The effect of exercise training on skeletal muscle GDNF content and neuromuscular physiology in a mouse model of amyotrophic lateral sclerosis.

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THE EFFECT OF EXERCISE TRAINING ON SKELETAL MUSCLE AND LUMBAR SPINAL CORD GDNF CONTENT AND NEUROMUSCULAR PHYSIOLOGY IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS.

by

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The effect of exercise training on skeletal muscle and lumbar spinal cord GDNF content and neuromuscular physiology in a mouse model of amyotrophic lateral sclerosis.

Nicole C. Carpp

Western Michigan University, 2015

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease accompanied by the loss of motor neurons, leading to paralysis and death. Glial cell line-derived neurotrophic factor (GDNF) promotes neuron health and function and has been proposed as a therapeutic treatment for ALS. GDNF protein expression in skeletal muscle is regulated by physical activity. The aim of this study was to determine if low intensity exercise would increase GDNF expression in skeletal muscle and slow degeneration of motor neurons in a mouse model of ALS. Following the first sign of disease onset, transgenic ALS mice were randomly assigned to one of three groups: sedentary control, involuntary low intensity exercise, and involuntary low intensity exercise with anti-GDNF injections, twice daily. Anti-GDNF injections were administered to determine if neutralizing GDNF inhibited the beneficial effects of exercise on the motor nervous system. Neurological score was tested daily throughout the exercise protocol, and animals were euthanized at 115 days of age. Lumbar spinal cord, soleus, and pectoralis major muscles were removed and analyzed for GDNF content by enzyme-linked immunosorbant assay. Immunohistochemical analysis of spinal cord was performed to assess motor neuron cell body count. Histological analysis of skeletal muscle was performed to examine endplate area and location of GDNF. Onset of neurological symptoms appeared to be delayed in the exercise group, when compared to the sedentary control and exercise with anti-GDNF treatment groups, although this was determined not to be statistically significant. GDNF content was not significantly affected in spinal cord, soleus, or pectoralis major, although there was a trend towards an increase in the exercised group compared to the control and anti-GDNF group. Histological analysis of spinal cord sections revealed a significant (p<0.05) increase in
motor neuron number in the exercised animals compared to the anti-GDNF treated animals. Histological analysis of soleus and pectoralis revealed a significant (p<0.05) increase in stained endplate area in the exercise group, compared to the sedentary and anti-GDNF treated groups. These results suggest that exercise protects against motor neuron loss and that neutralization of GDNF blocks this protective effect. This suggests that the neuroprotective effect of exercise may be related to the activity-dependent expression of GDNF and that GDNF may have implications as a therapeutic agent in neurodegenerative disease.

**Introduction**

Amyotrophic lateral sclerosis (ALS) is a progressive neurological disorder marked by the degeneration of motor neurons, leading to respiratory failure, paralysis, and death (Brown, 1997). The innervated skeletal muscles atrophy in response to the degeneration and death of their innervating motor neuron. The α-motor neurons of the peripheral nervous system are particularly vulnerable, and often decline significantly in ALS models (Mohajeri et al., 1998).

While the majority of ALS cases are sporadic with an unknown cause, an estimated 5-10% of the cases are familial, passed on as an autosomal dominant allele (Robberecht et al., 2000). An estimated 20% of the familial cases are due to a family of mutations in a superoxide dismutase, coded for by the SOD1 gene, which is involved in prevention of oxidative stress in the cell (Rosen et al., 1993). Previous studies have utilized transgenic mice with the mutated SOD1 G93A gene to model human genetic ALS cases (Gurney et al., 1994). While the exact mechanism that contributes to the neuronal damage in these models is unknown, current hypotheses include oxidative damage, altered metabolism, inflammatory processes, and alterations in growth factor signaling (Leitner et al., 2009).

Glial cell line-derived neurotrophic factor (GDNF), a neurotrophic factor involved in normal nerve growth and development, has been proposed as a therapeutic agent for ALS. Many neurotrophic factors, including GDNF, are target-derived (Henderson et al., 1994) in that the tissue expressing GDNF promotes
survival and maintenance of the neuron innervating it. Through retrograde transport of the protein up the axon, the target tissue contributes to maintenance of its own normal neurological function. Exogenous treatment, or endogenous production of GDNF in the target tissue have been shown to increased nerve branching and promote hyperinnervation (Nguyen et al., 1998).

In ALS models, treatment with GDNF has been shown to slow disease progression (Lu et al., 2004) and injections with GDNF have prevented motor neuron loss in mice (Wang et al., 2002). Treatment with GDNF and stem cells reduced motor neuron death and improved functioning in the SOD1 G93A rat model of ALS (Suzuki et al, 2008). Previous studies in wild type rats have indicated that GDNF expression was increased in rat soleus and gastrocnemius with walk-training (Spitsbergen et al., 2002).

Unlike other neurodegenerative diseases such as Parkinson’s disease, the role of exercise as a therapeutic intervention for ALS subjects is uncertain. Studies have indicated that exercise can slow the progression of ALS in humans (Drory et al., 2001), protect motor neurons in mouse models (Deforges et al, 2009; Kirkinezos et al, 2003; Liebetanz et al., 2004), and increase lifespan in mouse models (Lui and Byl, 2009). However, high-intensity endurance training has been shown to hasten motor neuron death and onset of neurological symptoms through proposed overwhelming of oxidative repair mechanisms in SOD1 G93A mice (Mahoney et al., 2004), suggesting that appropriate intensity may be crucial to harnessing the beneficial effects of activity.

This study aimed to examine the effect of low intensity exercise-induced GDNF expression in a transgenic mouse model of ALS. Thus far, no studies have linked upregulation of GDNF with activity-based adaptations in the genetic model of ALS. The mouse model of ALS was used in an attempt to observe changes similar to human pathology, in a shorter time frame. Our hypothesis is that low intensity exercise will increase GDNF levels and promote corresponding physiological adaptations in spinal cord and skeletal muscle of transgenic mice; blocking GDNF with anti-GDNF antibodies will prevent the beneficial effect of exercise.
Materials and Methods

All experiments were conducted in compliance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council) and the testing protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University.

Male transgenic B6SJL-Tg(SOD1*G93A)1Gur/J mice were selected for the study, as they exhibit an alteration in a superoxide dismutase that is similar to that observed in genetically-derived human ALS subjects (Gurney et al., 1994). All mice were given access to food and water \textit{ad libitum}. At 10 weeks of age, animals were randomly assigned to one of three experimental groups: a sedentary, age-matched control group (SED, n=3), an involuntary running group (EX, n=3), or an involuntary running group that received anti-GDNF injections (ANTI-GDNF, n=3). The ANTI-GDNF group received twice daily, subcutaneous injections of human anti-GDNF (R&D Systems) for the duration of the study. The animals received 25ng anti-GDNF/gram of animal weight every 12 hours throughout the duration of the study, which was similar to the regimen developed by Ferrer-Alcon et al. (2008).

The treatment protocol was initiated at the first sign of disease (day 77), as indicated by the inability of the mouse to fully extend its hind limbs. The EX and ANTI-GDNF mice were placed in individual forced running wheels, at a speed of 8m/min, intermittently, to accumulate 30min/day. The speed was set at 8m/min, as previous studies have indicated that velocities lower than 10m/min coincide with low-intensity exercise for mice (Bey and Hamilton, 2003). The protocol also included brief warm ups and cool downs daily. The animals were allowed access to the wheels for a 1 week period prior to the start of the study to facilitate acclimation to the running apparatus.

Neurological scores of disease progression were monitored daily. At 115 days of age, all animals were euthanized via CO$_2$ asphyxiation followed by thoracotomy. Lumbar spinal cord, soleus, and pectoralis were removed and stored at -80°C until processed.

GDNF Content Analysis
A section of lumbar spinal cord from L1-L3 and whole soleus and pectoralis from the animal’s right side were removed and frozen on dry ice for protein quantification. The samples were individually dipped in liquid nitrogen and pulverized into a fine powder. 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) was added and the solution was homogenized on ice. Samples were centrifuged at 14000g for 30 minutes at 4°C. Following centrifugation, the supernatant was removed and stored at -80°C until further processing.

GDNF protein content was measured using and enzyme-linked immunosorbant assay (ELISA). Ninety-six well plates were incubated overnight with a monoclonal antibody against GDNF (R&D Systems). The following day, the plates were washed with wash buffer and blocked for 1 hour with a PBS solution containing 1% bovine serum albumin (BSA) and 5% sucrose at room temperature. Plates were then washed and a GDNF standard (R&D Systems) or tissue samples were added to the wells. A GDNF standard curve, ranging from 1000pg/mL to 2pg/mL, was calculated from the GDNF standards. Plates were incubated for 2 hours at room temperature, then washed with wash buffer. A secondary antibody (R&D Systems) was added to each well and allowed to incubate at room temperature for 2 hours. The plates were then washed and a solution containing β-galactosidase conjugated to streptavidin (Molecular Probes) was added for 20 minutes at room temperature. A final wash step was conducted prior to addition of a chlorophenol red-β-D-galactopyranoside (CPRG) substrate was added with BSA in a solution of PBS. The plates were incubated at room temperature until the color had developed, then read at an absorbance of 575nm.

**Spinal Cord Histological Analysis**

The L4-L5 section of spinal cord was removed, fixed overnight in 4% paraformaldehyde at 4°C, and cryoprotected overnight in 30% sucrose made in PBS. Tissues were embedded in optimal cutting temperature (OCT) compound, cut into 40µm transverse sections on a cryotome, and thaw mounted onto
Histobond® slides (VWR International). Slides were incubated overnight with primary antibodies that were diluted 1:200 in PBS containing 1% BSA and 0.1% triton X-100. Sections were exposed to rabbit-anti GDNF (Santa Cruz Biotechnology), mouse anti-choline acetyltransferase (Millipore), rabbit anti-activated caspase-3 (Cell Signaling Biotechnologies), or mouse anti-GFRα1 (Santa Cruz Biotechnologies). Slides were washed in PBS before incubation with secondary antibodies of donkey anti-mouse conjugated to AlexaFluor 568 or donkey anti-mouse conjugated to AlexaFluor 647 and donkey anti-rabbit conjugated to AlexaFluor 488 that were diluted 1:500 in PBS containing 1% BSA and 0.1% triton X-100 for 2 hours at room temperature. Slides were mounted in a 1:1 solution of PBS and glycerol and visualized using a Zeiss laser scanning confocal microscope at a magnification of 63x.

ChAT-positive motor neuron cell body numbers were measured in Lamina IX of the spinal cord. Three random sections, 100µmx100µm, were selected from within the lumbar spinal cord and were evaluated by counting ChAT-positive cells in the ventral horn of the L4-L5 segment. Images were examined using Zeiss LSM 5 Image Examiner program.

**Skeletal Muscle Histological Analysis**

Whole soleus and pectoralis from the animal’s left side were removed, flash frozen in cold 2-methylbutane, and stored at -80°C until processed. Tissues were embedded in optimal cutting temperature (OCT) compound, cut into 50µm longitudinal sections on a cryotome, and thaw mounted onto Histobond® slides (VWR International). Slides were incubated overnight with primary antibodies that were diluted 1:200 in PBS containing 1% BSA and 0.1% triton X-100. Sections were exposed to rabbit-anti GDNF(Santa Cruz Biotechnology), mouse anti-neurofilament (2H3 Developmental Studies Hybridoma Bank), and mouse anti-SV2 (Santa Cruz Biotechnology). Slides were washed in PBS before incubation with secondary antibodies of donkey anti-mouse conjugated to AlexaFluor 568 and donkey anti-rabbit conjugated to AlexaFluor 647 and α-bungarotoxin that was directly conjugated to AlexaFluor 488. Secondary antibodies were diluted 1:1000 in PBS containing 1% BSA and 0.1% triton X-100 for 2
hours at room temperature. Slides were mounted in a 1:1 solution of PBS and glycerol and visualized using a Zeiss laser scanning confocal microscope.

α-bungarotoxin stained slides were used for endplate area quantification. Twenty randomly selected endplates from each muscle were selected for quantification. Endplates that were aligned with the plane of the section were selected, scanned, and stored for quantification. Images were examined using Zeiss LSM 5 Image Examiner program. The circumference of the stained endplate was traced and the Image Examiner software facilitated calculation of the stained area.

Statistical Analysis

All data values are reported as mean ± the standard error of the mean (SEM). Data was analyzed using one-way ANOVA and Tukey’s post-hoc comparison, testing for difference among the independent groups. Statistical significance was set at p≤0.05.

Results

Disease Progression

The initial exercise protocol was set at 8m/min. However, due to progression of neurological symptoms, the running protocol was adjusted to prevent injury. The adjusted protocol started at 8m/min for the first 3 weeks and was adjusted to 5m/min for the last week of the protocol for all of the EX and 2 of the 3 ANTI-GDNF animals. One of the ANTI-GDNF mice required speed reduction to 4m/min in the second week and 2.5m/min for the third and fourth weeks of the study.

Symptoms of neurological disease, as indicated by hind limb weakness (Table 1), began to appear first among the ANTI-GDNF group at 92 days of age. This was followed by the SED mice at 97 days of age and the EX mice at 101 days of age (Table 2), although this difference was not statistically significant.
Table 1: Neurological Score

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Full extension of hind legs</td>
</tr>
<tr>
<td>1</td>
<td>Trembling of hind legs during suspension</td>
</tr>
<tr>
<td>2</td>
<td>Toe curl or foot dragging</td>
</tr>
<tr>
<td>3</td>
<td>Rigid paralysis or minimal joint movement</td>
</tr>
<tr>
<td>4</td>
<td>Inability to right self within 30 seconds</td>
</tr>
</tbody>
</table>

Table 2: Symptom Onset

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Age at Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>SED</td>
<td>97 days</td>
</tr>
<tr>
<td>EX</td>
<td>101 days</td>
</tr>
<tr>
<td>ANTI-GDNF</td>
<td>92 days</td>
</tr>
</tbody>
</table>

Table 1. Scale of neurological symptom manifestation used to monitor disease progression. Table 2.

A trend toward a delay in onset of neurological symptoms was observed in the EX group, when compared to the SED and ANTI-GDNF group. Disease progression was assessed daily and quantified using the neurological score outlined in Table 1.

**GDNF Protein Content**

Exercise (22.0 ± 1.5pg GDNF/mg tissue weight) had no significant effect on GDNF protein content in the lumbar spinal cord compared to SED mice (49.1 ± 11.2pg GDNF/mg tissue weight) (Figure 1). Exercise (236.4± 97pg GDNF/mg tissue weight) had no significant effect on GDNF protein content in soleus compared to SED mice (10.7 ± 6.1pg GDNF/mg tissue weight) (Figure 2). In addition, exercise (18.3 ± 9.1pg GDNF/mg tissue weight) had no significant effect on GDNF protein content in pectoralis compared to SED mice (1.2 ± 0.5pg GDNF/mg tissue weight) (Figure 2).
Figure 1. Glial cell line-derived neurotrophic factor (GDNF) content, measured via ELISA, is not altered in lumbar spinal cord of ALS mice following low intensity exercise. The lumbar spinal cord (L1-L3) was harvested from B6SJL-Tg(SOD1*G93A)1Gur/J mice randomly assigned to a sedentary (SED) group (n=3) and exercise group (n=3). Values are representative of mean ± SEM.

Figure 2. Glial cell line-derived neurotrophic factor (GDNF) content, measured via ELISA, is not altered in soleus or pectoralis of ALS mice following low intensity exercise. The soleus and pectoralis
were harvested from B6SJL-Tg(SOD1*G93A)1Gur/J mice randomly assigned to a sedentary (SED) group (n=3) and exercise group (n=3). Values are representative of mean ± SEM.

**Histological Analysis: Spinal Cord**

A significant increase was observed from the EX mice (6.7 ± 0.7 ChAT-positive cells per section) compared to the ANTI-GDNF mice (4.0 ± 0.6 ChAT-positive cells per section). No difference in cell number was observed in the SED mice (5.3 ± 0.3 ChAT-positive cells per section) compared to the EX and ANTI-GDNF mice (Table 3, Figure 3).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Average Motor Neuron Cell Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary Control</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>Exercise</td>
<td>6.7 ± 0.7*</td>
</tr>
<tr>
<td>Exercise &amp; Anti-GDNF</td>
<td>4.0 ± 0.6</td>
</tr>
</tbody>
</table>

Table 3. A significant increase in motor neuron cell body counts was observed in the EX group, when compared with ANTI-GDNF group. Choline acetyltransferase (ChAT) positive neurons in three randomly assigned 100µm x 100µm boxes were counted from Lamina IX in the ventral horn of grey matter from the lumbar spinal cord to determine motor neuron cell number. Images were captured with a Zeiss laser scanning confocal microscope.
Figure 3. Cell counts of choline acetyltransferase (ChAT) positive motor neurons in the mouse lumbar spinal cord. Sections were labeled with antibodies against ChAT. Images were captured with a Zeiss laser scanning confocal microscope. A 100µm x 100µm box was randomly placed in three areas within Lamina IX in the ventral horn of the grey matter. Cells were counted from (A) SED mice, (B) EX mice, and (C) ANTI-GDNF mice. Scale bar represents 100µm

**Histological Analysis: Skeletal Muscle**

A significant increase in stained endplate area was observed from the soleus of EX mice (325.8 ± 1.9µm²) compared to the ANTI-GDNF mice (283.2 ± 3.3 µm²) and SED mice (248.1 ± 2.8µm²). A significant increase in stained endplate area was also observed from the pectoralis of EX mice (318.1 ±
4.1µm²) compared to the ANTI-GDNF mice (281.0 ± 1.6 µm²) and SED mice (251.0 ± 3.6µm²; Figure 3, Figure 5).

Figure 4. A significant increase in stained endplate area in pectoralis and soleus was observed in the EX group, when compared with the ANTI-GDNF group and SED group. Motor endplates were labeled with α-bungarotoxin, and images were captured with a Zeiss laser scanning confocal microscope. Zeiss Image Examiner software was used to outline motor endplates and calculate endplate area in the plane of the tissue.
Figure 5. Histological analysis of pectoralis (SED group) for endplate area. Sections were stained for synaptic vesicles and neurofilament (Red), acetylcholine receptors (Green), and GDNF (Blue). Images were captured with a Zeiss laser scanning confocal microscope. The areas of endplates in the plane of the tissue were calculated using Zeiss Image Examiner software. Scale bar represents 10µm.

**Discussion**

The purposes of this study were to examine if low intensity walk training would affect symptom onset, GDNF expression, or neuromuscular physiology in a transgenic model of ALS, and if treatment with anti-GDNF would block any changes seen with exercise. The results indicated that walk training increased motor neuron cell body survival and stained endplate area; these results were accompanied by a trend towards an increase in GDNF content in muscle and a trend towards a delay in neurological symptom onset. Treatment with anti-GDNF did prevent the exercise induced adaptations of endplate augmentation, motor neuron survival and symptom onset.
Currently, a consensus on the role of exercise in ALS populations has not been reached. Some researchers have demonstrated detrimental effects with exercise in ALS mice (Mahoney et al., 2004) and have suggested that physical activity may lead to increased oxidative damage resulting in motor neuron degeneration (Harwood et al., 2009). However, other studies have demonstrated that exercise can be beneficial. Utilization of resistance exercise in human ALS patients led to improved physical function (Bello-Hass et al., 2007). In rodent models, ALS mice that were exercised via treadmill running had a significantly longer lifespan than their sedentary counterparts (Kirkinezos et al., 2003). Swim training led to improved motor neuron survival, selective protection of fast-twitch motor units, and maintenance of astrocyte and oligodendrocyte numbers in spinal cord of ALS mice (Deforges et al., 2009). In our study, we saw a trend toward a delay in neurological symptom onset, further suggesting a beneficial effect of physical activity. Although this effect was not statistically significant, which may be due in part to our small sample size (n=3), it was accompanied by significant neural adaptations with an increase in motor neuron survival and motor endplate area.

GDNF has also been explored as a possible therapeutic intervention for ALS. Direct treatment with GDNF and progenitor cells to spinal cord in ALS mice led to preservation of motor neurons (Suzuki et al., 2007). In addition, neurotrophic factor produced in musculature of transgenic ALS mice overexpressing GDNF was found to delay onset of disease, increase life span, and improve motor performance (Li et al., 2007). Previous studies have shown that GDNF content is up-regulated in recruited musculature of rodents using similar exercise protocols (Wehrwein et al., 2002, McCullough et al., 2011). GDNF content in our study showed a trend towards an increase in muscle with exercise, although we did not see a significant effect, likely due in part to variability within our small sample size (n=3).

Interestingly, GDNF content showed a trend towards a decreased in spinal cord. A possible explanation for this is the duration of exercise used in the study. Since the mice were exercised until they displayed severe hind limb weakness, our total exercise protocol was almost five and one half weeks in duration. Previous studies on activity-dependent GDNF expression in rats have demonstrated that protein
content in spinal cord is not significantly affected by longer exercise durations ranging from 6 weeks to 6 months. This effect may be due to the increased activity of the motor neurons leading to an increased rate of neuronal uptake of GDNF, translating to a decrease in GDNF content in spinal cord.

Blocking GDNF with anti-GDNF injections blocked the neuromuscular effects observed with exercise, further supporting the conclusion that GDNF plays a role in exercise-induced neuroprotection. Several studies have used addition of antibodies against neurotrophic and growth factors to prevent exercise-induced neural effects. The effect of insulin-like-growth factor 1 (IGF-1) was prevented with daily injections of antibodies against IGF-1 (Ferrer-Alcon et al., 2008). Injections with BDNF inhibitor prevented the neuroprotective effects observed with exercise after traumatic brain injury (Greisbach et al., 2009) and in Parkinson disease models (Real et al., 2013). Using a similar design, we observed a counteraction of exercise induced neuroplasticity in the anti-GDNF group. The anti-GDNF treatment could be the result of a negative effect on the motor neurons that counteracts the beneficial effect of exercise. However, another explanation is that exercise increases GDNF expression leading to exercise induced neuroprotective adaptations.

In this study, low intensity exercise elicited neuromuscular adaptations including increased motor neuron survival and motor endplate area, accompanied by trends toward increased GDNF content in muscle and delay in neurological disease symptoms. Treatment with anti-GDNF blocked all neuroprotective effects, suggesting that exercise-induced GDNF expression is integral to activity-based neuroprotection. If a defined exercise program can be developed that balances increased GDNF expression with minimal activity based oxidative stress, physical activity could be a potential therapeutic intervention in ALS patient populations.
References


