Title: Forced Exercise Increases Muscle Mass in EAE despite Early Onset of Disability

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SUMMARY

INTRODUCTION: We aimed to determine whether 10 days of treadmill exercise can increase skeletal muscle mass and intramuscular concentrations of brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in experimental autoimmune encephalomyelitis (EAE). **METHODS**: Forty female Lewis rats were randomly assigned to either EAE sedentary (EAE^{Sed}), EAE exercise (EAE^{Ex}), Control sedentary (Con^{Sed}) and Control exercise (Con^{Ex}). Exercising animals completed a 10 day forced exercising training program. Hind limb skeletal muscles were excised and weighed with soleus muscle used for BDNF and NGF quantification. Statistical analysis was done using a one-way analysis of variance. **RESULTS**: Disability was more pronounced in the EAE^{Ex} group than in the EAE^{Sed} group. Exercising animals (EAE^{Ex} and Con^{Ex}) had significantly greater bilateral EDL, plantaris and gastrocnemius muscle mass compared to their sedentary animals ($p=0.01$). The EAE^{Ex} group had significantly higher NGF concentrations (1.98 ± 0.3 pg/mg) compared to Con^{Ex} (0.96 ± 0.07, $p=0.003$) and Con^{Sed} (1.2 ± 0.2; $p=0.04$) groups. The main effect of exercise represented a significantly lower BDNF concentrations in the soleus of exercising animals compared to sedentary animals ($p=0.03$). **CONCLUSIONS**: Our study provides preliminary evidence that exercise increases skeletal muscle mass despite the early onset of disability in EAE animals.

Keywords: Multiple Sclerosis, muscle adaptation, running, neuroprotection, neurotrophins

INTRODUCTION

Neurotrophins, a family of proteins, are recognized for their role in the development and maintenance of the central nervous system (CNS) in multiple sclerosis (MS). They have been shown to promote neuronal plasticity, neuronal regenerative capabilities and protection from degrading effects of inflammatory cytokines. A seminal study by Le Page (1996) concluded that 10 days of high-intensity exercise delays the onset and severity of experimental autoimmune encephalomyelitis (EAE) in inoculated rats (Le Page *et al.* 1996). Though mechanisms were not presented in that manuscripts, literature directs us to hypothesize that an increase in brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) play a significant role. Previous work by our group replicated Le Page's protocol and found that NGF concentrations were elevated in the CNS of exercising mice. In the periphery, muscle derived neurotrophic factors have been reported to preserve motor unit integrity, thus, protecting the peripheral nervous system from MS related attacks (Sakuma and Yamaguchi 2011). Recently, Wens and colleagues (2015) found on two difference occasions that exercise can delay the occurrence of hindquarter disease symptoms (Wens *et al.* 2015, Wens *et al.* 2015). The purpose of this pilot study was to investigate whether a forced high-intensity treadmill exercise program, implemented previous by Le Page (Le Page *et al.* 1996), modulates protein levels of BDNF and NGF in soleus muscle of EAE rats. We hypothesized that 10-day progressive treadmill training would preserve skeletal muscle mass and increase skeletal muscle BDNF and NGF concentrations in exercising EAE animals.

MATERIALS AND METHODS

ANIMALS

Forty, 8 week old (150-175 g), female Lewis rats were used for this study and maintained traditional 12:12 light:dark cycle with chow and water provided *ad libitum*. Animals were randomly assigned to one of four groups: EAE-Exercise (EAE^{Ex}), EAE-Sedentary (EAE^{Sed}), Control-Exercise (Con^{Ex}) and Control-Sedentary (Con^{Sed}). Chronic-relapsing EAE was induced as previously described (Patel and White 2013). Briefly, 50 ug of purified myelin oligodendrocyte glycoprotein (MOG_{33-55}) was dissolved in 50 uL saline and homogenized (1:1 v/v) with Freund's Complete Adjuvant (with mycobacteria) and injected intradermally at the tail base (Patel and White 2013). For the EAE^{Ex} group, inoculation occurred the day before the start of the exercise intervention. Body weight and clinical scores (Scale 0-5; 0=normal, 5=death) were recorded daily as previously described (Le Page *et al.* 1996). This project was part of a larger study investigating the impact of exercise on neuroprotection in the EAE model of MS (Patel and White 2013). The use of animals in this study was approved by the University of Florida Institutional Animal Care and Use Committee.

PROGRESSIVE TREADMILL EXERCISE PROTOCOL

Training and habituation to training was completed as previously described (Patel and White 2013). Beginning on Day 1, animals completed 10 consecutive days of treadmill running which coincided with the induction phase of EAE. Exercise training bouts began with 30 minutes of running at 15 m/min then increased to 30 m/min for the remaining period of the exercise session. Rodents ran for 60 minutes on days 1 and 2; 90 minutes on days 3-10, following the protocol previously published by Le Page (Le Page *et al.* 1996), with low grade electrical impulse provided as encouragement.

ANESTHESIA, EUTHANASIA and TISSUE COLLECTION

Approximately 24 hours after the final exercise session, rats were euthanized under isoflurane anesthesia. Hind limb muscles were removed intact and weighted. The soleus muscle was used to assess BDNF and NGF concentrations based on previous reports of muscle activity in treadmill running (Gómez-Pinilla *et al.* 2001, Jiménez-Maldonado *et al.* 2016). The soleus muscle was homogenized then centrifuged at 100xg for 5 mins at 4°C. Supernatant was removed and centrifuged again for 5 minutes to remove contaminants, and frozen at -80°C.

MEASUREMENT OF NEUROTROPHINS IN MUSCLE

BDNF and NGF concentrations were analyzed using the BDNF Emax ImmunoAssay System and NGF Emax ImmunoAssay System, (Promega, Madison, WI), respectively, according to manufacturer's instructions. The sensitivity of the BDNF Emax assay is 15.6 pg/ml, while for NGF Emax assay the sensitivity is 7.8 pg/ml.

STATISTICAL ANALYSIS

A 2x2 analysis of variance (ANOVA) was used to measure the effect of exercise and disease state on muscle mass and neurotrophin concentrations. Post hoc analysis was conducted using Tukey's post hoc test. Data are presented as mean ± standard error (SE). A value of $p<0.05$ was considered statistically significant.

RESULTS

Thirty-nine of the 40 animals completed the experimental protocol. One animal in the Con^{Sed} group died while under anesthesia during the administration of the sham injection and was excluded. Disability was more pronounced in the EAE^{Ex} group than in the EAE^{Sed} group (Figure 1a). Significant differences in disability were observed between EAE^{Ex} and EAE^{Sed} groups on days 5-9 ($p<0.001$). No differences were observed between groups at the conclusion of the study (day 10). Significant differences in body weight were observed between groups on day 4 [$F(3,35) = 3.89$; $p=0.17$], day 6 [$F(3, 35) = 4.206$; $p=0.12$], day 7 [$F(3,35)=7.167$; $p=0.001$], and day 8 [$F(3,35)=3.073$; $p=0.04$]. Post hoc analysis revealed that the Con^{Ex} group had significantly greater body weight compared to the Con^{Sed} group on Days 6 and 7 ($p=0.01$ and $p=0.002$, respectively). The EAE^{Ex} group had significantly greater body weight compared to the EAE^{Sed} group ($p=0.041$) and Con^{Sed} ($p=0.049$) on days 4 and only Con^{Sed} ($p=0.043$) on day 6. (Figure 1b).

TREADMILL EXERCISE INCREASES MUSCLE MASS IN EAE ANIMALS

Significantly greater plantaris mass [$F(3,35) = 5.38$; $p=0.003$] and gastrocnemius mass [$F(3,35) = 4.63$; $p=0.008$] were observed. Plantaris mass was significantly greater in the Con^{Ex} group compared to the Con^{Sed} ($p=0.03$) and the EAE^{Sed} ($p=0.02$) groups. Likewise, EAE^{Ex} had significantly greater plantaris mass compared to EAE^{Sed} ($p=0.03$). Gastrocnemius mass was significantly greater in the Con^{Ex} ($p=0.01$) and EAE^{Ex} ($p=0.04$) compared to the EAE^{Sed} group. No differences were seen in EDL or Soleus mass between groups.

When comparing conditions, exercising animals (EAE^{Ex} and Con^{Ex}) had significantly greater EDL ($p=0.04$), gastrocnemius ($p=0.001$), and plantaris ($p=0.0003$) muscle mass compared to their sedentary counterparts (EAE^{Sed} and Con^{Sed}). No difference was seen in soleus mass between EAE and Control animals.

FORCED EXERCISE DECREASES BDNF IN EAE

No significant differences in soleus BDNF concentrations were observed between the 4 groups The main effect of exercise represented a significantly lower BDNF concentration in the soleus of exercising animals compared to sedentary animals (Ex: 8.5 ± 0.5 pg/ml; Sed: 10.3 ± 1.1; p=0.03; Figure 2).

NGF CONCENTRATION GREATER WITH EXERCISE IN EAE

Significant differences were found in soleus NGF concentrations [$F(3,32)=5.38$; $p=0.004$] with greater concentrations in the EAE^{Ex} group compared to the Con^{Ex} ($p=0.002$) and the Con^{Sed} group ($p=0.04$; Figure 3). By comparison, the main effect of the EAE showed that EAE animals (1.73 ± 0.2 pg/ml) had significantly greater soleus NGF concentrations compared to control animals (1.1 ± 0.1; $p=0.001$).

DISCUSSION

The results of our study suggest that 10 days of progressive treadmill exercise can increase ambulatory muscle mass in EAE animals despite the earlier onset of clinical symptoms. However, contrary to our hypothesis, we did not see any significant impact of exercise on soleus BDNF or NGF concentrations in the EAE groups. The benefits of increased muscle mass and soleus NGF are limited by the mild onset of disability in the EAE^{Ex} animals.

The results of our study contradict the general understanding of the benefits of exercise in delaying clinical onset in EAE animals. In a previous study, onset of clinical disability in EAE

mice was delayed with voluntary wheel running (Pryor *et al.* 2014). While clinical onset of disability in sedentary animals was similar with our results (day 10) (Pryor *et al.* 2014), forced treadmill running might evoke clinical symptoms earlier in EAE, particularly during the induction phase of EAE. . This leads us to believe that the protocol selected may be too intense for this model. Future research should consider the use of voluntary wheel running or less intensive treadmill exercise.

The effects of exercise on BDNF have been thoroughly investigated in circulation and in CNS of MS and EAE, but little is known regarding exercise effect in muscle. Contrary to our hypothesis, we did not observe an exercise effect on soleus BDNF concentrations in EAE^{Ex} rats. Multiple studies cited in this paper suggest that exercise can increase intramuscular BDNF concentrations, leading us to hypothesize that our protocol would do the same. Studies in other animal models have reported BDNF to be substantially influenced by exercise with one report suggesting a 138% increase compared to baseline immediately following exercise after 5 days of training (Gómez-Pinilla *et al.* 2001).

We have demonstrated, similar to previous findings, forced high intensity exercise may diminish protein concentration of BDNF in soleus (Jiménez-Maldonado *et al.* 2016). One hypothesis for decreased BDNF concentrations in activated muscle is the possibility that neuromuscular activity might increase retrograde transport of BDNF from the muscle (Gómez-Pinilla *et al.* 2001) or possibly translocate into circulation . Secondly, stress response through elevated cortisol has been reported to impact BDNF concentrations (Jacobsen and Mork 2006). Although stress hormones were not measured in this study, previous studies have reported stress hormone upregulation with exercise (Jacobsen and Mork 2006).

EAE^{Ex} group had significantly higher soleus NGF concentrations compared to the two non-EAE control groups. However, the lack of significant difference between the EAE^{Ex} and EAE^{Sed} group limit the potential benefits of exercise in EAE animals, contrary to our hypothesis. Thus, elevations in NGF might be due to a neuroprotective response in the EAE group not seen in the control animals. The source of the increased NGF in the EAE^{Ex} group needs to be further investigated. NGF has been reported to be released by a number of cells, including fibroblasts, epithelial cells and smooth muscle cells. NGF has also been reported to be upregulated at the site of neuronal injury by glial cells. Future research to determine the major source of NGF in skeletal muscle is warranted.

In muscle, NGF has been associated with improving regenerative capacity (Qu-Petersen *et al.* 2002). NGF is clinically relevant as it may promote remyelination by stimulating the expression of 2', 3' cyclic nucleotide 3'-phosphodiesterase (CNPase), an enzyme associated with myelin found in muscle derived stem cells (Qu-Petersen *et al.* 2002). Increasing CNPase may promote the production of new myelin which may prevent further neural degradation. However, is it unknown whether muscle derived NGF may translocate into the CNS where it has been reported to delay the onset of EAE.

LIMITATIONS

This study has limitations that should be taken into consideration when designing future studies. The analysis of neurotrophic proteins in the soleus may be a limiting factor because there was no difference observed in soleus mass between the 4 groups. The focused analysis in the soleus limits our understanding of protein concentrations in other larger muscles. We are limited by the fact that we did not measure BDNF mRNA which has been reported in the literature to be

upregulated with exercise. Finally, the protocol chosen provides an inherent limitation. Forced exercise may evoke a stress response in animals that has been associated with suppressed BDNF concentrations (Jacobsen and Mork 2006). Future research should consider the use of voluntary protocols.

CONCLUSIONS

Taken together, data presented in this study indicates that forced exercise may help preserve muscle mass in EAE rats, at the expense of whole body disability. Future research should focus on the transport of BDNF and NGF from muscle to the CNS of EAE animals to determine the true neurprotective capabilities of these muscle derived proteins.

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FIGURE CAPTIONS

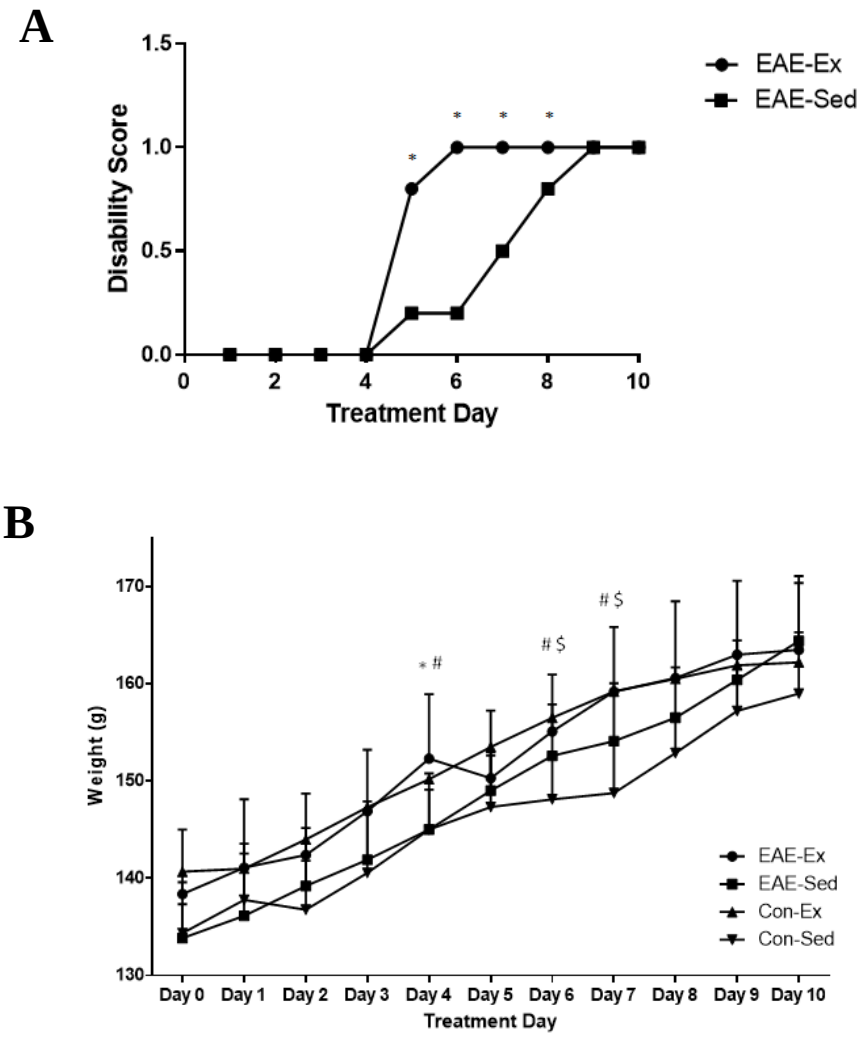

FIGURE 1. Clinical disability in EAE animals. (A) Clinical disability scores for EAE-Ex and 5 EAE-Sed groups. * indicates significant differences between group ($p<0.001$). (B) Body weight 6 associated with protocol day. * indicates significant difference between EAE-Ex and EAE-Sed 7 ($p=0.041$); # significant difference between EAE-Ex and Con-Sed ($p<0.05$); $ significant 8 difference between Con-Ex and Con-Sed ($p<0.01$)

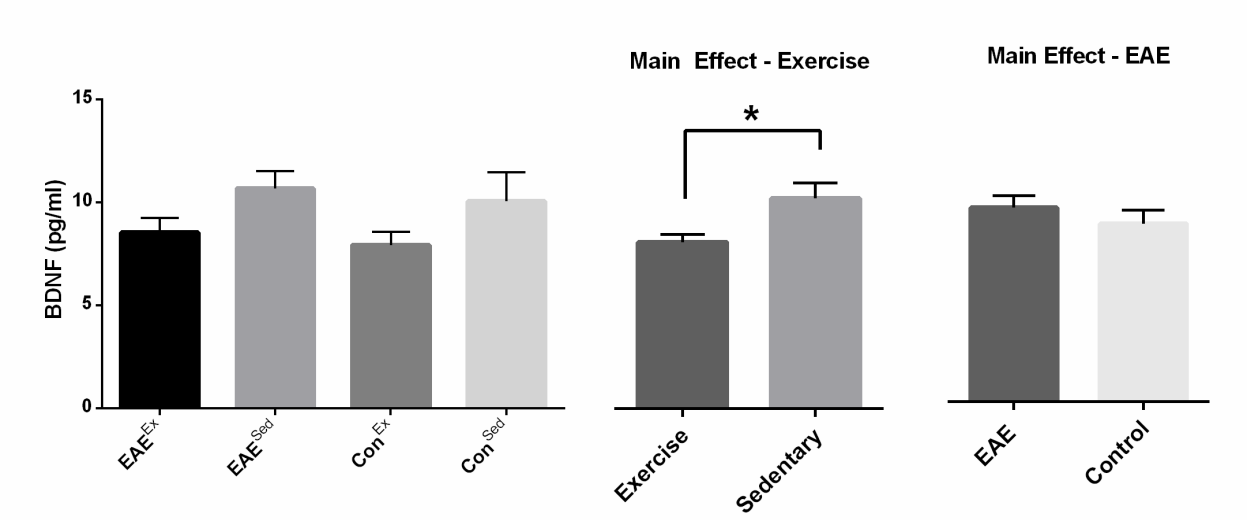

FIGURE 2. Intramuscular BDNF concentrations. No significant differences are seen between the four groups following the completion of the 10-day protocol. A significant reduction in BDNF was seen (p=0.03) due to the main effect of exercise. Finally, EAE animals and control animals, when grouped for the main effect of the treatment, showed similar concentrations of BDNF. Data expressed as pg/mg wet soleus mass. Data presented as mean ± SE.

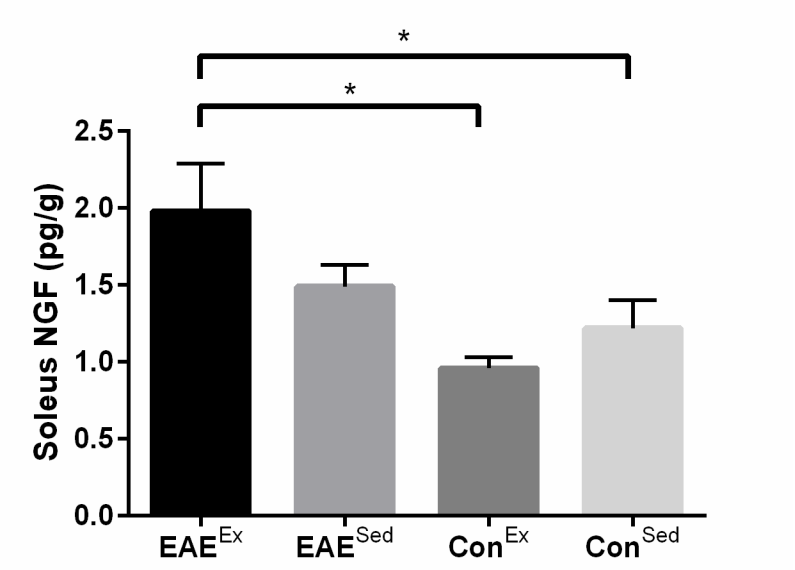

FIGURE 3. Nerve Growth Factor concentrations in the soleus after the 10-day experimental 12 **period.** * Significant differences between groups. Data expressed as pg/mg wet soleus mass. 13 Data presented as mean ± SE.