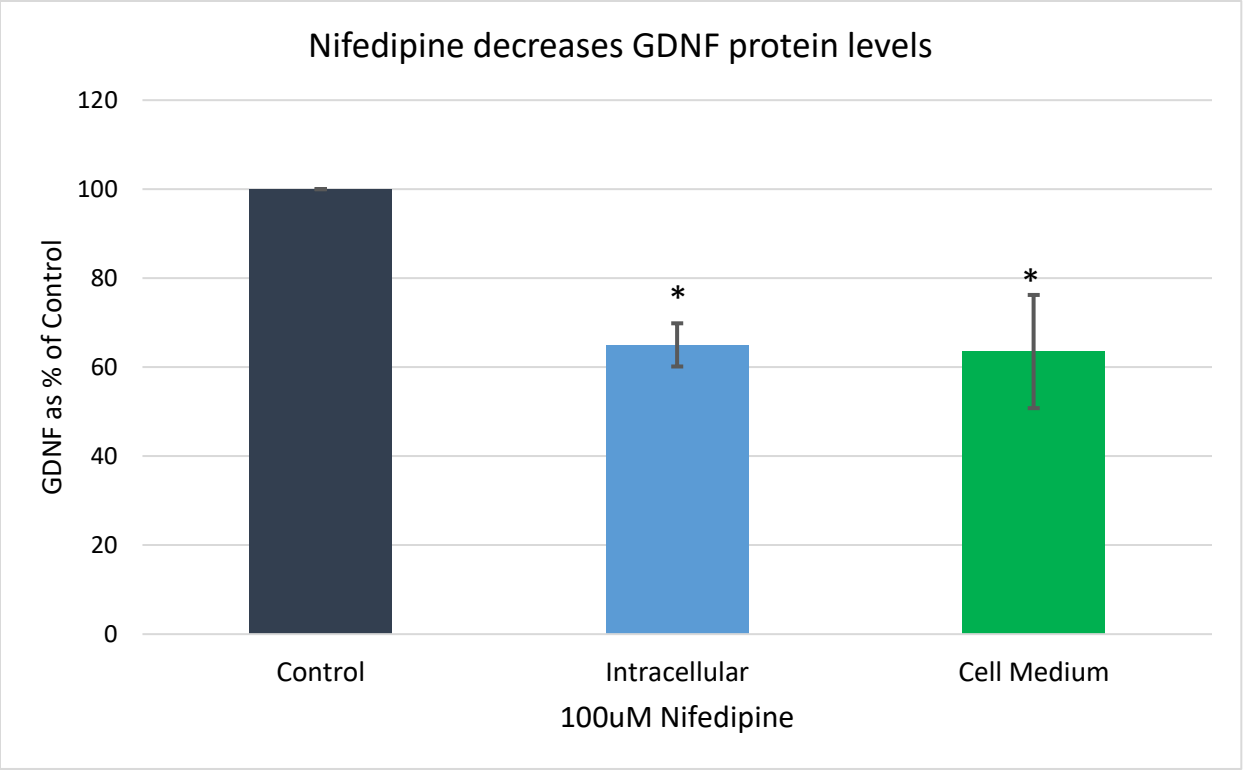

Figure 3: Effect of nifedipine on GDNF protein levels. C2C12 myotubes were treated with nifedipine (100μM) and GDNF was measured with ELISA. Results show that the intracellular GDNF protein levels were decreased to 65.0% of control (n=16). Extracellular GDNF protein levels were decreased to 63.5% of control myotubes (n=8). The values shown here indicate the sample mean ± SEM. The ***** indicates significance of $p<0.05$.

Role of RyR Ca^{2+} channels on myotube GDNF protein levels

Dantrolene is a known antagonist of ryanodine (RyR) channels within the sarcoplasmic reticulum. When RyR channels are inhibited there is a lower probability of calcium release from internal stores. Typically following the depolarization of voltage gated L-type calcium channels the RyR channels will open to increase intracellular

calcium levels. Here we demonstrate the effect of a RyR channel antagonist on intracellular and secreted GDNF protein levels (figure 4).

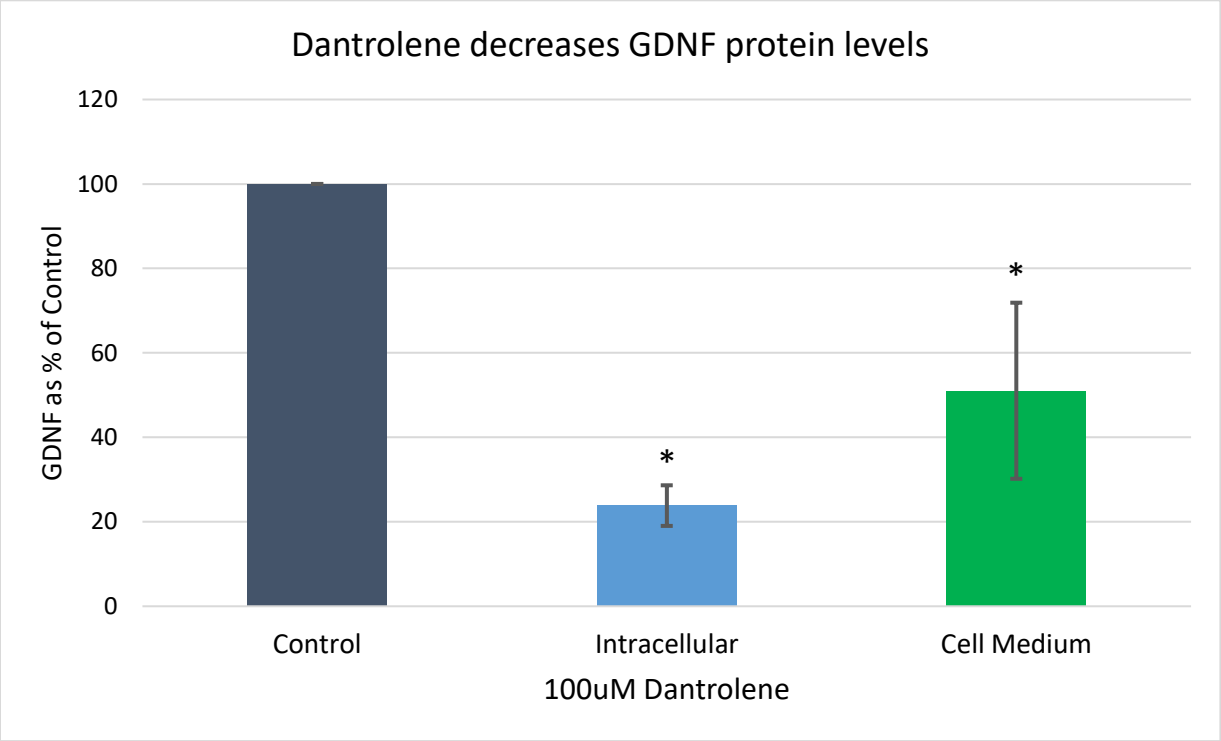

Figure 4: Effect of dantrolene on GDNF protein levels. C2C12 myotubes were treated with dantrolene (100μM) and GDNF was measured with ELISA. Results show that the intracellular GDNF protein levels were decreased to 23.8% of control (n=5). Extracellular GDNF protein levels were decreased to 51.0% of control myotubes (n=3). The values shown here indicate the sample mean ± SEM. The ***** indicates significance of $p<0.05$.

Treatment with dantrolene (100uM) decreased intracellular GDNF protein levels to 24% of control ($p<0.01$). Similarly, results showed a significant decrease in extracellular levels of GDNF protein to 51% of control ($p<0.05$).

mRNA levels of GDNF

In addition to examining GDNF protein levels, the expression of GDNF mRNA was also examined. Real time polymerase chain reaction (qPCR) was used to detect the levels of GDNF mRNA in untreated control myotubes and in myotubes treated with calcium channel agonists and antagonists. As predicted, results showed an increase in GDNF mRNA expression following treatment with Bay K8644 (figure 5). We observed a significantly increased level of GDNF protein compared to controls and as expected there was a significant ($p<0.01$) increase in mRNA. Myotubes treated with the L-type calcium channel agonist displayed an increase in expression to 211% of control GDNF mRNA levels (figure 5).

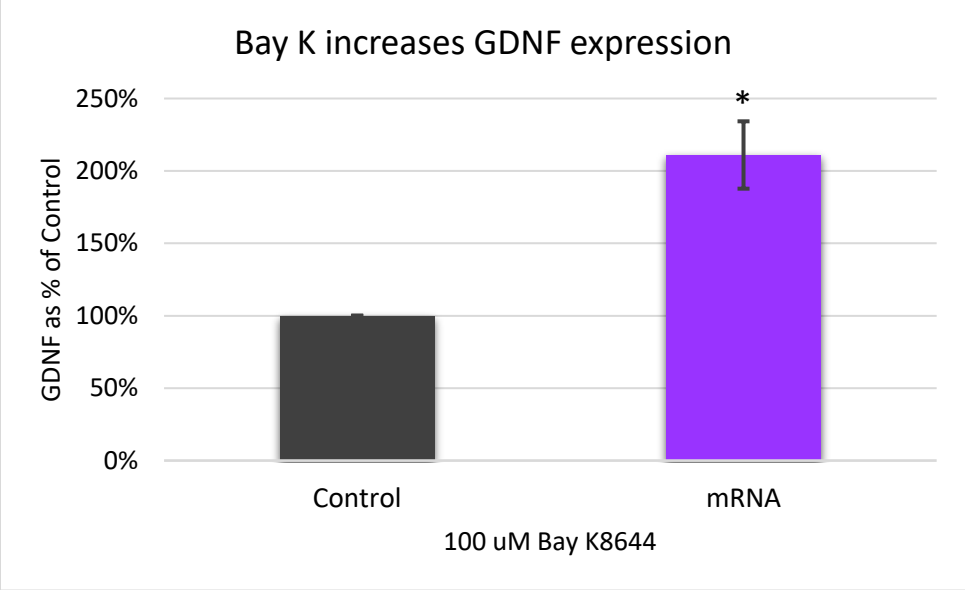

Figure 5: Effect of Bay K8644 on GDNF mRNA levels. Real time PCR was used to identify changes in GDNF transcription in C2C12 myotubes following treatment with Bay K8644 (100μM). Results showed that GDNF mRNA levels were significantly increased to 211% of control (n=4). The values shown here indicate the sample mean ± SEM. The ***** indicates significance of $p<0.05$.

As predicted, treatment of myotubes with the L-type Ca^{2+} channel antagonist, nifedipine resulted in a decrease in GDNF mRNA compared to control (figure 6). Results show that the mRNA is significantly decreased following a decline in calcium flux, these findings support the observed decrease regarding the protein levels. There was a significant ($p<0.01$) decline in GDNF mRNA levels to 50% of control mRNA following inhibition of voltage gated L-type Ca^{2+} channels (figure 6).

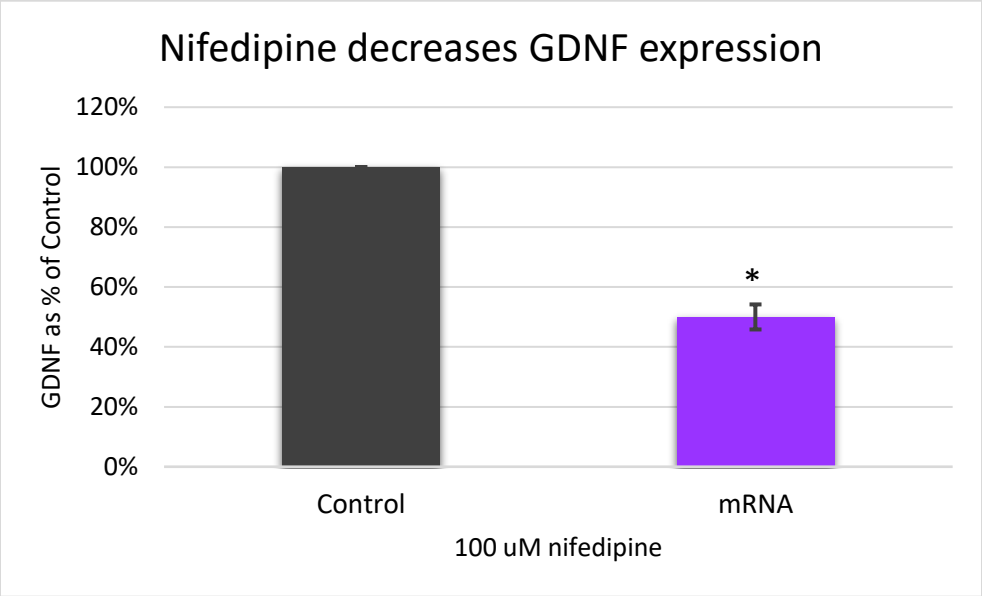

Figure 6: Effect of nifedipine on GDNF mRNA levels. Real time PCR was used to identify changes in GDNF transcription in C2C12 myotubes following treatment with nifedipine (100μM). Results showed that GDNF mRNA levels were significantly decreased to 50% of control (n=4). The values shown here indicate the sample mean ± SEM. The ***** indicates significance of $p<0.05$.

While there was a strong decline in GDNF protein levels following treatment with dantrolene, there was no statistically measureable decline in GDNF mRNA (figure 7). Included in the graph is the sample size of 3 (n=3) with one being 187% of control and the other 29% of control. The other n was beneath the detection limit of our qPCR

despite having normal and detectable levels of the two housekeeping genes (Gapdh and beta-actin). For this reason the graph shows the value for the sample that was below the detection limit of the assay as 0% of control GDNF mRNA. This very low mRNA level for GDNF could suggest that there may be a decline in GDNF transcription following inhibition of RyR receptors but further experiments are necessary (figure 7).

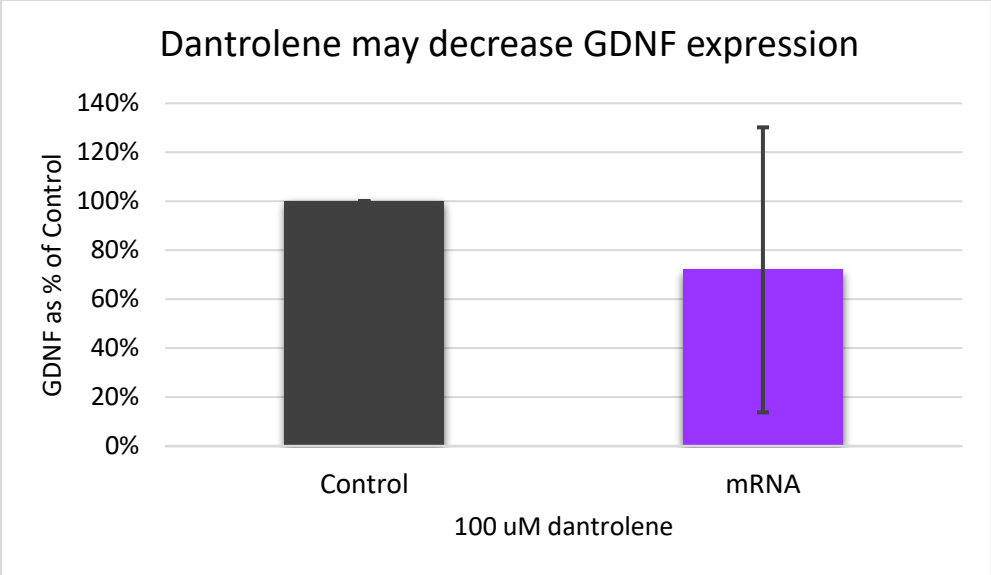

Figure 7: Effect of dantrolene on GDNF mRNA levels. Real time PCR was used to identify changes in GDNF transcription in C2C12 myotubes following treatment with dantrolene (100μM). Results showed that GDNF mRNA levels were an average of 72% from control (n=3). The values shown here indicate the sample mean ± SEM. The results were inconclusive and there was no significant effect of treatment, likely due to a low sample size and high variability.

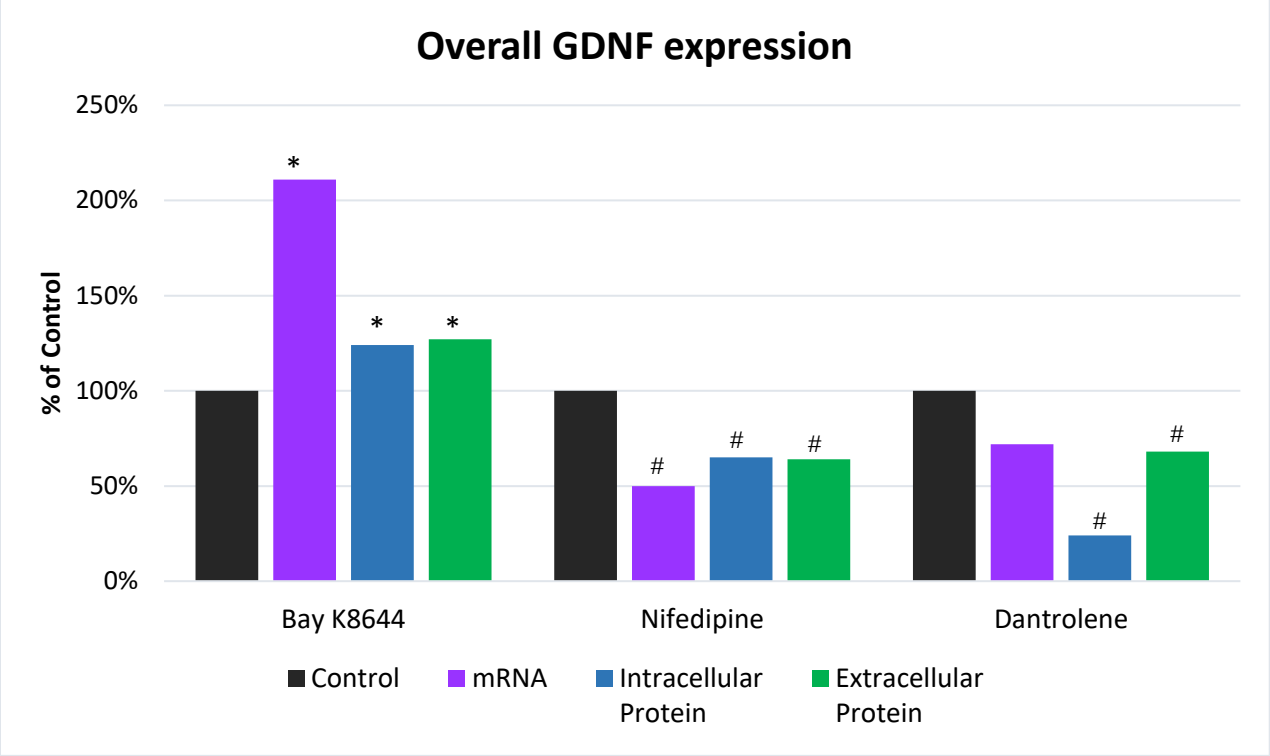

Figure 8: Overall effects of manipulation of Ca^{2+} channels on GDNF mRNA. Bay K8644 caused an increase in expression (* indicates $p<0.05$ increase from control). Nifedipine caused a decrease in expression (# indicates $p<0.05$ decrease from control), together these show the agonistic and antagonistic effects on L-type Ca^{2+} channels respectively. Dantrolene was used as an RyR channel antagonist and GDNF protein levels were significantly decreased below control. Values in the graph are expressed as sample means ± SEM.

DISCUSSION

Calcium is a known ion critical for excitation-contraction coupling and has the ability to act as a potent secondary messenger. The objective of this study was to investigate the effect that manipulation of calcium flux through L-type Ca^{2+} channels and RyR channels has on GDNF expression. Results from these studies suggest a link between Ca^{2+} and GDNF expression in myotubes.

Calcium channel agonist increases GDNF protein levels

Previous studies from our laboratory documented an increase in GDNF protein in skeletal muscles following exercise in rats (Gyorkos et al., 2014). Similarly, when C2C12 myotubes were subjected to electrical stimulation GDNF protein levels were altered (Vianney et al., 2014). Our study used manipulation of L-type Ca^{2+} channels in resting myotubes to simulate the calcium flux seen with exercise in skeletal muscle and observe changes in GDNF.

Bay K8644, a known agonist of L-type Ca^{2+} channels, has been shown to promote Ca^{2+} entry in C2C12 myotubes (Jorquera et al., 2013). Through the use of this channel agonist, our goal was to determine if increasing calcium flux in resting myotubes would increase GDNF levels. Other researchers have shown that in primary cortical neurons and in the PC-6.3 neuroendocrine cell line, GDNF is secreted in a calcium dependent manner (Lonka-Nevalaita et al., 2010). Our results showed that the use of a calcium channel agonist significantly increased both intracellular and extracellular GDNF protein levels (figure 2). This indicates that perhaps the entry of Ca^{2+} through L-type channels is similar to the calcium flux seen with excitation-contraction coupling in skeletal muscle and induces similar changes in levels of GDNF protein. Exploration of the effect of L-type Ca^{2+} channels on GDNF expression corroborates findings for an exercise-dependent production of neurotrophic factors by skeletal muscles (Vianney et al., 2014; McCullough et al., 2011). Physical exercise in skeletal muscle promotes calcium flux through L-type calcium channels and increases

GDNF protein levels, a similar effect was observed in the current study though the use of Bay K.

Lonka-Nevalaita et al. (2010) determined that when neurons were subjected to KCl-induced depolarization there was an increase in secretion of mature GDNF protein. This effect was inhibited by use of an extracellular calcium chelator, BAPTA-AM, which was sufficient to block the increase in GDNF protein secretion (Lonka-Nevalaita et al., 2010). That study suggests that calcium influx from external stores is necessary to increase GDNF protein levels in muscle cells, which is consistent with our results regarding the use of Bay K. Our current study indicates even in the absence of myotube stimulation, the alteration of calcium channels with agonists created an increase in GDNF protein levels. Our examination GDNF protein levels did not vary between intracellular and extracellular levels, compared to control, which suggests that GDNF may be regulated at the transcription level in resting myotubes as opposed to the posttranslational secretion of GDNF that Lonka-Nevalaita et al. observed in stimulated neurons (2010). Following our experiments with a Ca^{2+} channel agonist, we then utilized calcium channel antagonists to determine if GDNF protein levels would be decreased when calcium flux is inhibited in myotubes.

Calcium channel antagonists decrease GDNF protein levels

We examined the effect of inhibiting Ca^{2+} entry through L-type calcium channels with nifedipine. As expected, results showed decreased intracellular and extracellular GDNF protein levels in myotubes following treatment with nifedipine (figure 3). Results

suggest that when voltage-gated L-type Ca^{2+} channels are inhibited, there is a significant decrease in the amount of GDNF protein found within cells and in cell medium. This is consistent with studies which demonstrated that inhibition of L-type Ca^{2+} channels caused decreased transcription of neurotrophic factors (Jorquera et al., 2013). Our results suggest that when calcium flux through L-type channels is limited, the effect on GDNF protein levels is similar to the decreased muscular GDNF levels seen with inactivity (Vianney et al., 2014; Wehrwein et al., 2002). Our study demonstrated that manipulation of the voltage gated L-type Ca^{2+} channels with Bay K and nifedipine was sufficient to alter the protein levels of GDNF by mature myotubes. As predicted in the hypothesis, the greater Ca^{2+} flux allowed by open Ca^{2+} channels increased GDNF and the decline in Ca^{2+} mobility created a subsequent decline in GDNF protein levels.

One of the main influences of L-type Ca^{2+} channels is to open RyR channels in the sarcoplasmic reticulum, which release calcium from internal stores (Tao et al., 1998; West et al., 2001). Release of Ca^{2+} from the sarcoplasmic reticulum through RyR channels further increases intracellular calcium levels (Cardenas et al., 2004). We examined the effect of inhibiting RyR channels using the antagonist, dantrolene, on GDNF production in myotubes. Other researchers studied the effect of dantrolene in electrically stimulated hippocampal neurons and determined that dantrolene decreased extracellular BDNF protein levels (Balkowiec and Katz, 2002). Our results demonstrate that when RyR calcium channels are inhibited there is a significant decrease in extracellular GDNF protein levels and a similar decrease in intracellular GDNF protein

levels (figure 4). Similar to the earlier study our results indicate that inhibition of calcium release from internal stores can have an effect on neurotrophic factor protein levels.

Balkowiec and Katz (2002) noted that following electrical stimulation there was a decrease in the amount of BDNF protein secreted into cell medium when dantrolene was administered to neurons. Their findings suggest that Ca^{2+} flux through internal calcium channels plays a role in the secretion of BDNF and our results show an overall decreased level of GDNF protein in resting myotubes. We utilized a post hoc one-way ANOVA to determine if there was a significant difference between intracellular GDNF protein and GDNF protein found in cell medium. Our observations following manipulation of L-type calcium channels, and with manipulation of RyR channels, found no significant difference between intracellular and extracellular GDNF protein levels. A significant difference between intracellular and extracellular GDNF protein levels may suggest that Ca^{2+} flux from internal stores could have a role in regulating the secretion of GDNF protein from skeletal muscle cells.

Calcium channel agonist increases GDNF mRNA

There are many intracellular signaling pathways by which calcium flux can significantly alter gene expression (West et al., 2001; Nikoletopoulou and Tavernarakis, 2012). Furthermore, the calcium flux observed during depolarization in skeletal muscle, has the ability to activate a variety of intracellular calcium dependent pathways (Cardenas et al., 2004). In order to determine if the increase in GDNF protein following treatment of myotubes with an L-type Ca^{2+} channel agonist was accompanied by an

increase in GDNF transcription, we examined levels of GDNF mRNA. Our results demonstrate that manipulation of calcium flux with a channel agonist is sufficient to increase GDNF mRNA in myotubes (figure 5). A recent study examined the effects of electrical stimulation in skeletal muscles following nerve injury and discovered an upregulation of both GDNF and BDNF mRNA (Willand et al., 2016). This study suggests that neurotrophic factor transcription may be regulated in an activity dependent manner and supports our findings that an increase in calcium flux increased GDNF mRNA levels. Additionally, the study by Willand et al. (2016) suggests that GDNF and BDNF are similar in that both neurotrophic factors displayed an activity-dependent increase in mRNA levels.

Consistent with our results regarding GDNF mRNA, other researchers discovered that BDNF displays activity- dependent gene transcription via Ca^{2+} entry through L-type Ca^{2+} channels (Tao et al., 1998). The researchers examined BDNF expression in cultured neurons and found that calcium influx was able to activate the CREB signaling pathway and induce BDNF gene transcription. Our results show a similar calcium dependent effect that GDNF expression can be increased in myotubes by utilizing an L-type Ca^{2+} channel agonist.

Calcium channel antagonists decrease GDNF mRNA

We discovered that inhibition of calcium flux in myotubes was able to decrease GDNF mRNA levels. Other researchers have demonstrated the importance of muscle depolarization and the activity of L-type Ca^{2+} channels in downstream gene transcription

(Yamamoto et al., 1996; Yu et al., 2001). Our results show that the use of nifedipine to inhibit calcium flux through L-type Ca^{2+} channels significantly decreased levels of GDNF mRNA (figure 6). These results showed that in the absence of depolarization, or electrical stimulation, the inhibition of calcium flux was sufficient to decrease GDNF mRNA in myotubes. Our results suggest that since alterations of voltage-gated Ca^{2+} channels have an effect on GDNF mRNA that this decrease in expression would be further amplified following depolarization in myotubes. Gyorkos et al. (2014) demonstrated that skeletal muscles not recruited during physical exercise failed to increase GDNF protein content when compared to more heavily recruited muscles. Other research suggests that RyR receptors within the sarcoplasmic reticulum of muscle cells play an even more prominent role in gene transcription than L-type Ca^{2+} channels (Andersson et al., 2013).

Alterations of Ca^{2+} flux from internal stores can play a role in levels of neurotrophic factor mRNA. Thapsigargin was used to increase calcium release from the endoplasmic reticulum in C6 glioblastoma cells (Oh-hashi et al., 2006). Their results showed that an increase in calcium flux from internal stores caused an increase in GDNF mRNA. Oh-hashi et al. (2006) demonstrated the potential for Ca^{2+} flux to regulate GDNF expression in C6 cells, an observation consistent with our results in C2C12 cells.

In order to investigate the effect of a RyR channel antagonist on GDNF mRNA we utilized dantrolene in myotubes. Our results indicate that there was a nonsignificant effect of dantrolene on production of GDNF mRNA (figure 7). It is possible that a

greater sample size would lead to a significant decline in GDNF mRNA when intracellular calcium flux is inhibited. One sample of dantrolene treated myotubes displayed GDNF mRNA levels beneath the detection limit of our qPCR which suggests a large downregulation of GDNF without altering mRNA levels of housekeeping genes. Other researchers have examined the role of calcium flux through RyR channels and its effect on neurotrophic factors. Within hippocampal neurons inhibition of calcium release from internal stores, utilizing dantrolene, decreased expression of BDNF (Balkowiec and Katz, 2002). The significant decrease in GDNF protein following inhibition of RyR channels, suggests that with additional experimentation there may be a decrease in GDNF mRNA. Our results suggest that calcium flux through RyR channels in skeletal muscle may regulate GDNF expression.

Alterations in calcium homeostasis may contribute to neuromuscular dysfunction

A disruption in calcium homeostasis is implicated with sarcopenia. Researchers discovered that alterations to RyR channel functionality caused changes in intracellular calcium levels and lead to a decline in force production of skeletal muscle (Andersson et al., 2011). Our research suggests that calcium flux in myotubes may regulate GDNF expression. In combination with other studies demonstrating the importance of GDNF in regulating functionality of the neuromuscular junction it is possible that declines in regulation of Ca^{2+} homeostasis contribute to decreased levels of GDNF (Lin et al., 1993; Morcuende et al., 2013).

With age there is a decline in the intracellular buffering capacity of neurons and this disruption of Ca^{2+} homeostasis can lead to neuronal cell death (Mattson, 2007). Hormesis is the process of exposing cells to moderate stressors that will result in adaptive changes for the purpose of protecting the organism from damage associated with more severe stress. Calcium has the ability to activate hormetic pathways by activation of CREB by calcium and calmodulin (Mattson, 2007). CREB activation can then induce expression of neurotrophic factor proteins which will promote neuronal survival. Our data suggests that the use of the calcium channel agonist, Bay K, causes an increase in GDNF transcription as evidenced by the increase in GDNF mRNA in myotubes. A previous study examined the activation of CREB in its ability to regulate gene expression of brain derived neurotrophic factor (BDNF) in neuronal cells (Tao et al., 1998). In its inactive form CREB is not phosphorylated and gene expression of BDNF was inhibited (Tao et al., 1998). The increase of intracellular calcium levels can activate transcription by phosphorylation of the CREB transcription regulating proteins followed by expression of BDNF in neurons.

Exercise has been shown to increase both neuronal and muscular levels of GDNF, however more research into the physiological pathway responsible for this effect is necessary. It is possible that exercise provides a hormesis effect by enhancing the ability of muscle cells to regulate Ca^{2+} homeostasis. The research described herein demonstrates that the use of Ca^{2+} channel agonists and antagonists in the absence of electrical stimulation was sufficient to alter GDNF protein expression in skeletal muscle cells.

Other research indicates an age-dependent change in RyR Ca^{2+} flux (Andersson et al., 2013). Jimenez-Moreno et al. studied the effect of age on calcium ion release from the sarcoplasmic reticulum (2008). They discovered that elderly mice exhibited decreased levels of calcium mobility through RyR channels in the flexor digitorum brevis muscle when compared to young mice. Similarly, Wang et al. (2000) investigated the effect of calcium flux in the same muscle in mice following electrical stimulation. Researchers noted a significant reduction in peak Ca^{2+} concentration following electrical stimulation in elderly mice compared to middle and young aged mice. The decrease in regulation of calcium flux in aged muscle may be due to calcium channel dysfunction. Calcium leak through RyR channels has been observed in aged muscle leading to declines in the ability of muscle cells to regulate Ca^{2+} homeostasis (Andersson et al., 2013). Our research suggests that dysregulation of normal calcium channels could contribute to alterations in neurotrophic factor protein and mRNA. Examination of the extensor digitorum longus muscle in 24 month old rats demonstrated an age-dependent decline in RyR channel function and a reduction in calcium release from internal stores (Andersson et al., 2013). It is possible that the reduced skeletal muscle force production that accompanies RyR channel dysfunction is the result of decreased neuromuscular plasticity caused by inadequate levels of GDNF protein. Our results indicate that calcium flux has the ability to regulate GDNF production in skeletal muscle.

Potential limitations of study

There are a few potential limitations to the previously described studies. Inhibitors for calcium channels such as nifedipine and dantrolene have been extensively studied for their ability to limit Ca^{2+} ion movement in skeletal muscle cells by increasing the probability of a closed channel state. Similarly, Bay K8644 is an extensively studied and publicized Ca^{2+} channel agonist so experimentation to validate its effect on Ca^{2+} ion mobility was not performed in this case. A potential limitation to this study could be the lack of quantification of Ca^{2+} ion mobility since actual Ca^{2+} ion levels in cells or extracellularly were neither measured nor visualized. Another limitation of this study was the time course for experimentation. At early time points such as 2-6 hours the levels of GDNF protein were often below the detection limit of our ELISA. For this reason all samples were taken 24 hours following change of cell medium with chemical agents and protein levels were within our standard curve even when decreased below control myotubes GDNF levels. In order to be consistent with the protein data, GDNF mRNA was extracted from myotubes 24 hours after treatment. It is possible that GDNF mRNA levels can oscillate in a matter of hours which would not be captured by this protocol but could be remedied by creating a time course for GDNF expression in C2C12 myotubes.

Recommendations for future study

One area for future study would be an examination of the time course for GDNF expression in human skeletal muscle and how this corresponds to *in vitro* studies.

Gathering data regarding the normal fluctuations of GDNF during inactivity and physical exercise in adult mammals would result in a better understanding of the production and regulation of GDNF over time. This would enable researchers to examine the skeletal muscle activity dependent alteration of GDNF.

While this study suggests that GDNF expression is a calcium dependent process in skeletal muscle cells more research is necessary to determine the upstream regulatory pathway for GDNF expression. This could be accomplished by treating cells with various signaling molecule inhibitors and monitoring the downstream effects of such treatments. The study described here suggested that a rise in intracellular Ca^{2+} ions may result in increased neurotrophic factor expression but further research on which signaling pathway mediates this effect is necessary to fully document how physical activity in skeletal muscle alters GDNF production. Since activity-dependent BDNF transcription occurs through the CREB pathway in neurons, it is possible that GDNF transcription in myotubes uses the same pathway (Tao et al., 1998). In order to test the involvement of the CREB pathway in GDNF expression, researchers could utilize an inhibitor of CREB phosphorylation and activation in skeletal muscle cells, while quantifying GDNF mRNA and protein.

CONCLUSION

In summary, the results demonstrate that calcium in skeletal muscle cells has the ability to regulate expression of GDNF. Calcium ions play a large role in skeletal muscle contraction and Ca^{2+} flux may regulate GDNF expression. The current studies

demonstrate that when myotubes are treated with a calcium agonist there is a significant increase in GDNF mRNA and protein. Similarly, treatment with Ca^{2+} antagonists resulted in a significant decrease in GDNF mRNA and protein levels. After examination of L-type channels and RyR channels it appears that a rise in intracellular Ca^{2+} levels is necessary to stimulate upregulation of GDNF above vehicle control levels. Similarly, a decline in intracellular Ca^{2+} levels was determined to correlate with a significant decrease in GDNF expression. Considering the highly regulated Ca^{2+} homeostasis that occurs in all cell types and the novel information presented here it is perhaps unsurprising that disruptions in Ca^{2+} ion levels may alter the structure and function of the mammalian neuromuscular junction by altering levels of GDNF.

In aging mammals there is often progressive muscle weakening and neuronal degeneration. These unfortunate effects of aging are often times accelerated in people with neurodegenerative diseases. Disruptions in Ca^{2+} homeostasis in neurons is associated with a variety of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Bezprovanny, 2010; Mattson, 2007). Research on alterations of neurotrophic factors with neurodegenerative disorders corroborates the current findings that Ca^{2+} ion mobility can alter the amount of GDNF produced by skeletal muscles. If GDNF is not made by the skeletal muscle and transported to a nearby motor neuron denervation of the muscle may occur resulting in a decline in muscle strength and coordination.

There are many physiological changes that occur with age and recent research suggests that dysregulation of Ca^{2+} homeostasis may be one factor contributing to

sarcopenia. Age-dependent skeletal muscle weakness can result from leaky RyR channels in the sarcoplasmic reticulum of rodents (Andersson et al., 2011). Disruptions of calcium homeostasis in skeletal muscle can lead to muscle weakness and, as the current study determined, can also lead to alterations in neurotrophic factor production.

Fortunately, researchers have studied mechanisms to prevent or delay the negative age-related neuromuscular changes typical in mammals. Physical exercise in rats provides a delay in the onset of sarcopenia and has neuroprotective effects (Deschenes et al., 2010; Tintignac et al., 2015). Furthermore, exercise helps to preserve the integrity and functionality of the neuromuscular junction and research from our laboratory indicates an increase in GDNF protein with exercise (Cheng et al., 2013; McCullough et al., 2011; Gyorkos et al., 2014). In addition to measuring GDNF protein, current research from our laboratory has provided information regarding GDNF mRNA. The overall goal of the study described here was to gain a better understanding of the regulation of GDNF expression in target tissues. Through investigation of Ca^{2+} channel manipulation it appears that there is now an understanding for why physical exercise in skeletal muscle cells would have a positive downstream effect on GDNF production. Further research will be required to determine the exact signaling pathway for GDNF expression in skeletal muscle cells however the current experiments suggest that the activated pathway is calcium dependent. Understanding GDNF and how cell populations can be induced to create more GDNF, is a stepping stone which will hopefully provide a therapeutic effect on preserving the functionality of the human neuromuscular system in the future.

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