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Walk-Training Increases Expression of GDNF in Pectoralis Muscle but not Diaphragm from Mouse

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Walk-training Increases Expression of GDNF in Pectoralis Muscle but Not Diaphragm from Mouse

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Abstract

Neurotrophic factors are proteins that play an important role in the development and maintenance of neurons. Recent studies have shown that neurotrophic factors may hold promise for treating damage to the nervous system caused by trauma or diseases, such as amyotrophic lateral sclerosis or Parkinson's disease. Glial cell line-derived neurotrophic factor (GDNF) is expressed in skeletal muscles and affects peripheral motor neurons. Results of previous studies have demonstrated that exercise can increase GDNF content in skeletal muscle of rat. The goal of the current study was to examine whether expression of GDNF in skeletal muscle of mouse is also regulated by physical activity. Our hypothesis states that muscles undergoing higher levels of contractile activity will express GDNF at higher levels than muscle exhibiting lower levels of contractile activity. For these studies we examined GDNF protein content in tissues from sedentary control mice and in mice following exercise. Muscles examined included the diaphragm (DIA), an involuntary slow twitch muscle and pectoralis major (PEC), a voluntary slow twitch muscle. Treatment groups included 7 control mice and 6 mice that underwent walk training. The treatment group had a one-week training period, and then exercised for 30 minutes a day, at 8 meters/minute, five days a week. Following completion of the exercise regimen muscles were removed and processed for determination of GDNF protein content using enzyme-linked immunosorbant assay, GDNF protein localization and end plate morphology. The results show that DIA from control animals contains more GDNF than PEC, while exercise increased GDNF protein content in PEC but not DIA. These findings suggest that GDNF production is effected by exercise in voluntary slow-twitch muscles, but not involuntary slow-twitch muscles. These results contribute to understanding the mechanisms underlying the normal control of GDNF expression in skeletal muscles. It is important to develop a more complete understanding of normal expression

of GDNF in skeletal muscle before we can determine whether GDNF-based therapies may be used in the treatment of traumatized or diseased neural tissues.

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Keywords: GDNF, Exercise, Skeletal Muscle

Introduction

Skeletal muscles are the striated muscles in our body which act as the engine of the body. They are used in everything from moving, eating, breathing, and standing still[4]. A skeletal muscle is made up of many individual muscle fibers. These fibers contract as a result of the motor neuron that innervates them. This connection occurs at the neuromuscular junction, which is defined as where the nerve meets and innervates the muscle[5]. The neuromuscular junction is composed of the motor neuron, the synaptic cleft, and the endplate on the muscle fiber. The synaptic cleft is a gap between the membranes of the nerve cell and the muscle cell that spans about 50 nm wide. The end plate is the region of the muscle fiber with which the terminals make contact[4]. In order for the muscle contraction to occur, an action potential must travel down the axon of the motor neuron. This electrical conduction causes the release of neurotransmitter, most importantly acetylcholine, across the synaptic cleft. As the acetylcholine diffuses across the cleft and binds to the acetylcholine receptors on the muscle cells, the muscle undergoes ionic changes which cause the fibers, and the muscle as a whole, to contract.

Neurotrophic Factors are the functional group of growth factors that specialize in acting on the cells of the nervous system. As the name indicates, these growth factors are considered trophic agents, meaning they can only stimulate hypertrophic growth. This is due to the fact that the target neuronal cells are post mitotic[2]. Some of the responses invoked by neurotrophic factors include neurite proliferation, maintenance of viability, and induction of neural-specific genes. Neurotrophic factors are divided into families of several homologous members. These members have similar structure, amino acid sequences, and tissue specificity. Also, each family of neurotrophic factors has a corresponding family of receptors of related proteins. There is a growing body of research on the how neurotrophic factors impact the nervous system but there are still gaps in our understanding of all the functions neurotrophic factors provide.

Glial cell line-derived neurotrophic factor (GDNF) was first discovered as a growth factor that was secreted from glial cells, capable of supporting survival of dopaminergic neurons in the embryonic ventral midbrain, and later the CNS[1,13]. Since its discovery, the known functions of GDNF have increased significantly. Not only does GDNF promote the survival of additional central neurons, but is thought to play a role in the survival of some peripheral ganglia as well, such as spinal motor neurons[1]. Also, GDNF is not only found in a wide variety of neuronal tissues, but also found in non-neuronal cells, such as skeletal muscle[13].

In order to understand how GDNF affects the neuromuscular system, we must first know where GDNF comes from and the exact target that it acts on. Although these issues are still somewhat debated, there has been a lot of recent research done to find out more. We know that GDNF can be synthesized by both the muscle cells and Schwann cells [6]. However, regarding the GDNF that localizes at the neuromuscular junction, many studies suggest that the neurotrophic factor is secreted by the skeletal muscle. The GDNF is then taken up by the axon of the motor neuron and transported to the cell body[12,13].

At this point in time, the affects that GDNF has on the neuromuscular system seem limitless. Originally, GDNF was thought to be specific for dopaminergic neuron growth[3]. However, now it is known to have a variety of effects, including increasing muscle innervation at the neuromuscular junction and neurotransmitter release. In one study on neonatal mice there was significant increase in the number of innervating motor axons to a neuromuscular junction after elevated GDNF levels in muscle cells[6]. Other studies have shown enhancement of neurotransmitter release at immature mammalian neuromuscular synapses after increases in GDNF[7].

Although the exact mechanism of action may be uncertain, there is no question that GDNF is the most potent neurotrophic factor in the survival of motor neurons, both in vitro and in vivo[3,6]. Research using GDNF knockout mice found a significant loss of motor neurons, showing that the survival of motor neurons is dependent on GDNF[1]. Not only does GDNF rescue motor neurons from naturally occurring cell death, but it can prevent the wasting away of motor neurons when they are injured through axotomy[3,13].

Knowing how beneficial GDNF is to the neuromuscular system, it is no surprise that problems can arise with low levels of GDNF. These problems can occur with the usual aging and inactivity that comes along with it, or with something more threatening, such as disease. It is believed that GDNF is important in maintaining motor function with age, and the deficit of GDNF can fast-track age-related motor problems. One study showed that motor function improved with the administration of GDNF to aged rats[13]. Also, GDNF levels are crucial to maintaining dopaminergic neurons during the aging process[8]. Just as dangerous as aging is inactivity, as GDNF expression shows significant differences when inactive[12].

The capabilities of GDNF cause researchers to believe that it could play a vital role in fighting against the degeneration and death of motor neurons that come with neuromuscular diseases such as amyotrophic lateral sclerosis (ALS) and Parkinson's Disease (PD)[3]. PD is due to the loss of dopamine neurotransmission. In a recent study, treating PD model rats with GDNF injections resulted in significant reduction of motor deficits, as well as increases in dopaminergic neurons[9]. ALS is caused by the death of large motor neurons in the brain and spinal cord, potentially as a result of glial dysfunction. With ALS patients, GDNF is a successful therapeutic measure in vitro, however it has not been able to be put to the test in vivo due to difficulties with crossing the blood-brain barrier[11].

Although these outcomes of low GDNF can be frightening, research has shown that there may potentially be ways to increase GDNF levels. One of the most promising methods is exercise, as GDNF is believed to be produced in skeletal muscle[6,10,13]. It is well known that physical activity can alter the neuromuscular junction, but it is still unknown how and if GDNF plays a role in these alterations. However, research in Dr. Spitsbergen's WMU lab has been convincing that GDNF has a role due to the increases of GDNF we see with exercise. Not only do we see increases in GDNF content in skeletal muscle, but have also been able to observe alterations in acetylcholine and acetylcholine receptor activation[12]. In addition, we have seen increased size and degree of branching of motor nerve terminals at the NMJ. Notably, differences in GDNF content were observed based on the level of activity[14]. Forced wheel running increases GDNF levels in rats but hind limb suspension decreases GDNF in the hind limb muscles[12]. These observations lead us to believe that exercise could be used as a therapeutic measure against motor neuron deficiencies.

Hypothesis

Through my research I attempt to address a couple of important ideas for continuing the research on the relationship between GDNF and exercise. The first goal of my study was to determine whether our lab can use the same methods in mice as we used in rats. If this is true, we can expand our research by using more genetic variations that are only available in mouse models. Specifically, I will look at whether we can use the same enzyme-linked immunosorbant assay (ELISA) method that has been used in rat models, to measure GDNF content in mouse muscle. I will also explore if we can use the immunocytochemical staining methods previously described in rat to visualize GDNF in mouse muscle. Related to this, I will confirm our prediction that exercise increases GDNF content in mouse skeletal muscle, as we have seen occur in rat skeletal muscle. Beyond this research, I will explore my own hypothesis about whether a tonically active skeletal muscle, such as diaphragm, contains more GDNF protein than phasically active muscle, such as

the pectoralis major. I predict that muscles showing higher levels of contractile activity will produce higher levels of GDNF protein.

Methodology

I. Subjects

Thirteen young adult mice of 3 months of age were used in this experiment. Treatment groups include 7 control mice and 6 exercise mice that underwent walk training. The control group had no access to a running wheel. The exercise group had a one-week training period, and then exercised on the wheel for 30 minutes a day, at 8 meters/minute, five days a week. All animal experiments were performed in accordance with the "Guide for the Care and Usage of Laboratory Animals" (National Research Council) and all protocols have been approved by the Institutional Animal Care and Usage Committee at Western Michigan University.

II. Tissue Processing

After the last scheduled exercise session, mice were euthanized within 72 hours. Muscles from one side of the mouse were removed, and used for GDNF protein quantification. Muscles were removed and promptly dipped into liquid nitrogen. Tissues were then smashed into a fine powder and mixed with sample processing buffer until homogenous. Samples were then centrifuged for 30 minutes at 4°C. The supernatant was stored at -80°C. The muscles from the other side of the mouse were used in immunohistochemical staining. Muscles examined included the diaphragm (DIA), an involuntary slow twitch muscle, and pectoralis major (PEC), a voluntary slow twitch muscle.

III. GDNF Protein Quantification

Quantification of GDNF protein in skeletal muscle tissue was accomplished using an enzyme-linked immunosorbant assay (ELISA). A 96-well plate was used to assess the GDNF standard and the tissue supernatants. A standard curve was created from the known GDNF

standard concentrations. Using this standard curve, the tissue samples could be analyzed for GDNF protein content. These data values are expressed as pg GDNF/ mg Tissue Weight and reported as a mean with a standard error of mean (SEM). The treatment groups are compared using a t-test, with p values ≤ 0.05 considered statistically significant.

IV. Immunocytochemistry

In order to localize GDNF in skeletal muscle samples, immunocytochemical staining was used. Tissues were fixed overnight in 4% paraformaldehyde at 4°C then rinsed with phosphate buffer saline (PBS). The pectoralis muscles were embedded in mounting medium and cut into 40 µm sections using a cryostat. These sections, as well as the already thin sections of diaphragm, were then mounted on slides and vacuum sealed overnight. Primary antibodies were applied to the slides and incubated overnight at 4°C. Then secondary antibodies conjugated with fluorescent dyes were added for easy visualization. Rabbit anti GDNF (Santa Cruz Biotechnology, Santa Cruz, CA) was bound to a secondary antibody conjugated to AlexaFluor 596 (Life Technologies) in order to see GDNF and its location. α-Bungarotoxin was directly conjugated to AlexaFluor 488 (Life Technologies) to show the location of nicotinic acetylcholine receptors, which in turn localizes the endplate of neuromuscular junctions. Mouse neurofilament was bound to a secondary antibody conjugated to AlexaFluor 647 (Life Technologies) to view the axon of the motor neuron making the neuromuscular junction with the muscle fiber. Tissues were then mounted onto the slides using a 1:1 solution of PBS:glycerol and sealed with a cover slip. Slides were then visualized using a Zeiss LSM 510 confocal laser scanning microscope and LSM510 software.

Results

Exercise had no effect on GDNF content in the diaphragm.

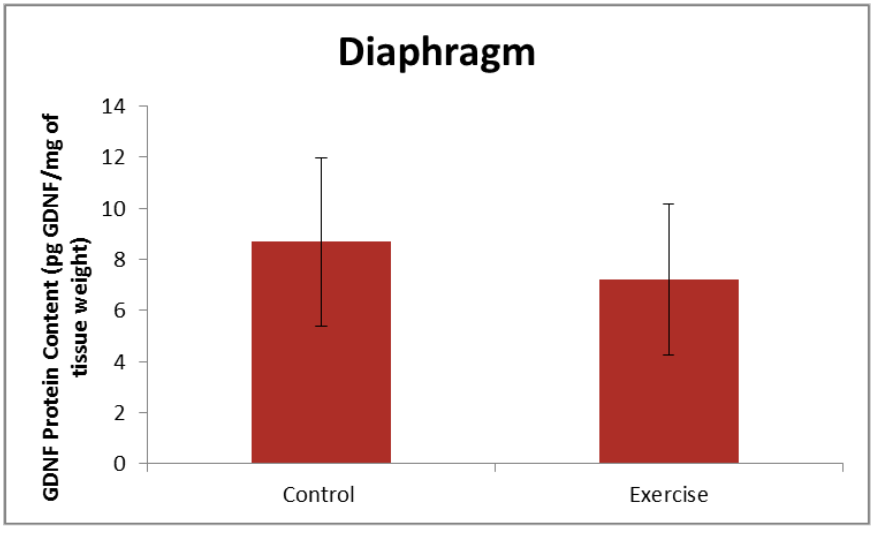

FIG. 1 Results show that in tonically active diaphragm, there is no significant change in GDNF protein content following 2 weeks of involuntary exercise. GDNF protein content of muscles was measured via ELISA. Data represents the mean ± SEM from control and exercised groups. Control animals averaged 8.685 pg GDNF/mg of tissue weight, while exercise animals averaged 7.217 pg GDNF/mg of tissue weight, values that were not significantly different ($p = 0.645$).

Exercise increases GDNF protein content in the pectoralis major.

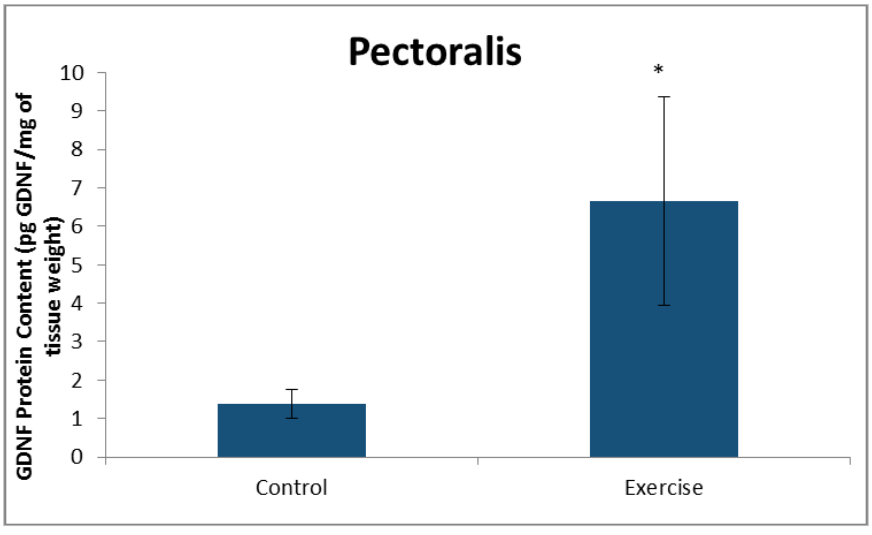

FIG. 2 Results show that in phasically active pectoralis, there is a significant increase ($p = 0.049$) in GDNF protein content following 2 weeks of involuntary exercise. GDNF protein content of muscles was measured via ELISA. Data represents the mean ± SEM from control and exercised groups. Control animals averaged 1.390 pg GDNF/mg of tissue weight, while exercise animals averaged 6.655 pg GDNF/mg of tissue weight. The asterisk (*) denotes a significant difference from control.

GDNF staining is localized to the endplate region in muscle.

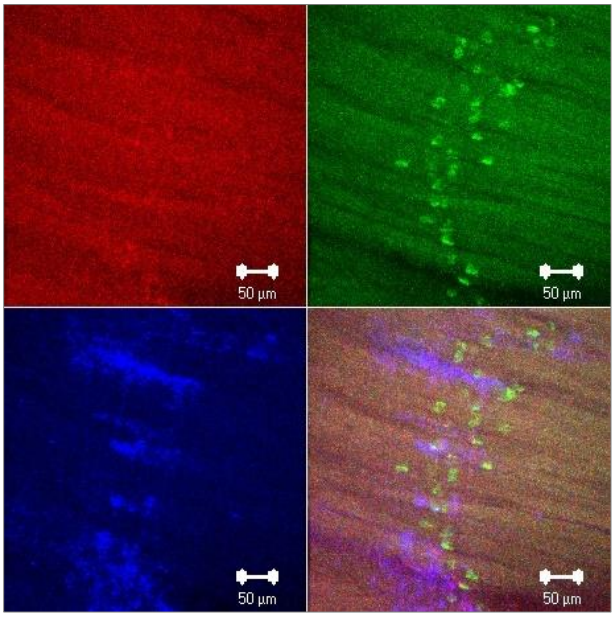

FIG. 3 Exercised diaphragm at 20x magnification. Sections were immunolabled using antibodies against GDNF (blue), α-Bungarotoxin to identify acetylcholine receptors (endplate, green), and antibodies against neurofilament to identify motor axons (red). GDNF is found in the same regions as endplates. This supports the theory that GDNF is made in the muscle fiber and is expressed in higher levels at the point where the nerve makes contact with the muscle.

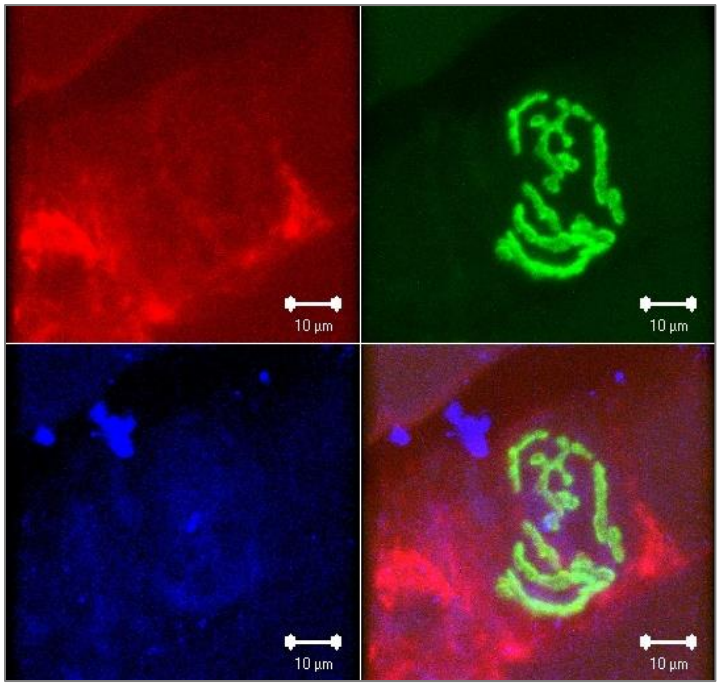

FIG. 4 Exercised pectoralis at high magnification. Sections were immunolabled using antibodies against GDNF (blue), α-Bungarotoxin to identify acetylcholine receptors (endplate, green), and antibodies against neurofilament to identify motor axons (red). There is positive staining for GDNF in the region of the endplate. Once again this localization of GDNF suggests that the protein is synthesized in the muscles around the neuromuscular endplate.

Discussion

Our results show that we were successful in using rat methods to measure GDNF in mice. We can measure GDNF in mouse muscle using ELISA, allowing us to distinguish between tissues with high v. low protein content. We can also detect GDNF in mouse muscle using immunohistochemical methods. In using these methods, we are able to see correlations between endplate and GDNF localization. Being able to use the same methods in mouse muscle that have been previously used in rat muscle allows for expansion of research in several new directions. Most importantly it will allow for the use of many genetic models that are available in mice.

As previous studies have shown that exercise increases GDNF in rat muscles[12, 13], my study shows that exercise increases GDNF in mouse skeletal muscle as well, especially in a voluntary muscle such as the pectoralis major. Although there was no increase in GDNF after exercise in the diaphragm, this is not surprising since the muscle is involuntary, and continually in use while the mouse is breathing. In the control animals, we can see that the involuntary diaphragm starts with more GDNF than the voluntarily-used pectoralis major. Our results from the ELISA support our hypothesis that tonically active diaphragm muscles produce more GDNF protein than phasically active pectoralis major muscle. The results show that DIA from control animals contains more GDNF than PEC.

However, after the exercise regimen, the treatment mice have a significant increase of GDNF protein content in the pectoralis major, but not the diaphragm. Notably, the exercise brings levels of GDNF in the pectoralis close to the control level of GDNF in the diaphragm. These findings suggest that GDNF production is affected by exercise in voluntary slow-twitch muscles, but not involuntary slow-twitch muscles. These results contribute to understanding the mechanisms underlying the normal control of GDNF expression in skeletal muscles.

When looking at our results from immunohistochemical staining, we were successful in seeing the endplate and localizing the GDNF around the endplate. This supports the theory that GDNF is produced in the muscle and then shipped up the axon of the motor neuron. Although we had good results for endplate and GDNF protein staining, our staining of the neurofilaments did not produce good results. This could be due to many factors in our immunohistochemical staining methods, however one likely explanation is that the antibody used to identify rat neurofilament does not recognize mouse neurofilament.

Recent studies have shown that GDNF is synthesized by the skeletal muscle cells, and taken up by the neurons[12,13]. It is also believed that the GDNF synthesis may decrease due to the cholinergic motor neurons regulating their own supply of GDNF, resulting in the inhibition of excessive GDNF secretion from the muscle cells. The feedback loop providing the regulation of GDNF production involves the ACh receptors on the muscle cells[13]. My observations of the differences in the changes of GDNF content between pectoralis and diaphragm during an increased exercise regime provides further support of a regulated GDNF system, one in which increased neural/muscle activity increases GDNF content in muscle.

My staining shows GDNF localization around the endplates of the NMJ, supporting the idea that GDNF is produced in the muscle around the NMJ. The increase in GDNF we observed in the skeletal muscle of exercised mice could be due to an increase in muscle growth (hypertrophy). Voluntary muscles, such as the pectoralis, have nearly unlimited growth potential, and exercise would increases GDNF production in order to support the need for innervation of the growing muscle. Once the muscle stops growing and is fully innervated the cholinergic neurons would start regulating the production of GNDF through a mechanism involving the ACh receptors on the skeletal muscles. My results support the regulated system hypothesis in that my exercised mice had growing pectoralis muscles. Involuntary skeletal muscles, such as the diaphragm were under a constant state of exercise in both the control group and the exercised group, and the difference in growth of the diaphragm between the exercised mice and the control mice was not detectable in GDNF production because the diaphragms of both groups of mice were already close to maximum innervation due to their constant contractile activity. Therefore, the cholinergic motor neurons and the ACh receptors on the muscle were already exhibiting equilibrium of GDNF secretion due to constant activity.

Conclusion

It is important to develop a more complete understanding of normal expression of GDNF in skeletal muscle before we can determine whether GDNF-based therapies may be used in the treatment of traumatized or diseased neural tissues. The success of our mouse studies allows us to expand our research into a variety of genetic models not available in rats. This expansion in research will allow us to learn more about GDNF and neurotrophic factors. Through investigating neurotrophic factors further, we can also expand our knowledge of neurons, glial cells, neurotransmitters, and the electrical physiological properties of the nervous system.

Our observation that exercise increases GDNF in skeletal muscles may help explain the beneficial effects of exercise on motor neurons. A better understanding of exercise's effects on GDNF production can aid the medical world with a preventative and possibly therapeutic measure against fatal neuromuscular diseases, such as ALS, as well as typical aging. In continuing with this GDNF research we hope to enhance the quality of life for those with neuromuscular deficits due to motor neuron problems.

If given more time with this project, I would have liked to expand it in a couple different ways. First, I would have explored deeper into the histology of the tissues using immunohistochemical staining and analysis of factors such as GDNF localization in the neurofilaments and endplate size. Using quantitative statistical analysis of these factors could tell us more about both where GDNF is produced (muscle or neuron), as well as the effect that exercise has on endplate size and synaptic strength. Also, I would have liked to use more tissues from the mouse to confirm my hypothesis about the differences in GDNF production in involuntary v. voluntary slow twitch muscles. Having more tissues that followed my hypothesis would make for a stronger argument. I am anxious to see how GDNF research evolves in the future, as I believe it could possibly be the answer to many neuromuscular deficits and disease.

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