4-22-2016

Effects of Creatine, Leucine, and Ethanol on Glial Cell Line-Derived Neurotrophic Factor in C2C12 Skeletal Muscle Cells

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May 2016.

A Thesis Submitted in Partial Fulfillment of the
Requirement for the Degree of
Bachelor of Science
At Western Michigan University

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Acknowledgments

Working in the laboratory of my mentor Dr. John Spitsbergen has been a tremendous opportunity and a wonderful privilege. The encouragement to ask a new question, the guidance in designing an experiment, and patience when my designs and ideas needed revision, extended to me by Dr. Spitsbergen have been instrumental in my growth as a scientific thinker and as a person. For this experience, I am deeply grateful.

I would also like to express my appreciation to Dr. John Jellies for his assistance in writing this thesis. His insights and attention have greatly improved my scientific writing and sharpened my mind.

Additionally, I would like to thank Dr. Sister John-Mary Vianney for all of her help during this research and other activities within the Spitsbergen lab. From Dr. Vianney, I have learned to address scientific questions with joy and compassion.

Finally, I would like to thank the Lee Honors College, and the College of Arts and Sciences for providing additional financial support for my research. The opportunity to design and conduct an independent experiment is unusual for an undergraduate and I am profoundly grateful for the support I have received.

Abstract

Glial Cell Line-Derived Neurotrophic Factor (GDNF) is a protein expressed throughout the mammalian nervous system that serves several protective functions. Originally discovered in the brain, where it supports the survival of dopaminergic neurons, GDNF has since been shown to be expressed in skeletal muscle and is vital for the maintenance of motor neurons. The relationship between muscle hypertrophy, and GDNF is not well understood. The purpose of this study was to examine the relationship between hypertrophy and GDNF by inducing hypertrophy in C2C12 skeletal muscle cells using leucine and creatine, inhibiting hypertrophy using ethanol and measuring GDNF content. As measured by enzyme linked immunoabsorbent assay, GDNF content was reduced following all treatments, therefore a clear relationship between hypertrophy and GDNF content could not be established.

Introduction

Glial Cell Line-Derived Neurotrophic Factor (GDNF) was discovered because of its importance in the maintenance of dopaminergic neurons, specifically in the putamen, an area of the mammalian brain responsible for coordinating fine movements (Lin 1993). Since the isolation of GDNF, much has been learned about both the structure and function of this protein. Early analysis of GDNF revealed a familiar structural pattern. A specific series of seven cysteine residues, known as a "cysteine-knot motif" was found in the amino acid sequence of GDNF and is also present in many proteins belonging to the Transforming Growth Factor Beta (TGF-β) family (Saarma 2000). These cysteine residues are capable of forming disulfide bridges, connecting parallel beta-sheet secondary structural components. However, further analysis has revealed that the relationship between GDNF and other TGF-β family members is more complicated. The TGF-β family is composed of dozens of proteins, organized into several subfamilies (Wakefield et al 2013). These subfamilies include the Activins, the TGF-βs, Growth and Differentiation Factors (GDF) and Bone Morphogenic Proteins (BMP) (Wakefield et al. 2013). Several pieces of evidence suggest that GDNF belongs to a subfamily within the TGF-β with a small number of other proteins, the Glial Derived Family of Ligands (GFL). First, the amino acid sequence of GDNF is only approximately 20% congruent with that of other TGF-β family members (Saarma 2000). Three other proteins have since been discovered that show the same level of congruence with other TGF-β family members, but are approximately 40-50% congruent with GDNF (Saarma 2000).

These proteins are neurturin (NRTN), artemin (ARTM) and persephin (PSPN) (Kotzbauer et al. 1996; Baloh et al. 1998; Milbrandt et al. 1998). Second, the GFL proteins induce signaling cascades by interacting with a different set of receptors than those used by other members of the TGF-β family (Takahashi 1998). Each of the four members of the GFL family bind first with one of the Glial Family Receptors (GFRα-1, GFRα-2, GFRα-3, GFRα-4), which is bound to glycosylphospatidylinosol (GPI), a molecule attached to a lipid raft on the surface of a cell (Saarma 2000). The GFL-GFRα-GPI complex then binds to the tyrosine kinase receptor RET causing autophosphorylation of RET and the activation of several other pathways, including RAS/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, and p38 mitogen activated protein kinase (MAPK) (Takahashi 2001). In contrast, the TGF-β family proteins bind to serine threonine kinase receptors (O'Grady et al 1992). Downstream of these receptors, several of the same pathways, such as p38 and MAPK are activated by the TGF-β family proteins and GFL proteins (Horbelt et al 2012).

While the fact that GDNF is vital for the survival of dopaminergic neurons in the putamen is well established, (Lin et al. 1993; (Clarkson et al. 1997; Granholm et al. 2000) evidence exists that GDNF may perform other functions throughout the mammalian body. GDNF has been identified in several other regions of the mammalian central nervous system (CNS) including the hippocampus, striatum, cortex and the spinal cord (Springer et al. 1994). GDNF has also been found to exert effects in several places throughout the peripheral nervous system, (PNS), in various classes of neurons, including sympathetic neurons (Moore et al. 1996)

parasympathetic neurons (Ebendal et al. 1995), sensory neurons (Buj-Bello et al. 1995) and motor neurons (Zurn et al. 1994). The role played by GDNF in the survival of motor neurons is of particular interest to researchers as GDNF has been shown to be the most effective survival factor for motor neurons (Henderson et al. 1994a). Many studies have shown GDNF to reduce programmed cell death in motor neurons after axotomy in mouse and rat models *in vivo* (Giehl et al. 1997a; Leffler et al. 2002; Morcuende et al. 2013; Munson and Mcmahon 1997; Oppenheim et al. 1995). GDNF has also been shown to protect against the oxidative stress that is characteristic of spinal cord injury (Li et al. 2016) and the neurodegenerative diseases Parkinson's disease (PD) (Smith and Cass 2007) and amyotrophic lateral sclerosis (ALS) (Barber and Shaw 2010). GNDF has also been shown to aid in growth of new neurons, specifically after persistent axotomy (Boyd and Gordon 2003).

GDNF is expressed in skeletal muscle (Henderson et al. 1994; Nagano and Suzuki 2003) although its role and factors regulating its expression are not fully understood. GDNF is known to have some effects on the neuromuscular junction as overexpression of this protein leads to hyperinnervation and the formation of multiple motor end plates (Nguyen et al. 1998). Although, it is unclear if this effect is the result of the growth of new neurons or the preservation of existing neurons that would ordinarily be removed during the neuronal pruning that takes place during development (Schuldiner and Yaron 2015). GDNF may also function as a signaling molecule, attracting axons from nearby neurons to the muscle fiber (Dudanova, Gatto, Klein 2010). Previous studies from our lab have established a few

factors that appear to regulate the production of GDNF. Various forms of exercise, such as walking, running and swimming have been examined using mice and rats. In muscles that are composed primarily of slow-twitch fibers, periods of walking increased GDNF expression in those muscles (Gyorkos, et al. 2010). In fast-twitch muscles, another relationship emerged, as walking lead to decreases in GDNF content of the muscles (Gyorkos et al. 2010). When resistance was added, and the fast-twitch dominant muscles were recruited sufficiently to induce hypertrophy, GDNF content of the muscles increased (Gyorkos and Spitsbergen 2014). The relationship between muscle hypertrophy and GDNF is not well understood. Many physiological changes occur during exercise, so the possibility that muscle growth is sufficient to induce changes in the nervous system via GDNF cannot be currently stated. The purpose of this experiment was to examine this relationship by measuring the content of GDNF in skeletal muscle after treatment with substances known to affect hypertrophy. The organic acid creatine, and the branched chain amino acid leucine have been shown to increase hypertrophy (Anthony et al. 2000; Buse and Reid 1975; Deldicque et al. 2007) and ethanol has been shown to inhibit hypertrophy (Arya et al. 2013; Dasarathy et al. 2013). If a clear relationship between hypertrophy and GDNF does in fact exist, these treatments should illicit different responses. It is my hypothesis that muscle cells treated with creatine or leucine will produce higher levels of GDNF than control and muscles treated with Ethanol will produce lower levels of GDNF.

Materials and Methods

C2C12 cell culture

C2C12 skeletal muscle cells were acquired from Sigma Aldrich (Sigma Aldrich USA). First, these cells were grown in proliferation medium composed of Dulbecco's Modified Eagle Medium (DMEM) (ATCC Manassas, VA) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich F0926) and 1% Penicillin/Streptomycin (Sigma Aldrich A5595). Cells were allowed to proliferate in a 100mm dish (Cyto One CC7682-3394) then transferred to 12-well plates (Cyto One CC7682-7512). Medium was replaced every 24 hours. After reaching confluence (90%), proliferation medium was removed and differentiation medium composed of DMEM, 10% Horse Serum (HS) (Sigma Aldrich H1270) and 1% Penicillin/ Streptomycin was added. Experimental treatments containing leucine (Sigma Aldrich L8912) 0, 5mM and 10mM, ethanol (Acquired from Chemistry Stockroom, WMU) 0, 25mM and 50mM or creatine (Sigma Aldrich C3630) 0mM, 25mM and 50mM were syringe-filtered using a 0.20µm filter (VWR 28145) added to the differential media. 500µL samples were removed from each well and stored in 1.5mL microcentrifuge tubes at -20°C. Incubation conditions, 37°C, 5% CO_2 were maintained throughout the experiment using a Thermo Scientific Hareaus incubator.

GDNF Content

GDNF content was measured using a two-site, sandwich Enzyme Linked Immunoabsorbent Assay (ELISA). Briefly, primary anti-GDNF antibody (R&D

Systems MAB 212) was bound to 96 well plates (Thermo Scientific 8004LE0910) non-specific biding was prevented by blocking the plate using phosphate buffered saline (PBS) pH 7.4 containing 1% Bovine Serum Albumin (BSA) (Amresco, Solon, Ohio) and 5% sucrose (MP Biomedicals, Solon, Ohio) for one hour. Samples and standards were introduced and allowed to incubate for 2 hours. A biotinylated secondary anti-GDNF antibody (R&D Systems BAF 212) was added and allowed to incubate for 2 hours. Streptavidin Horse Radish Peroxidase conjugate (Strep-HRP) (Thermo Fischer Scientific PI-21134) was diluted 1:200 from stock solution in PBS added and allowed to incubate for 30 minutes. Pierce substrate, TURBO TMB-ELISA (Thermo Fischer Scientific PI-34022) was applied and the reaction proceeded for 40 minutes. Finally, the reaction was stopped using 1M HCl and the plate was analyzed by measuring absorbance at a wavelength of 450nm using a plate reader (Biotek Epoch) and Gen51 Software. A standard curve was constructed using Microsoft Excel.

Quantification of Differentiation (Future Studies)

Jenner's stain working solution was prepared by diluting Jenner's stain stock solution (ScyTek JSS500) 1:3 in PBS (pH 5.6). Giemsa stain working solution was prepared by diluting Giemsa stain stock solution (ScyTek GSS500) 1:20 in PBS (pH 5.6). Cell culture medium was removed from cells by aspiration. Cells were washed 3 times with PBS (pH 5.6). To fix the cells, 100% methanol was added and allowed to stand at room temperature for 5 minutes. Methanol was removed by aspiration and cells were allowed to dry for 10 minutes. Jenner's stain working solution was

added (0.5mL/well) for 5 minutes and removed by aspiration. Cells were washed 3 times with PBS (pH 5.6). Giemsa stain working solution was added (0.5mL/well) for 5 minutes and removed by aspiration. Cells were washed 3 times using PBS (pH 5.6) and then allowed to dry at room temperature. Cells were stored at room temperature. Cells were photographed using a Nikon inverted microscope at 100x total magnification and images were recorded using Metamorph software.

Results and Discussion

GDNF protein was detected in cell culture medium from all control wells as expected. All treatments resulted in decreases in GDNF protein content measured. GDNF content in cell culture medium from cells treated with leucine at both low and high concentrations was below the detection limit for the assay as performed. GDNF content in cell culture medium from cells treated with creatine at both low and high concentrations was also below the detection limit for the assay. GDNF content in cell culture medium from cells treated with ethanol at low and high concentrations was detectable but lower than control. Myotubes in the wells treated with creatine or leucine did appear larger, indicating higher levels of differentiation, which was consistent with the hypertrophy-inducing effects reported in previous studies (Anthony et al. 2000; Buse and Reid 1975; Deldicque et al. 2007). Myotubes in the wells treated with ethanol appeared smaller, showing lower levels of differentiation; this was consistent with previous studies that show ethanol having a negative correlation with differentiation and hypertrophy (Arya et al. 2013; Dasarathy et al. 2013). These effects were not measured quantitatively.

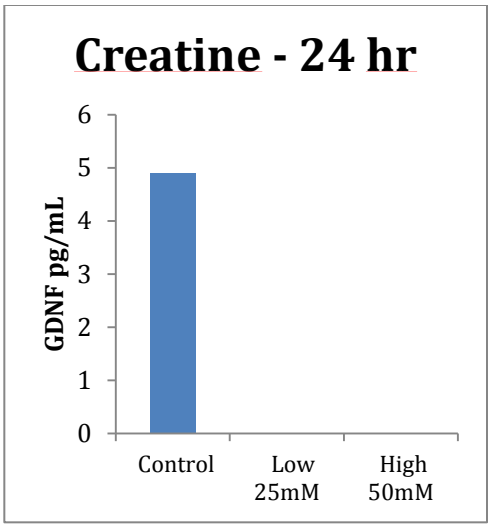

Figure 1: C2C12 skeletal muscle cells were treated with 0mM, 25mM and 50mM Creatine in differential medium. 24 hours after treatment, samples were taken and frozen at -80°C. GDNF protein content was measured using ELISA. GDNF was detected and measured in the control wells, but below the detection limit for the assay in wells treated with both low (25mM) and high (50mM) concentrations of Creatine.

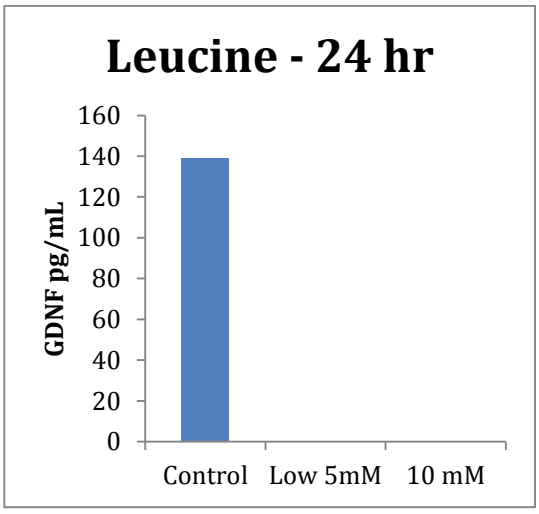

Figure 2: C2C12 skeletal muscle cells were treated with 0mM, 5mM and 10mM Leucine in differential medium. 24 hours after treatment, samples were taken and frozen at -80°C. GDNF protein content was measured using ELISA. GDNF was confirmed to be present and

measured in control wells, but below the detection limit for the assay in the treatment wells.

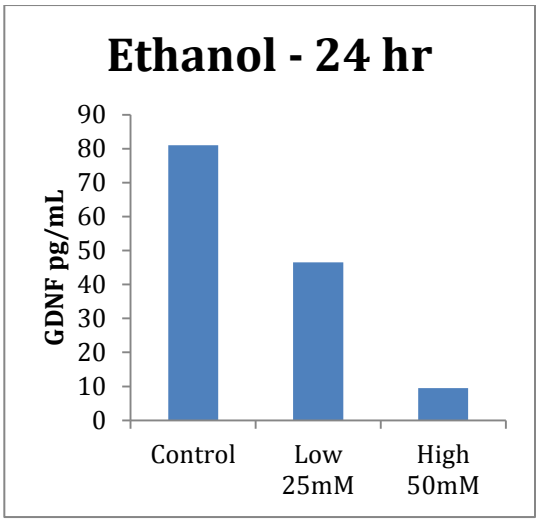

Figure 3: C2C12 skeletal muscle cells were treated with 0mM, 25mM and 50mM Ethanol in differential medium. 24 hours after treatment, samples were taken and frozen at -80°C. GDNF protein content was measured using ELISA. GDNF content was determined to be present in all wells. GDNF was measured to be lower in wells treated with low (25mM) and high (50mM) concentrations of ethanol.

Because GDNF was originally discovered in the brain, where it is vital for the survival of dopaminergic neurons, much of the research on it has focused on possible applications for the treatment of Parkinson's disease. GDNF is now known to exert effects in many other areas of the body. The fact that GDNF is secreted by skeletal muscle and is the most significant protective factor for motor neurons has several potential therapeutic applications. The neuromuscular degenerative disease amyotrophic lateral sclerosis (ALS) is characterized by the death of several classes of motor neurons and muscular atrophy, resulting in paralysis, respiratory failure and death (Jerusalem et al. 1996). One possible cause of ALS is an insufficiency of

neurotrophic factors (Jerusalem et al. 1996). Several GDNF delivery methods have been explored including direct infusion of GDNF into the target muscle (Giehl et al. 1997b) and mesenchymal stem cell injection (Brian 2008). GDNF content in skeletal muscle decreases with age (Buj-Bello et al. 1995) and may be a therapeutic treatment for age-related muscle loss. Examining the normal regulation of GDNF could provide additional information vital to understanding these diseases and developing future treatments.

The hypothesis tested in this experiment was that treating skeletal muscle cells with compounds known to affect hypertrophy would result in changing levels of GDNF secreted into the culture medium. Specifically, that leucine and creatine, substances known to increase hypertrophy, would result in an increase in GDNF protein content and treatment with ethanol, a substance known to decrease hypertrophy would result in lower levels of GDNF. The results from this experiment do not support this hypothesis very well.

Skeletal muscle cells treated with ethanol did produce less GDNF than untreated controls. This finding is consistent with the hypothesis that inhibiting hypertrophy would be associated with lower levels of GDNF. This correlation is insufficient to answer the question of causality. Whether GDNF is a cause of or a result of hypertrophy is not possible to infer from this data.

In contrast, treatment with Leucine and Creatine resulted in levels of GDNF that were too low to be measured with the existing assay. These results directly refute the hypothesis. Again, these results are insufficient to indicate causality between GDNF and hypertrophy.

The fact that treatments with opposite effects on hypertrophy did not result in opposite effects on the quantity of GDNF present in the culture medium does not support the hypothesis that a simple relationship, either positively or negatively associated, exists between GDNF and hypertrophy. Other experimental factors should be considered. For example, the samples in this experiment were collected at a time interval of 24 hours. It may be possible that GDNF levels increase quickly after treatment with either leucine or creatine and then decrease sharply as a result of a negative feedback system. If this is indeed the case, collecting samples from earlier time points, perhaps 4 hours and 8 hours after treatment, would reveal higher levels of GDNF and samples collected from later time points, perhaps 36 hours and 48 hours after treatment would show GDNF levels returning to control levels over time.

Another possible explanation for these results is that inducing hypertrophy in the absence of significant muscular contraction, may be insufficient to induce increases in GDNF, regardless of an abundance of hypertrophy-inducing compounds. Experiments currently underway examining the effects on GDNF of myostatin inhibition could help to address this issue, as the inhibition of myostatin is known to increase muscle hypertrophy in the absence of exercise (Wang and Mcpherron 2012). Previous work from our lab that found a relationship between GDNF and hypertrophy (Gyorkos et al. 2014; McCullough et al. 2011) tested the effects of exercise and would be consistent with this hypothesis.

The causal relationship between GDNF and hypertrophy could also be explored in future studies. Treating C2C12 skeletal muscle cells with GDNF, and

blocking the action of GDNF using an antibody, and measuring hypertrophy would be a way to test this hypothesis and add clarity to the relationship between hypertrophy and GDNF.

Measuring the degree to which C2C12 cells are differentiating, forming myotubes, could also add information to the relationship between GDNF and hypertrophy. Observations were made regarding the size of myotubes in each of the wells, but the accuracy and precision of these evaluations cannot be asserted with confidence. One method that can be used is a Jenner-Giemsa staining procedure (Veica and Bunce 2011). This method includes two histological stains, Jenner's stain and Giemsa stain that are applied to the cells. These stains are absorbed by the myoblasts and myotubes differently. As a result, undifferentiated myoblasts appear lighter and myotubes appear darker. Image analysis software can then be used to quantify the proportion of darker myotubes and lighter myoblasts.

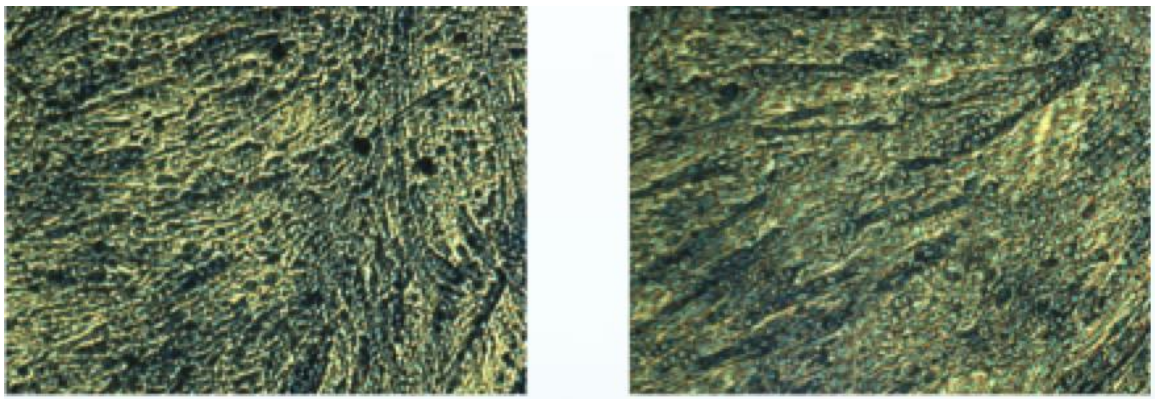

Figure 4: A sample image created using the Jenner-Giemsa staining protocol. C2C12 cells were stained with Jenner's stain and Giemsa stain, and photographed using an inverted microscope. Cells treated with leucine (10mM) (RIGHT) show myotubes that appear larger than controls (LEFT).

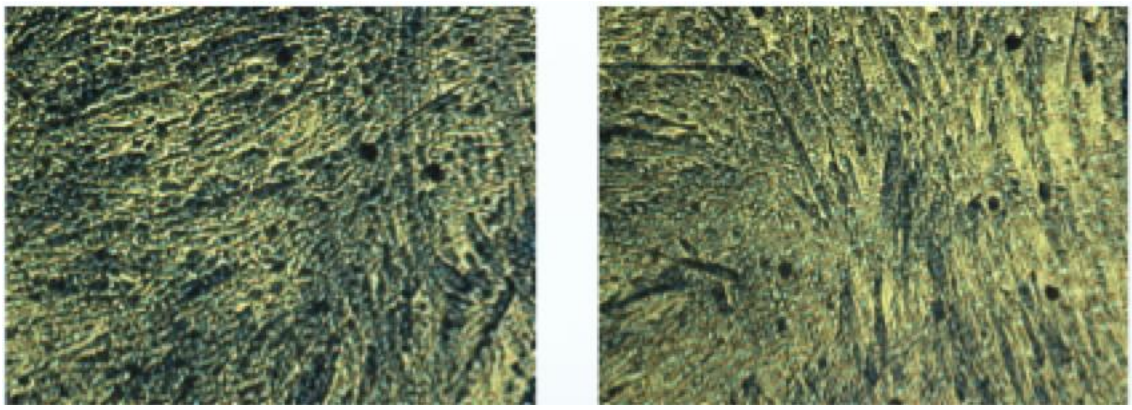

Figure 5: A sample image created using the Jenner-Giemsa staining protocol. C2C12 cells were stained with Jenner's stain and Giemsa stain, and photographed using an inverted microscope. Cells treated with ethanol (50mM) (RIGHT) show myotubes that appear to be fewer and smaller than controls (LEFT).

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