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Effects of Muscle Activation on GDNF Protein Content in Skeletal Muscle

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Abstract:

Impairment of neuromuscular function as a result of aging is primarily associated with degeneration of the peripheral nervous system and loss of type II muscle fibers (Lexell et al, 1988). Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor found in skeletal muscle that has been shown to rescue motor neurons from axotomy-induced cell death (Oppenheim et al., 1995) and to induce NMJ plasticity (Keller-Peck et al., 2001). The aim of this study was to examine GDNF protein content following muscle activation in vitro and in vivo models. GDNF protein content was measured in rat gastrocnemius muscles following differing levels of activity. GDNF protein content was measured in culture medium of C2C12 skeletal muscle cells following electrical stimulation for 2 and 48 hours. Quantification of GDNF protein was done with ELISA. Exercise did not have a significant effect on GDNF protein content in skeletal muscle but there was a negative trend toward a decrease in GDNF protein content. Forty-eight hours of electrical stimulation of C2C12 skeletal muscle decreased GDNF protein content within cell culture medium. These results suggest that muscle activation inhibits GDNF protein content in rat gastrocnemius muscle and decreases protein content in C2C12 skeletal muscle cells**.** Linking GDNF production to physical activity may help explain the benefits of exercise for the peripheral nervous system.

Introduction:

According to the UN, by year 2100 the population above age 60 is predicted to be 3.2 billion. Between ages 30-80 there is a tendency for a 30-50% decrease in muscle mass and strength (Daley & Spinks, 2000). Loss of strength from aging is associated with a reduction in the number of skeletal muscle fibers and degeneration of the peripheral nervous system. Loss of motor neurons and skeletal muscle fibers, especially Type II muscle fibers, results in decreased strength and ultimately decreased quality of life (Lexell et al, 1988). Motor neuron loss is followed by expression of proteins that stimulate dendrite formation from residual motor neurons and with aging this mechanism becomes less efficient (Gonzalez-Freir et al, 2013). While the mechanism behind age-related sarcopenia and deterioration is unknown, current theories suggest that sedentarism and impairment of trophic signaling in the neuromuscular junction (NMJ) may be involved (Gonzalez-Freir et al, 2013).

Humans become more sedentary with age. Lack of exercise is associated with loss of muscle strength, increased frailty, and increased fatigue (Gonzalez-Freir et al, 2013). Strength training has been shown to stop or reverse these effects (Hurley & Roth, 2000). Exercise benefits the nervous system by increasing the plasticity of the neuromuscular junction (NMJ) (Andonian & Fahim, 1987) and inducing release of neuronal survival factors (Corman & Berchtold, 2002). The NMJ is a synaptic interface which transmits signals from the motor neuron to the skeletal muscle. Exercise increases plasticity of the NMJ by increasing the size and degree of branching motor nerve terminals (Andonian & Fahim, 1987), increasing acetylcholine (ACh) release (Dorlöchter et al., 1991) and increasing the area of pre and postsynaptic elements (Deschenes et al., 1993).

Neurotrophic factors are proteins that regulate critical aspects of neurons such as adult synaptic plasticity, neurite branching, synaptogenesis, and ontogeny of electrophysiological properties (Sariola & Saarma, 2003). Neurotrophic factors such as the glial cell line-derived neurotrophic factor (GDNF) family, which includes GDNF, neurturin (NTRN), artermin (ARTN), and persephin (PSPN) were all suggested to play a role in the maintenance and development of central and peripheral neurons (Airaksinen & Saarma, 2002).

Glial cell line-derived neurotrophic factor (GDNF) was initially discovered in midbrain cultures and was found to increase dopamine uptake and play a role in dopaminergic neuron survival (Lin et al.,1993). Since its discovery, GDNF has been categorized as a survival factor for spinal motor neurons and has been shown to exert effects on the NMJ that are similar to exercise (Suzuki et al., 1998). GDNF has been shown to rescue motor neurons from axotomy-induced cell death (Oppenheim et al., 1995), facilitate synaptic transmission (Wang et al., 2002), maintain synaptic activity (Zwick et al., 2001), induce NMJ plasticity (Keller-Peck et al., 2001), and enhance nerve recovery after injury (Oppenheim et al., 1995). Mechanisms that mediate GDNF expression are poorly understood.

Previous research suggests that increased activity led to increased levels of GDNF content in rat skeletal muscle depending on mode of exercise and fiber composition of the muscle (McCullough et al, 2010). Modes of exercise similar to endurance training increased levels of GDNF content in Type I slow twitch muscle fibers (McCullough et al, 2010). Modes of exercise similar to sprinting increased GDNF content in Type II fast twitch skeletal muscle fibers (Gyorkos et al, 2014).

Research examining effects of electrical stimulation suggest a relationship between duration of electrical stimulation and content of GDNF within cultured C2C12 cells. Short

durations (30 minutes) of electrical stimulation at 24 V led to decreased levels of GDNF protein content while long durations (24 hours) of electrical stimulation led to increased levels of GDNF protein content within C2C12 skeletal muscle cells (Vianney et al, 2014).

Hypothesis:

This study has two aims. First, to determine if changes in GDNF protein content in skeletal muscle are linked to exercise. Second, to determine if GDNF protein content in C2C12 skeletal muscle cells is linked to electrical stimulation. My first hypothesis is that 24 weeks of exercise will decrease GDNF protein content in rat gastrocnemius a predominantly type II muscle. My second hypothesis is that long term (48 hour) stimulation at a lower voltage (15 V) will decrease GDNF protein content in C2C12 skeletal muscle cells.

Methodology:

I. Subjects:

Animal subjects:

All experiments were conducted in compliance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council). Experimental protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University. 18 Sprague Dawley rats were used for this study. All rats had access to food pellets and water *ad libitum*.

II. Training Protocol:

Rats were randomly divided into three groups: Day 0 (n=6), sedentary (n=6), and exercise (n=6). Exercised rats participated in voluntary exercise in a running wheel (Lafayette Instruments, Lafayette, IN) for the 24-week duration of the study. Distance run by each exercised rat was

collected via a scurry rat sensor (Lafayette Instruments, Lafayette, IN). The sedentary and Day 0 groups were housed in cages without access to running wheels.

III. Tissue Processing:

All rats were euthanized via CO2 asphyxiation and thoracotomy. Day O rats were euthanized at 24 weeks of age. Sedentary and exercised rats were euthanized at 48 weeks of age. Immediately following death, the gastrocnemius muscles were dissected from both hind legs. Muscles were washed with phosphate-buffered saline (PBS: 0.225 M NaCl, 0.02 M NaH2PO4, and 0.08 M Na2HPO4), frozen on dry ice, and stored at −80°C. For measurement of GDNF protein content, muscles from the right side of the body were dipped in liquid nitrogen, smashed into a fine powder, and homogenized in sample processing buffer (0.55 M NaCl, 0.02 M NaH2PO4, 0.08 M Na2HPO4, 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA, and 0.05% Tween-20). Homogenate was centrifuged at 4°C for 30 min at 13,000 RPM and the resultant supernatant was decanted, remixed, and stored at −80°C.

IV. C2C12 skeletal muscle cell culture

C2C12 cells are myoblasts capable of differentiating into myotubes and are commonly used as an in vitro model of skeletal muscle (Richler and Yaffee, 1970). C2C12 myotubes exhibit contractile phenotype and can express neurotrophic factors (Vianney et al, 2014). Passage 9 C2C12 cells were used in this study.

All chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) unless stated otherwise. For routine culturing, C2C12 skeletal muscle cells were grown in a 100-mm plates (Cyto One**)** and maintained in 10 ml of Dulbecco's Modified Eagle Medium (DMEM) (ATTC Manassas, VA) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-

antimycotic. Medium was replaced every 24-30 hours. After C2C12 cells reached 70-80% confluency in the 100-mm plates, both plates were split to 6-well plates (Cyto One) containing 2.5 ml per well of DMEM supplemented with 10% (FBS) and 1% antibiotic-antimycotic. Once each well reached 85-90% confluency medium was changed to 2.5 ml per well of DMEM supplemented with 10% Horse Serum (HS) and 1% antibiotic-antimycotic to encourage myotube formation. Medium was replaced every 24-30 hours.

V. Electrical Stimulation:

For each experiment three wells were stimulated while the other 3 wells served as controls. All stimulation was completed with 6 or 7-day old myotubes. The first 6-well plate containing C2C12 Skeletal Muscle cells was stimulated at 15 V for 2 hours. The second 6-well plate containing C2C12 Skeletal Muscle cells was stimulated at 15 V for 48 hours.

Semicircular electrodes were used as part of a culture dish lid along with a capacitor to block direct currents, and current sampling resistors. The electrodes were secured to the 6-well culture dish lid with bolts. Voltage was provided by a Grass Technologies S88 stimulator that was connected to a custom-made interface box. Electrical current was measured using a single oscilloscope channel that was connected to corresponding sampling resistors that were set at 100 Ω.

VI. Quantification of GDNF Protein Content:

GDNF protein content was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described by McCullough, et al (2010). 96-well plates were incubated overnight at room temperature using a monoclonal antibody raised against GDNF (R&D Systems). Remaining sites on the plates were blocked with PBS containing 1% BSA and 5% sucrose. Plates were rinsed

3 times in wash buffer and tissue supernatant or GDNF standards were added to each well and plates were incubated at room temperature for at least 2 hours. Wells were then washed 3 times and an anti-GDNF antibody conjugated to biotin (R&D Systems) was added and incubated for 2 hours at room temperature. Plates were washed again, and horseradish peroxidase conjugated to streptavidin (Pierce) was added for 20 min at room temperature. Plates had a final wash (3 times), and the tetramethylbenzidine color reagent (Sigma) was added according to manufacturer's specifications. The reaction was stopped with 0.1 M Hydrochloric acid and absorbance was measured at 450 nm.

VII. Statistical Analysis:

All data values are reported as mean ± the standard error of the mean (SEM). Data was analyzed using Students T-test. Statistical significance was set at $p \leq 0.05$.

Results:

Cell Culture Stimulation:

Electrical stimulation of C2C12 skeletal muscle cells caused a significant decrease in GDNF protein content within culture medium following 48 hours of electrical stimulation.

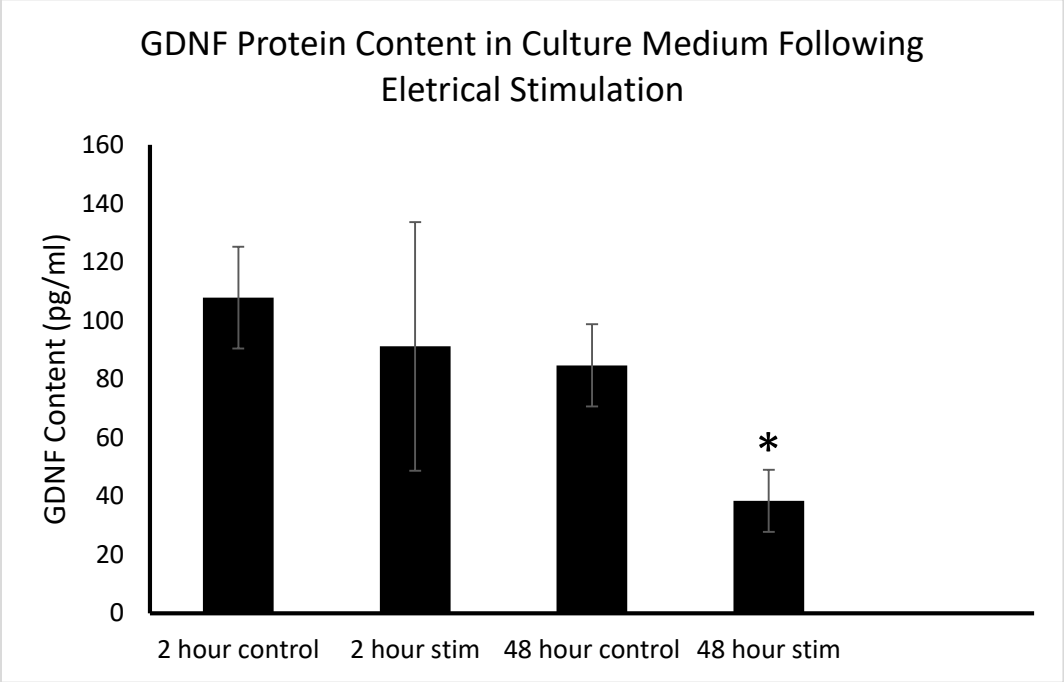

Figure 1. GDNF content in C2C12 skeletal muscle cell culture medium following electrical stimulation. Day 6 or 7 myotubes were stimulated at 15 V for 2 hours and 48 hours. Following 2 hours of electrical stimulation no difference in GDNF content is observed. The 48-hour stimulation group shows inhibitory effects on GDNF production. An asterisk (*) indicates a significant decrease from control. Values are presented as Mean ± S.E.M.

Exercise:

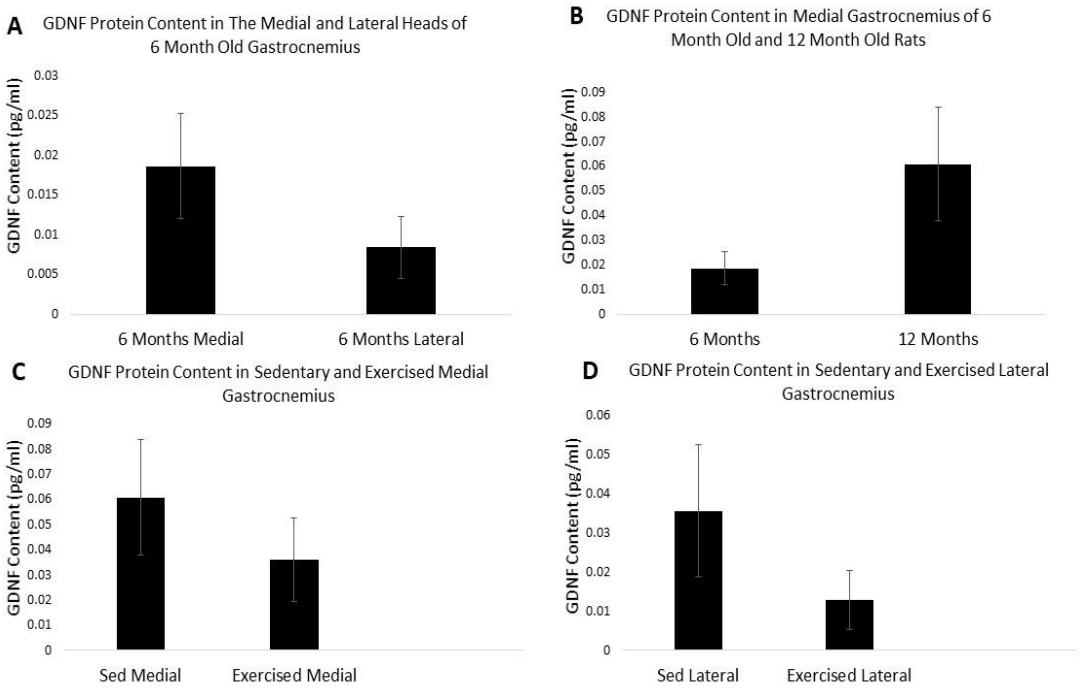

Figure 2. GDNF protein content in rat gastrocnemius. Panel A compares the medial and lateral head of the gastrocnemius in 6 months old rats. Panel B compares the medial heads of the gastrocnemius in 6-month-old and 12-month-old rats. Panel C compares the lateral gastrocnemius of the sedentary group and exercised group. No significant differences in GDNF protein content were found. All values are presented as Mean ± S.E.M.

Discussion:

The aim of this study was to determine if muscle activation causes changes in GDNF protein content in rat skeletal muscle in vivo, and C2C12 skeletal muscle cells in vitro. Previous research linked long term stimulation to increases of GDNF expression while exercise has been linked to varying levels of GDNF depending on activity intensity and muscle fiber type (McCullough et al, 2010; Gyorkos et al, 2014; Vianney et al, 2014). Since a type II muscle was used in this study, we expected a decrease in GDNF levels following voluntary exercise. Following 48 hours of electrical stimulation at 15 V we expected a decrease in GDNF protein content measured in C2C12 cell culture medium. Our results demonstrate that GDNF protein production was inhibited following long-term stimulation (48 hours) at a lower voltage (15 V). These results suggest that manipulation of voltage may cause differences in how GDNF protein expression is mediated in myotubes. Exercise showed no significate effect on GDNF protein content but there was a trend toward a decrease in GDNF protein content in exercised gastrocnemius.

Electrical stimulation of C2C12 skeletal muscle cells may effect changes in GDNF protein content:

Since direct electrical stimulation of C2C12 myotubes has been shown to elicit contraction and to alter protein expression in skeletal muscle (Thelen et al, 1997) it directly correlated with the aims of this study. Altering the magnitude of a voltage may allow us to see if stimulus intensity correlates with GDNF expression in skeletal muscle. Additionally, this study may provide insight on the potential benefits of electrotherapy on the peripheral nervous system.

Previous research showed inhibitory effects on GDNF protein content in C2C12 skeletal muscle cells following stimulation at 15 V for 30-90 minutes (Vianney et al, 2014). Stimulatory

effects on GDNF protein content in C2C12 cells were observed following 24 V of long term (24-48 hour) stimulation (Vianney et al, 2014). This study examined GDNF protein content in cell culture medium following stimulation at 15 V for 2 and 48 hours. Varying levels of stimulation result in differing levels of muscle fibers reaching threshold. If a muscle fiber does not reach threshold, that fiber will not contract. Stimuli near the minimal threshold of a muscle will cause a small percentage of muscle fibers to contract while stimuli near the maximal threshold will cause a large percentage of muscle fibers to contract. By lowering the magnitude of the stimulus, fewer muscle fibers may have reached threshold and contracted. While to my knowledge no studies show this, lower amounts of muscle fiber contraction may lead to decreases in GDNF expression. This may help to explain why differences in intensity of activity leads to different effects on GDNF expression in Type I and Type II muscle fibers.

It is important to note that during this study no obvious contractions were observed. This may indicate a loss in contractile phenotype within the C2C12 cell. Regardless, this would still support the theory that a lack of muscle fiber contraction lead to a decreased in GDNF protein content within the C2C12 skeletal muscle cell medium.

Exercise may inhibit GDNF production based on fiber type:

Previous research has shown that changes in GDNF protein expression in fast and slow twitch muscle differs depending on intensity of exercise. Low intensity activity decreased GDNF protein content in extensor digitorum longus (McCullough et al, 2010), a predominantly type II muscle (Alnaqeeb & Goldspink, 1987) and increased GDNF protein content in soleus, a predominantly type I muscle (McCullough et al, 2010). This study examined changes in GDNF protein content in the medial and lateral heads of rat gastrocnemius which have been shown to be predominantly Type II muscles (Cornachione et al, 2011). With the exercise regimen consisting

of voluntary exercise the rats likely performed low intensity exercise. Since low intensity exercise was shown to decrease GDNF protein content in Type II muscle we would expect to see a similar result in the rat gastrocnemius. When thinking about the results in terms functionality they may make sense. The rat gastrocnemius is a predominantly type II muscle which is characteristic for short and intense activity. If only low intensity activity is performed by an organism, the use of type II muscle fibers would be inefficient. As a result, survival factors may not be expressed in type II muscles.

Conclusion:

The goal of this study was to determine if muscle activation leads to changes in GDNF protein content in both skeletal muscle and C2C12 skeletal muscle cells. Our results show that a decrease in voltage during long term stimulation of C2C12 cells had an inhibitory effect on GDNF protein content. Additionally, low intensity exercise showed a negative trend for GDNF protein content in skeletal muscle. When thinking about the functionality of an organism these results may make sense. Gastrocnemius and C2C12 skeletal muscle cells are predominantly comprised of type II muscle fibers which are categorized by their ability to contract rapidly, to produce large amounts of power, and to quickly fatigue. With a reduction in voltage and a low intensity exercise regimen, the use of Type II muscle fibers would be inefficient. If type II muscle fibers become unneeded then survival factors may not be expressed in these tissues.

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