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Effects of Aging & Exercise on Production of Glial Cell Line-Derived Neurotrophic Factor Protein by Fast & Slow Skeletal Muscle

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**Effects of Aging and Exercise on Production of Glial Cell Line-Derived Neurotrophic
Factor Protein by Fast and Slow Skeletal Muscle**

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A Thesis Submitted to the Lee Honors College
in Partial Fulfillment for the Degree of
Bachelor of Science
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Abstract

Exercise is a positive form of reinforcing positive lifestyle choices. There are many benefits to exercising such as decreased risk of cardiovascular disease and improved mental condition (Centers for Disease Control and Prevention). In addition, exercise can serve a protecting role for the nervous system. One form in which exercise can protect the nervous system is by increasing the production of neurotrophic factors, specifically glial cell line-derived neurotrophic factor. Another way by which exercise can protect the nervous system is by preserving muscle tissue due to actively recruiting it. The goal of this study was to explore the effects of aging and exercise on extensor digitorum longus (EDL) from 4-week-old and 1-year-old rats. EDL is primarily a fast-twitch fiber (Staron et al., 1999). Enzyme-linked immunosorbent assay (ELISA) and Western Blot were used to compare GDNF protein content and phenotype switch between fast and slow-twitch muscle fibers between sedentary and exercised subjects. An increase in GDNF occurred from the young rats to 1-year-old sedentary rats. No significant differences in GDNF content were noted between sedentary and exercised 1-year-old rats. Fast-twitch muscle fiber was detected in all three groups of rats. Understanding molecular mechanisms can lead to insight on the protection of nervous system due to exercise.

Introduction

One of the goals of public health is to improve the overall health of all citizens. Improving the health of citizens can begin by reinforcing positive lifestyle choices. A choice that can be made by many citizens is to exercise. Exercise has many positive benefits. According to

the Centers for Disease Control and Prevention, exercise can reduce the risk of some cancers and of many diseases, including diabetes and cardiovascular issues. Exercise can also improve one's mental health and mood. Additionally, the effects of exercise on the nervous system have also been studied. The potential for exercise to protect the nervous system can have impactful consequences on the overall health of citizens, especially the aging population.

A particular area of interest is whether the nervous system can serve a protecting role in preserving muscle tissue. This study focuses on the role of exercise in the protection of the nervous system in conjunction with the muscular system. In order for the role of exercise to be understood, the link between the nervous system and muscles and the degeneration of the nervous system in response to aging must both be discussed first.

The interaction between muscular tissues and the nervous system has been highly investigated. The nervous system and muscle fibers are highly connected via neuromuscular junctions (NMJs). Neurons are specialized types of cells that are responsible for the communication between the central nervous system and muscles. Neurons interact with muscles at various NMJs. One neuron is capable of innervating various muscle fibers, or muscle cells, but a single muscle fiber can only be innervated by one neuron. The combination of a single neuron and all of the muscle tissues it innervates is referred to as a motor unit. The role of muscle fiber phenotype was another factor analyzed in this study. Muscle fibers can be categorized into two broad categories: fast and slow-twitch. The separation into these two categories is based on different characteristics, including the relative amount of mitochondria, the speed of ATPase activity and the amount of myoglobin found in the fibers. Proteins play special roles in providing support and nourishment for neurons. A particular group of proteins that was discovered are neurotrophic factors (Oppenheim, 1989).

As individuals begin to age, performing daily activities becomes more difficult. One possible reason for the difficulty in completing tasks can be linked to loss of communication between the nervous and muscular systems. One cause of the difficulties senior citizens face may be attributed to the defects in communication between the nervous and muscular system. Maintaining strong NMJs is necessary in order for the nervous system to be able to properly communicate with muscle tissue. There are various diseases directly or indirectly related to the detachment of neuromuscular junctions. Often as individuals age and begin to live more sedentary lives, muscle fibers stop being recruited. The lack of recruitment in muscle fibers results in the denervation of the neurons, thus a loss of muscle fibers. Not only do diseases have deteriorating effects on the nervous system but aging also contributes to the degeneration of NMJs.

Loss of muscle fiber mass is another problem that aging individuals face. This inevitable age-related condition is called sarcopenia. The loss of muscle mass contributes to difficulty in tasks requiring strength. Sarcopenia not only affects individuals physiologically, but it also has deteriorating effects on the freedom and quality of life of individuals. Thus, if the effects of sarcopenia can be slowed down, the impacts on public health will be decreased.

Changes in muscle fiber phenotype can also increase the risk for injury among the elderly. Research has shown that there is a relationship between the loss of power in the elderly and an increase in the number of falls they experience (Skelton, et al. 2002). Thus, finding mechanisms that slow the loss of fast-twitch muscle fibers is important to reduce the elderly's risk of injury.

Muscle fibers can be classified into two broad categories: slow-twitch (Type I) and fast-twitch (Type II). Slow-twitch fibers are more heavily recruited for longer-term, aerobic muscular

requirements due to the large number of mitochondria within the cells. In contrast, fast-twitch fibers are recruited for shorter-term anaerobic muscular movements. Interestingly, the switch between muscle fiber types is not solely an issue of the aging. Athletes also have to consider the switch from fast to slow muscle fiber types. Type II fibers are essential for athletes to be powerful and explosive.

In order to maintain healthy muscle tissue, proper innervation of the tissue must be maintained. How can muscle fiber loss be reduced? There are different approaches that have been considered. For example, caloric restriction has been shown to reduce deteriorating effects on NMJs, which in turn protects muscle fiber degeneration (Valdez, et al. 2010). Another approach is by considering the effects of exercise on molecular mechanisms in the body. A molecular mechanism of interest is the role of neurotrophic factors, specifically glial cell line-derived neurotrophic (GDNF) factor. Neurotrophic factors play a key role in the survival of neurons (Oppenheim, 1989). GDNF was first described as playing a survival role in midbrain dopaminergic neurons (Lin et al., 1993). Lin and his group (1993) thought that GDNF could have a therapeutic effect in the treatment of Parkinson's disease. The role of GDNF in the survival of motor neurons was first attributed to Henderson et al. (1994). The study completed by his group revealed GDNF to be the most potent survival factor for motor neurons that had been identified. The group identified GDNF as a great candidate to slow the degeneration of motor neurons and in turn protect the nervous system. The discovery of GDNF and its protective potential sparked many questions. A particular question of interest: if GDNF protects the nervous system, under what conditions does the human body produce more of this protein? From this question, the role of exercise arose.

Exercise may be a solution to increase GDNF protein content. Exercise results in the recruitment of muscle fibers. The signal for muscle recruitment comes from the nervous system so preservation of Previous studies in the Spitsbergen laboratory have found interesting effects of exercise on the nervous system. McCullough et al. (2011) showed that there might be an activity dependent relationship. That is, the amount of GDNF protein content that was measured depended on the type and amount of exercise that the rats were trained through. Gyorkos et al. (2014) showed an increase in EDL's GDNF content following swim training. This was the first time that an increase in GDNF protein content was observed in fast-twitch muscle fibers. Later, Gyorkos and Spitsbergen (2014) showed that high-intensity, resistance exercise training increased in GDNF protein content in plantaris (PLA), a primarily fast-twitch muscle. The rats were only exercised for a period of two weeks in this study. The purpose of this study was to examine the effects on aging and exercise on muscle fiber phenotype and GDNF protein content over a longer period of time than previous studies.

Extensor digitorum longus (EDL) of Sprague-Dawley rats was the system examined. EDL is primarily a fast-twitch muscle (Staron et al. 1999). Separate comparisons were carried out. First, the GDNF protein content of one-year old rats that had been sedentary for six months was compared to the content in one-month old rats. Aging was expected to decrease GDNF protein content. Secondly, the GDNF protein content in rats that were voluntarily exercised for six months was compared to the content in rats that were sedentary for six months. Aerobic exercise is expected to result in a decrease in GDNF protein content.

Thirdly, the muscle fiber phenotype was assessed for all three ages of rats. Aging is expected to result in a shift from fast-twitch muscle fibers to slow-twitch muscle fibers. Exercise is expected to decrease the shift from fast-twitch muscle fibers to slow-twitch muscle fibers.

Future studies may ensure that the exercise performed by the subjects actively recruits the specific muscle fiber type that is being studied. In our study, the training performed by the rats was mainly endurance-based. That is, the muscles recruited were mostly slow-twitch fibers. In a study by Gyorkos et al (2014), rats were exercised by involuntary running and swimming. The soleus (SOL), a primarily slow-twitch muscle, and the EDL of the rats were extracted for examination of GDNF protein content. The rats that had participated in swimming were found to have an increase in GDNF protein content compared to the rats that were sedentary. Thus, to ensure proper recruitment the exercise protocol can be tailored to the specific muscle fiber examined. Another area that can be further explored is how GDNF expression occurs. If exercise does result in increased GDNF protein content, is this increase due to the release of stored GDNF protein or an increase in protein synthesis processes? This question may begin to be answered by considering mRNA levels within cells. The factors that trigger its expression could also be considered.

Maintaining a healthy nervous system leads to the slowing of muscular-related diseases and losses in quality of life. The potential for exercise to serve as a positive factor in the protection of the nervous system adds to the list of its benefits. Participating in exercise can lead to an increase in the overall health of populations.

Methodology

I. Animal Subjects

Animals were maintained according to guidelines set forth by the National Research Council in “Guide for the Care and Use of Laboratory Animals”. Twelve six-month old Sprague-Dawley rats were housed in 12 hours light to 12 hours dark cycles. Food and water was accessible to the rats. The rats were randomly divided into two even groups: sedentary and exercised. The

exercised rats were given unlimited access to a running wheel. The rats were housed in clear chambers for six months before they were euthanized by carbon dioxide asphyxiation.

Six three-week old rats were received. The rats were housed in clear chambers for one week before they were euthanized by carbon dioxide asphyxiation. These rats were used as controls.

II. Tissue Collection and Processing

Before euthanizing the rats, each was weighed. The rats were then asphyxiated with carbon dioxide. Proceeding quickly the extensor digitorum longus (EDL) was removed bilaterally from all eighteen rats. The tissues were stored at regular length at -80°C. The right EDL from all eighteen rats was prepared for quantification by an enzyme-linked immunosorbent assay and Western blot. The left EDL was used for histology. Before processing, each tissue was weighed. The right EDL was prepared by dipping the tissue into liquid nitrogen, smashing it into a fine powder and homogenizing it in sample buffer with a tissue tearor. The sample buffer consisted of 0.55 M NaCl, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 2 mM ethylenediaminetetraacetic acid, 0.1 mM benzethonium chloride, 2 M benzamidine, 20 KIU/ml aprotinin, 0.5% bovine serum albumin (BSA), and 0.05% Tween-20. The amount of sample buffer used depended on the weight of each tissue sample. The ratio of 20 ml of sample buffer for every mg of tissue was utilized each time. 100µl of homogenate were added to microcentrifuge tubes and stored at -80°C for Western Blot analysis. The homogenate was centrifuged at 13,000 g for thirty minutes. The supernatant was collected and aliquoted into 450µl and stored at -80°C.

III. Enzyme-linked Immunosorbent Assay (ELISA)

96-well assay plates (COMPANY) were incubated with primary antibody, monoclonal GDNF antibody (COMPANY) overnight in a humidified chamber. The plates were washed two times with wash buffer (PBS containing 0.05% Tween-20). The plates were then blocked with PBS

containing 1% BSA and 5% sucrose. The plates containing the blocking solution were incubated for two hours. The plates were washed three times. 100µl of standard solution and processed tissue samples were added to each well. An anti-GDNF antibody was conjugated to biotin. The plate was incubated in a humidified chamber overnight at room temperature. Plates were washed three times. A 10ml solution of PBS with 0.1g of BSA and 50µl of Streptavidin HRP was prepared and added to each of the plates. The plate was incubated in a humidified chamber for thirty minutes. The plate was washed three times. With the lights off, 100µl of color reagent (Turbo) were added to each well. Once a color change had occurred, after about twenty to forty minutes, 100µl of 0.99M HCl was added to each of the wells. Absorbance was measured at 450nm.

IV. Western Blot

Homogenate of the tissue samples was mixed with 5µL reducing agent. The mixtures were heated at 95°C for ten minutes in a heating block. The samples were added to two 4-12% Bis-Tris Gel (Life Technologies). 15 to 18 µl of samples were added to the wells. 10 µl of Invitrogen™ Novex™ SeeBlue™ Plus2 Pre-Stained Protein Standard (LC5925, Invitrogen) was added to one of the wells. 1x MES running buffer was placed into the gel electrophoresis chamber. The samples were run for 35 minutes. The initial voltage used was 200V and then changed to 180 V after 15 minutes had elapsed. The gels were placed into transfer buffer. An Invitrolon™ PDVF membrane (Novex, Life Technologies) was soaked in 99.8% methanol for 1 minute. The membranes were placed between two filter papers and soaked in transfer buffer for 5 minutes. The gels were sandwiched separately with blotting pads, filter paper and a transfer membrane (**Figure 1**). The proteins from the gels were transferred onto a PDVF membrane using a semi-dry transfer machine. The transfer machine was run at 14V for 1 hour and 15 minutes.

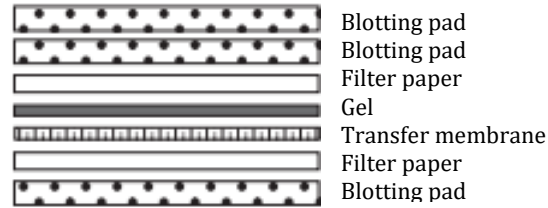


Figure 1: Description of how the electrophoresis gel was stacked and placed into the semi-dry transfer machine.

Blocking solution was prepared by adding 250 μ l of fetal bovine serum (FBS) to 10 ml of tris-buffered saline (TBS). Two blocking solutions were prepared and added to separate basins into which the gels were transferred. The basins containing the gels were placed onto a rocker for 30 minutes at room temperature. Two separate primary antibody solutions were prepared. The first contained 20 μ l of Troponin I Type 1 (slow skeletal) Antibody (Novus Bio). The second contained 20 μ l of Troponin I Type 2 (fast skeletal) Antibody (NBP2-26200, Novus Biologicals). Both antibodies were added to 250 μ l of FBS mixed into 10 ml of TBS. Blocking solution was drained and replaced with the respective primary antibody solution. The basin was placed onto a rocker for 5 hours. Secondary antibody solutions were prepared by adding 2 μ l secondary antibody to 10 ml of TBS. The primary antibody solutions were drained. The basins were washed 3 times for 10 minutes by adding TBS so that the membranes were submerged. The secondary antibody solution was added to each of the basins and the basins were placed onto a rocker for two hours. The membranes were washed 3 times for 3 minutes on a rocker. Developing solution was added and the basin was placed onto a rocker. When bands appeared the reaction was stopped by rinsing the membranes with DI water. An image of the membrane was taken.

V. Statistical Analysis

Significance of results of ELISA were determined with a student t-test. Significance was set to $p \leq 0.05$.

Results

GDNF Protein Content in EDL

GDNF protein content was analyzed by a sandwich ELISA. The GDNF protein content was significantly higher from EDL muscles of animals sedentary for 24 weeks compared to animals sedentary for only 4 weeks (Figure 2).

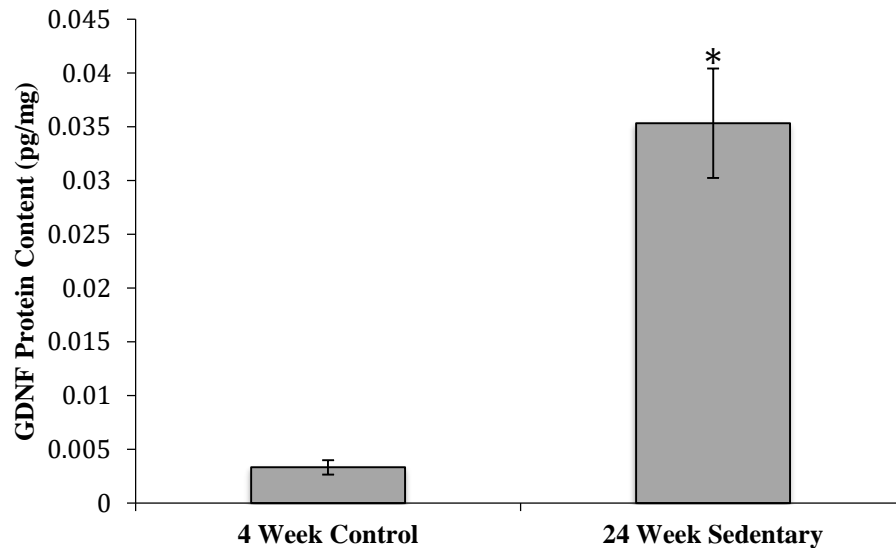


Figure 2: GDNF protein content comparing 4 week control rats to rats sedentary for 24 weeks.

The values are expressed in GDNF weight per weight of muscle tissue. Data is represented by the mean \pm SEM (n=6 for both groups). The difference in means was significant ($p < 0.001$).

There was no significance in the GDNF protein content of the EDL or rats that were exercised for 24 weeks compared to rats sedentary for 24 weeks. However, there was a trend towards greater GDNF protein content in the exercised rats.

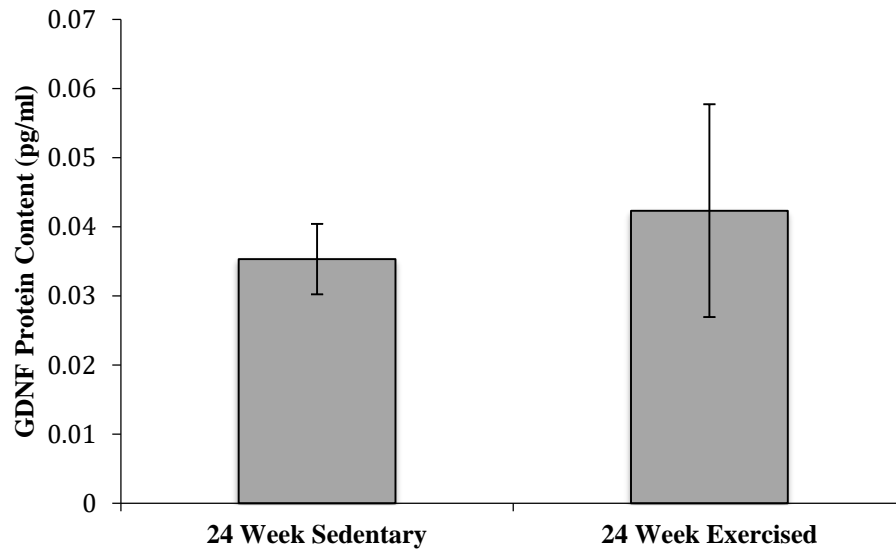


Figure 3: GDNF protein content comparing 24 week sedentary rats (control) to rats exercised for 24 weeks. The values are expressed in GDNF weight per muscle tissue weight. Data is represented by the mean \pm SEM (n=6 for both groups). The difference in means was not significant ($p>0.2$).

Muscle Fiber Phenotype Switch

EDL muscle fiber was analyzed by Western Blot. Western Blot was used to qualitatively assess whether tissue samples contained fast and slow muscle tissue. Primary antibody specific to different isoforms of troponin between fast and slow skeletal muscle was used to differentiate the different muscle fiber phenotypes. Primary antibody specific for fast skeletal muscle was added to the first membrane. The presence of bands around the 21kDa point (between the 14 and 28 band molecular markers) indicated the presence of Troponin specific to fast-twitch fibers (Novus Biologicals).

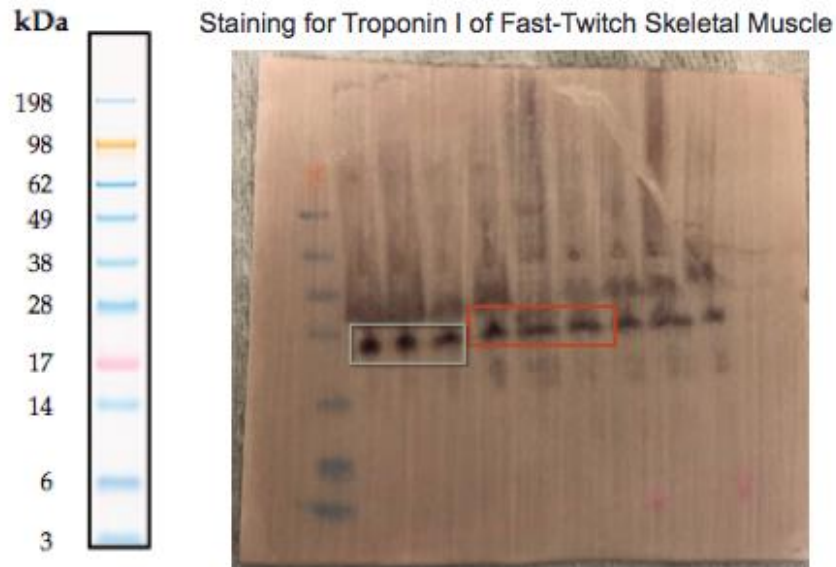


Figure 4: Western Blot membrane when stained with primary antibody The grey rectangle shows bands for the 4 week sedentary rats. The orange rectangle shows the bands for the 24 week sedentary rats. The presence of a band between the 14 and 28 kDa band indicates the presence of fast-twitch Troponin I. It can be noted that the bands had varying shapes which may have been due to improper transfer technique.

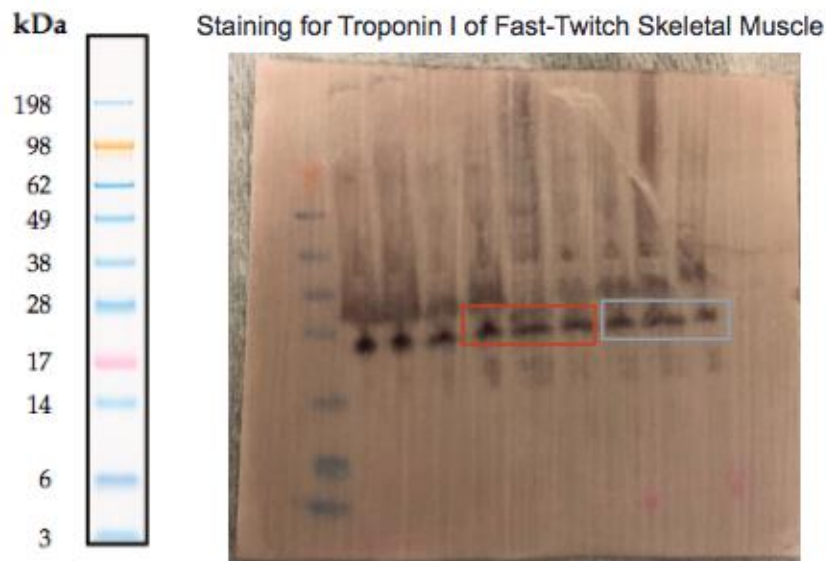


Figure 5: Western Blot membrane when stained with primary antibody for fast-twitch Troponin I. The orange rectangle shows bands for the 24 week sedentary rats. The grey rectangle shows the bands for the 24 week sedentary rats. The presence of a band between the 14 and 28 kDa band indicates the presence of fast-twitch Troponin I. The bands are slightly shifted upwards on the membrane which may have been due to a slight tear that occurred in the gel when it was being transferred onto the membrane.

Unfortunately, an issue was encountered when running the membrane to which antibody specific to slow-twitch muscle fibers was added. During the 5-hour incubation period, after the addition of the primary antibody, part of the solution dried up. This caused the membrane to dry slightly. Thus, when the membrane was covered in developing solution no bands appeared. A shorter incubation period and ensuring that the temperature of the room was correct could have resolved the issue. Due to time constraints, however, another Western Blot was not possible to run.

Discussion

The aim of this study was to determine the effects of aging and exercise on GDNF protein content and muscle fiber phenotype switch. McCullough et al. (2011) showed that there may be an activity-dependent model in rats where different types of exercise recruit different muscles. Her group showed that GDNF protein content increased in slow-twitch muscle (SOL) and decreased in fast-twitch muscle (EDL) after two weeks of involuntary exercise. Gyorkos et al. (2014) showed an increase in GDNF protein content in fast-twitch muscle fiber after swim training. This was the first time that an increase in GDNF protein content in fast-twitch muscle fiber had been observed. Thus, exploring the link between GDNF protein content and muscle

fiber phenotype can provide insight into the relationship between exercise and protection of the nervous system. GDNF protein content was analyzed with enzyme-linked immunosorbent assay. Muscle fiber phenotype was observed with Western Blot.

GDNF protein content may vary as rats develop

This assay provided insight about the amount of protein associated with each muscle fiber.

Aging resulted in an increase in GDNF protein content of EDL over a six-month period. This opposed the hypothesis and previous studies (McCullough et al., 2011). This result could be due to differences in GDNF protein content as rats age. GDNF plays a critical role during development (Nagano & Suzuki, 2002, Nguyen et al., 1998). At the early points of development, GDNF protein content tends to be high as muscles are being innervated (Nagano & Suzuki, 2002). Thus, if the time that the rats were evaluated occurred at different stages of development, the expression of the highest levels of GDNF may have been missed. Additional studies with rats of varying ages could be done to further explore the relationship between development and GDNF expression.

Exercise increasing GDNF protein content in fast-twitch muscle opens new possibilities

Although no significant difference was observed, a trend towards increase in GDNF protein content was found in the exercised rats. The exercise protocol of the rat was mostly aerobic running, likely recruiting slow-twitch muscle fiber instead of fast-twitch muscle fiber. Thus, our results for a trend towards increase in GDNF protein content after exercising the rats reveals new possibilities. Since EDL is not as highly recruited, there may have been a build up of GDNF protein that was not being used by the muscle. However, mRNA levels of the protein may have dropped. Analyzing mRNA expression could provide insight into whether there are translational controls affected by exercise. An alternative explanation is that although EDL is not as actively

recruited by aerobic exercise, there may have been a variety of anaerobic sprints by the rats over the six month period. These anaerobic sprints may have recruited the muscle to a level that caused an increase in GDNF protein content.

Slow-twitch muscle fiber may produce more GDNF than fast-twitch muscle fiber

Another plausible explanation for the results in both the aging and exercise comparisons is that slow-twitch muscle fibers produce more GDNF than fast-twitch muscle fiber. Since the fast-twitch muscle fibers are not recruited, there may have been a switch in the EDL muscle fibers from predominantly fast-twitch to slow-twitch fibers. If slow-twitch muscle fibers produce more GDNF and a phenotype switch was observed, then this could provide the additional explanation for the increase in GDNF protein content in older sedentary animals compared to younger sedentary animals and in aerobically exercised animals compared to age-matched sedentary animals. This possibility was explored in the muscle fiber phenotype switch aspect of this study.

Evidence of fast-twitch muscle fiber was seen in all three groups

In order to further examine what was occurring at the molecular level, the phenotype of the muscle fiber was assessed. By observing changes in phenotype, the hope was to see how aging and exercise affected a primarily fast-twitch muscle. There was a technical issue with the membrane probed for slow-twitch Troponin I drying up, and a time constraint. Due to this, the only conclusion that could be made about the muscle fiber phenotypes of EDL was that all three groups contained fast-twitch phenotypes. This was noted by the appearance of bands at the 22 kDa marker. Unfortunately, this does not allow for the assessment of whether a muscle fiber phenotype switch was observed in the muscle. Had there been more time, additional Western Blot assays would have been completed in order to fully answer the questions proposed in this study.

Conclusion

Exploring the role of exercise in the protection of the nervous system can provide valuable knowledge about possible ways to slow down the process of aging. This study showed that GDNF protein content of fast-twitch muscle can be affected by aging and aerobic exercise. In addition, the presence of fast-twitch muscle fibers was detected in young, aged and exercised tissue.

Future studies that can be done are Western Blot to detect slow-twitch muscle fibers, the quantification of fast-twitch and slow-twitch fibers using Western Blot, and analysis of mRNA expression. Additionally, it would be interesting to see the results of longer-term swim training in rats on GDNF protein content. Histological analysis of muscle fiber phenotypes could also be done.

The link between exercise and protection of the nervous system can be used to strengthen public health campaigns. Protecting the nervous system, and thus the identity of human beings, is an incredible motivation to participate in exercise.

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