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Research report

Voluntary exercise increases neurotrophin-3 and its receptor TrkC in the spinal cord

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Abstract

We have evaluated changes in the expression of neurotrophin-3 (NT-3) and its tyrosine kinase C (TrkC) receptor in the neuromuscular system as a result of voluntary physical activity. We assessed changes in the mRNAs and proteins for NT-3 and TrkC in the lumbar spinal cord and associated soleus muscle following 3 and 7 days of voluntary wheel running. We used quantitative Taqman RT-PCR to measure mRNA and ELISA to assess protein levels. NT-3 mRNA and protein levels increased in the spinal cord to reach statistical significance after 7 days of exercise compared to sedentary control rats. Immunohistochemical analyses localized the elevated NT-3 to the substantia gelatinosa (SG) and nucleus of the dorsal horn. TrkC mRNA levels were significantly elevated in the spinal cord after 3 and 7 days of running. In the soleus muscle, NT-3 mRNA levels and its receptor TrkC were elevated after 3 days, while NT-3 protein levels remained unaffected. The results demonstrate that voluntary exercise has a differential effect on NT-3 as well as its receptor TrkC in the neural and muscular components of the neuromuscular system, and emphasize the role of voluntary activity on the spinal cord and muscle.

Theme: Development and regeneration

Topic: Neurotrophic factors: expression and regulation

Keywords: Neuromuscular activity; Neurotrophin; Skeletal muscle; Spinal cord; NT-3; TrkC

1. Introduction

Physical activity is considered beneficial for overall neural function. In particular, a large volume of evidence has demonstrated the role of locomotor training in alleviating motor and sensory impairments in the injured spinal cord [5]. The mechanisms by which activity translates into structural and functional changes in the CNS, however, are poorly understood. Recent studies showing that neurotrophins are regulated in an activity-dependent fashion [23] indicate that select neurotrophins may play an important role in these exercise-induced effects on the CNS. For example, an increasing body of evidence shows that neuromuscular activity induces brain-derived neurotrophic factor (BDNF) in the brain [20] and spinal cord [11]. It is likely that exercise orchestrates the action of different trophic factors [20] with resulting effects on neural plasticity and regeneration. Therefore, it is critical to determine how exercise affects the expression of select neurotrophins that have recognized roles on CNS plasticity.

NT-3 plays a central role in mediating CNS plasticity and regeneration in the spinal cord and muscle. The spinal cord expresses NT-3 and its trkC receptor [4,19,27]. NT-3 plays an important role in the survival and function of sensory neurons, such that mice lacking the NT-3 gene show a severe loss of sensory neurons and concomitant gait abnormalities [7,8,31]. Application of NT-3 into the spinal cord of rodents can compensate for induced damage as shown by increased axonal regeneration, reduced atrophy, and increased functional recovery [2,12,21,24,29,30]. The action of NT-3 is also important for the regulation of synaptic transmission, such that NT-3 added to muscle culture potentiates synaptic transmission and promotes the

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maturation of the neuromuscular junction [16,33]. Membrane depolarization rapidly elevates NT-3 mRNA and secretion in myocytes, and these changes are accompanied by increased synaptic transmission between myocytes and motoneurons [35].

These results suggest that NT-3 may play an important role in plasticity of the neuromuscular system, and that physiological means to induce endogenous NT-3 production could have a significant impact on neuromuscular function. In the present study, we determined that voluntary activity could modulate the expression of NT-3 and its signal transduction receptor in the neuromuscular system. We have focused these studies on the lumbar region of the spinal cord based on its well-defined connectivity with the hindlimb musculature. The soleus muscle was chosen because of its high level of recruitment during postural and locomotor tasks [26].

2. Materials and methods

2.1. Animals and exercise

Male Sprague–Dawley rats, 3 months of age (Charles River, CA, USA) were used in this study. The rats were housed singly in standard polyethylene cages, provided food and water ad libitum, and kept under a 12-h light:12-h dark cycle starting at 07:00 h. After 1 week of acclimation, the animals were assigned randomly to a control ($n=5$) or exercise ($n=5$, each time point) group. Exercising animals were placed in standard cages equipped with running wheels (diameter, 31.8 cm, width, 10 cm; Nalgene Nunc International, NY, USA) with adjustable resistance. On the first day of exercise the animals were exposed to freely moving running wheels (no load) as an adaptation period. The load was increased to 100 g for all subsequent days to augment the number of motor units recruited during locomotion [14]. The number of wheel revolutions was recorded automatically by computer using VitalViewer Data Acquisition System software (Mini Mitter Company, Sunriver, OR, USA). Each revolution was counted irrespective of the direction of wheel rotation. Sedentary animals were left undisturbed in their home cages. Rats were killed by decapitation at 07:00 h after 3 and 7 days of exercise. The lumbar spinal cord enlargement and the soleus muscle were identified, rapidly dissected, frozen on dry ice and stored at −70 °C until processed. The lumbar region of the spinal cord was selected for this study because the motoneuron pools that innervate the hindlimb musculature are located in this region. The soleus muscle was selected because of its high level of recruitment during running [26]. All experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California at Los Angeles, Animal Research Committee.

2.2. Isolation of total RNA and real-time quantitative RT-PCR

Total RNA from tissue was isolated using TRIzol reagent (Life Technologies) as per the manufacturer's protocol. RNA samples were further purified by DNase digestion and extraction with phenol and chloroform. The mRNA for NT-3 was measured by real-time quantitative RT-PCR using PE Applied Biosystems prism model 7700 sequence detection instrument. The sequences of forward and reverse primers as designed by Primer Express (PE ABI) were: 5′-TGTGACAGTGAGAGCCTGTGG-3′ and 5′-TGTAACCTGGTGTCCCCGAA-3′, respectively, for NT-3; 5′-TTTGCCCAGCCAAGTGTAGTT-3′ and 5′-GCCCACATAGTCCAGCCAGA-3, respectively, for TrkC. The TaqMan™ fluorogenic probes used were 5′-TGACCGACAAGTCCTCAGCCATTGAC-3′ and 5′-TGGCGGATTTTCTTGCTGGGAAGC-3′, for NT-3 and TrkC, respectively. The TrkC Taqman RT PCR probe recognizes the full length TrkC receptor as well as the TrkC splicing form. During PCR amplification, 5′ nucleolytic activity of *Taq* polymerase cleaves the probe separating the 5′ reporter fluorescent dye from the 3′ quencher dye. Threshold cycle (C_t) which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increases above a threshold level. The NT-3 and TrkC mRNA levels were corrected for GADPH mRNA, which was measured using the probes previously described using Taqman™ [11].

2.3. Protein immunoassay

Lumbar spinal cord and soleus muscle samples were homogenized in 3 volumes of homogenization buffer (50 mM Tris–HCl, pH 8.0, 600 mM NaCl, 1% BSA, 0.1 mM PMSF, 10 μg/ml aprotinin, 0.1 mM benzethonium chloride, 1 mM benzamidine HCl, 4% Triton X-100). Homogenates were centrifuged and supernatants were collected. Protein concentrations were estimated with the MicroBCA procedure (Pierce, Rockford, IL) using bovine serum albumin (BSA) as standard. NT-3 protein was quantified using an enzyme linked immunosorbent assay (ELISA; NT-3 Emax ImmunoAssay System kit, Promega, Madison, WI). Briefly, Nunc MaxiSorp 96-well plates were coated with 0.1 ml of a polyclonal antibody against NT-3 in a buffer containing 0.025 M sodium bicarbonate and 0.025 M sodium carbonate (pH 9.7) for 16 h at 4 °C. After washing in TBS-T (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20), the wells were incubated with 0.2 ml of a blocking buffer at room temperature for 1 h and then washed in TBS-T again. Spinal cord and muscle samples, six serial dilutions of a NT-3 standard (300 pg/ml), and a blank (no NT-3) were added in duplicate into separate wells. Plates were incubated for 2 h at room temperature and washed five times in TBS-T. A mono-

clonal antibody against NT-3 (1:4000 dilution) was added into each well and the plates were incubated for 6 h at room temperature. After five washes in TBS-T, 0.1 ml of a secondary anti IgG antibody with a horseradish peroxidase conjugate was added to each well and the plates were incubated for 16 h at 4 °C. Wells were washed five times with TBS-T. A hydrogen peroxidase solution with a peroxidase substrate was added and incubated for 10 min at room temperature. Reactions were stopped with 1 M phosphoric acid and absorbance at 450 nm was measured using an automated microplate reader. Standard curves were plotted for each plate. Duplicates were averaged and values were corrected for total amount of protein in the sample.

2.4. Immunohistochemistry

The immunohistochemistry for NT-3 was performed as previously described [10]. A 1:1000 dilution was used for the goat polyclonal anti-NT-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For the analysis and photography we used an Olympus BX 51 microscope.

2.5. Statistical analyses

The mean values for the mRNA and protein levels were computed for the control and experimental groups. Overall differences were determined using a two-way ANOVA and individual group differences were detected with Fisher test using Statview software (Abacus Concepts, CA). The mean values for NT-3 mRNA and TrkC mRNA were expressed as a percent of control. The mean values for NT-3 protein measurements were expressed as pg of NT-3 per mg of total protein.

3. Results

Levels of NT-3 mRNA and protein and TrkC mRNA were measured in the lumbar enlargement of the spinal cord and the associated soleus muscle and compared in exercised and sedentary control rats.

3.1. NT-3 expression in the lumbar spinal cord

The levels of NT-3 mRNA in rodents that exercised for 3 days were 145% ($P>0.05$) of sedentary control values. NT-3 mRNA values increased to 187% ($P<0.05$) of control after 7 days of exercise (Fig. 1A). NT-3 protein levels reached a value of 117% of control ($P>0.05$) after 3 days of running and 144% ($P<0.05$) of control after 7 days of running (Fig. 1B).

We performed NT-3 immunohistochemistry in another set of animals to determine the localization of changes in NT-3 protein (quantified using ELISA, Fig. 1B) in the lumbar spinal cord. In sedentary animals, NT-3 immunoreactivity was light in ventral horn motoneurons and their axonal processes throughout the white matter (Fig. 2A,E). Fiber elements in the SG nucleus of the dorsal horn showed moderate to intense NT-3 staining (Fig. 2A). Small astrocyte-like cells in the white matter also were NT-3 positive. In exercised animals, NT-3 immunoreactivity appeared greater in the SG nucleus of the dorsal horn relative to control rats (Fig. 2C,D).

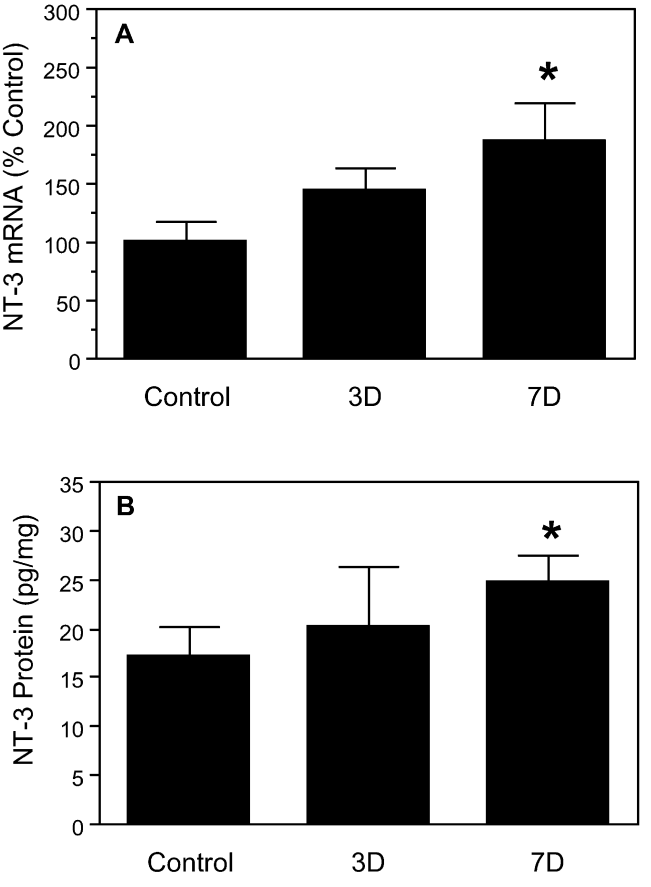

Fig. 1. NT-3 mRNA (A) and protein (B) in the lumbar spinal cord region after 3 or 7 consecutive days of voluntary wheel running exercise. NT-3 mRNA (A) and protein (B) increased to reach statistical significance after 7 days of exercise relative to sedentary control rats. *$P<0.05$ (ANOVA, Fisher test).

3.2. NT-3 expression in the soleus muscle

The levels of NT-3 mRNA in the soleus were 187% ($P<0.01$) and 139% ($P>0.05$) of controls, after 3 and 7 days of exercise (Fig. 3A). NT-3 protein levels in the soleus muscle were relatively unaffected by the exercise protocol (Fig. 3B). It can be noted that after 3 days of exercise, the levels of the protein were slightly elevated (~25%, $P>0.05$; Fig. 3B).

3.3. TrKC expression

Levels of TrkC mRNA in the lumbar spinal cord were

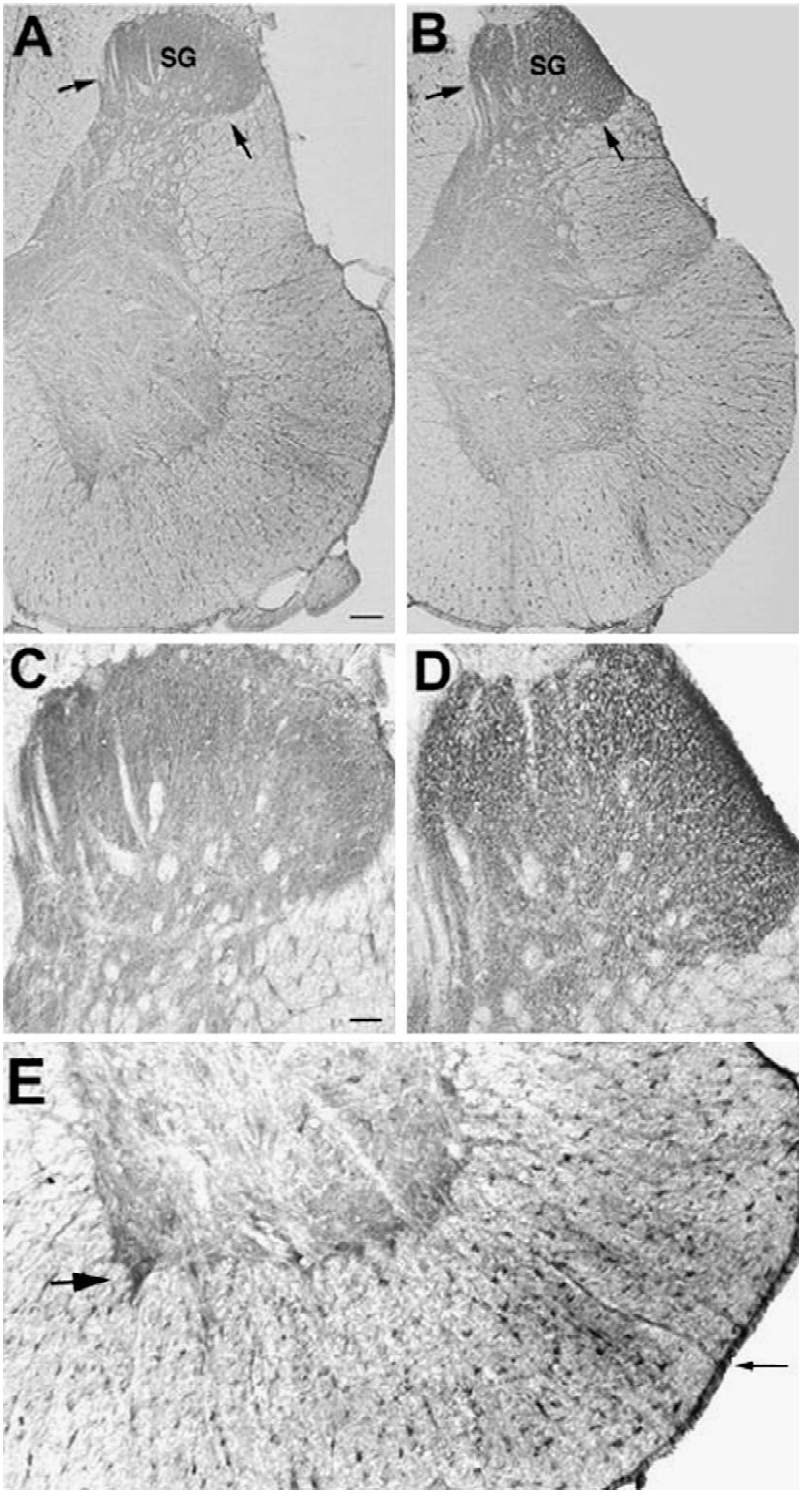

Fig. 2. Representative photomicrographs showing NT-3 immunoreactivity in the lumbar spinal cord region of sedentary rats and rats exercised for 7 days. (A) Normal NT-3 immunostaining was observed in neuritic elements of the SG in the dorsal horn, and motoneuron cell bodies in the ventral horn and their axonal processes through the white matter (shown at E, thin arrow). (B) Exercised rats showed a qualitative increase in NT-3 immunostaining that was mostly evident in the SG. High magnification photographs of the SG are shown for sedentary (C) and exercised rats (D). (E) High magnification photograph of the ventral aspect of the cord shows NT-3 immunostaining in motoneurons (thick arrow), and glial-like cells along axonal processes crossing the white matter (thin arrow). Scale bar: 100 μm (shown at A, for A and B), 20 μm (shown at C, for C, D, E).

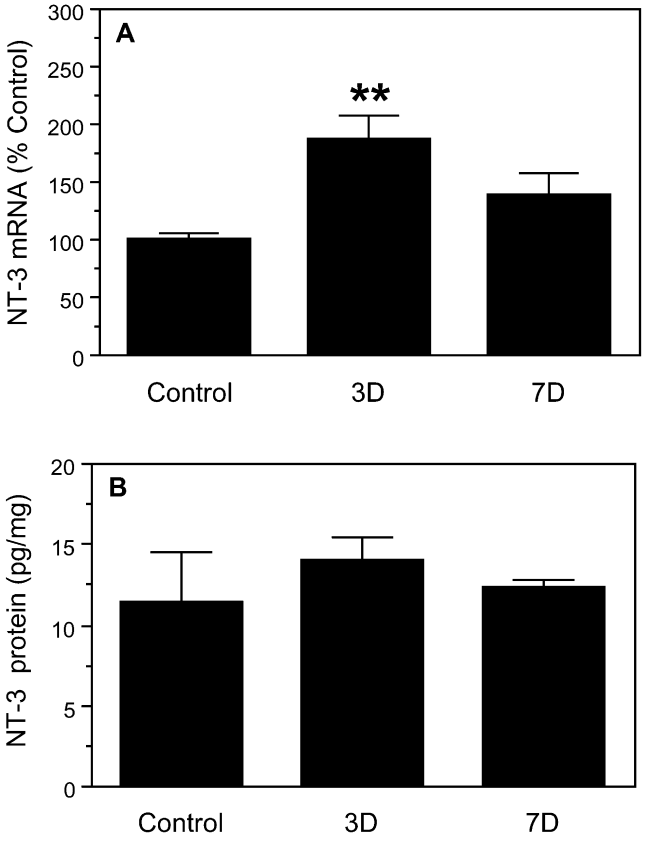

Fig. 3. NT-3 mRNA and protein in the soleus muscle after 3 or 7 consecutive days of voluntary running exercise. There was an increase in NT-3 mRNA (A) after 3 days of voluntary wheel running. NT-3 protein (B) levels remained unchanged. $^{**}P<0.01$ (ANOVA, Fisher test).

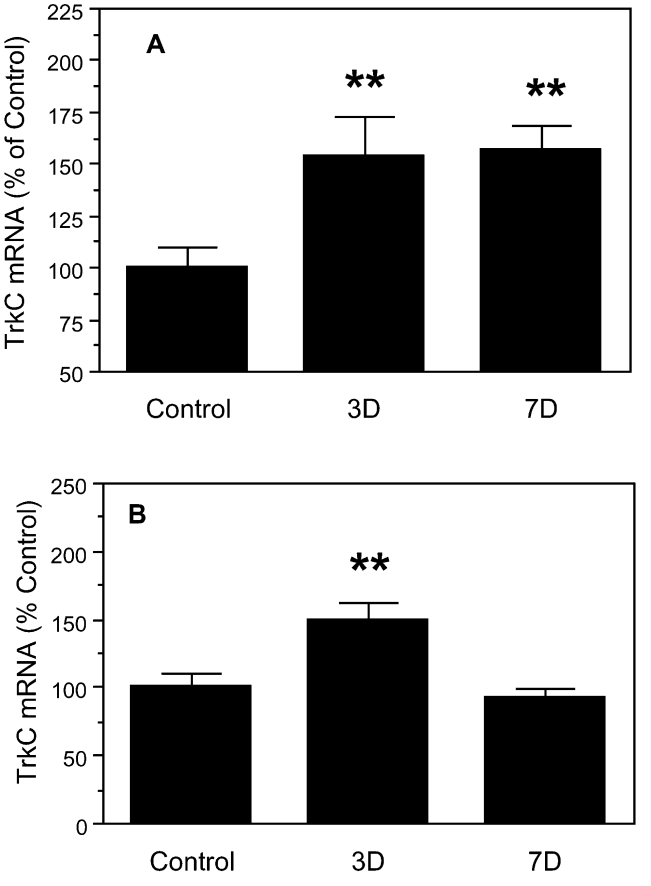

Fig. 4. TrkC mRNA in the lumbar spinal cord region (A) and soleus muscle (B) after 3 or 7 consecutive days voluntary wheel running exercise. (A) TrkC mRNA levels were significantly increased in the lumbar spinal cord after 3 and 7 days of exercise compared to sedentary control rats. (B) TrkC mRNA levels were significantly increased in the soleus muscle after 3 days of exercise. $^{**}P<0.01$ (ANOVA, Fisher test).

elevated ($P<0.01$) after both 3 (154%) and 7 days (157%) of running compared to sedentary control rats (Fig. 4A). TrkC mRNA levels in the soleus muscle were elevated (150%, $P<0.01$; Fig. 4B) after 3 days of running.

4. Discussion

Our results show that neuromuscular activity regulates the expression of NT-3 and its signal transduction receptor TrkC in the neuromuscular system of adult rats. Under our experimental conditions, voluntary exercise increased the mRNA and protein for NT-3 in the spinal cord and the soleus muscle. Exercise also increased the trkC mRNA levels in the spinal cord and the muscle. It is significant that changes in NT-3 mRNA in the spinal cord were accompanied by changes in NT-3 protein suggesting that the spinal cord produces its own NT-3 in response to exercise. Previous studies have demonstrated the beneficial value of NT-3 application for the survival and function of spinal cord cells. In the present study we report that running exercise provides a means of up-regulating this therapeutic neurotrophic factor via the pharmacology intrinsic to the spinal cord.

4.1. NT-3 mRNA and protein pattern of modulation

Results derived from NT-3 mRNA measurements demonstrate that NT-3 can be locally produced in the spinal cord in response to exercise. Our immunohistochemical analyses, supported by protein quantification using ELISA, showed that NT-3 was phenotypically expressed in the SG, and glial-like cells surrounding NT-3 immunopositive motoneuron axons (Fig. 2). The strongest NT-3 immunostaining, however, was observed in the SG that is in general agreement with previous reports showing the critical role of NT-3 for sensory function [15]. SG is a relay station for sensory neurons that have cell bodies located in the dorsal root ganglion. Adequate levels of NT-3 seem important for maintaining proprioceptive function, and for sensory perception [7,15]. The increase in NT-3 in the spinal cord as a result of exercise suggests that exercise could be a means of treatment of peripheral sensory dysfunction.

It is interesting that motoneuron axons projecting to muscle showed NT-3 immunostaining, suggesting that NT-

3 may be involved in spinal cord-skeletal muscle interactions. It is possible, that some of the NT-3 being produced in the periphery can support the spinal cord via retrograde transport. This possibility is in line with our results showing no exercise-related changes in NT-3 protein levels in the soleus, in spite of increased mRNA. It has been suggested that NT-3 can work as an activity-dependent retrograde signal during development of the neuromuscular connectivity [22]. The presence of NT-3 in glial-like cells in the white matter is in agreement with in situ hybridization studies [27] and with immunohistochemical studies [3]. Thus, glial derived NT-3 might provide trophic support to local neuronal elements via a paracrine mechanism, as it is known that neurotrophins can act via autocrine or paracrine mechanisms [27,28].

4.2. Consequences of NT-3 changes for muscle physiology

Exercise increased the mRNA for NT-3 and TrkC in the soleus muscle. The results of the ELISA showed that the soleus muscle contains NT-3 protein, but this level was approximately one-third less than that detected in the lumbar spinal cord. The interpretation of the effects of voluntary exercise on NT-3 or its receptor in skeletal muscle are complex particularly considering the lack of knowledge about the role of neurotrophins in skeletal muscle. NT-3 is expressed in muscle spindles of adult rodents [3], and considerable evidence exists demonstrating the effects of NT-3 on the electrophysiological properties of group Ia fibres [18]. Therefore, it appears that the main role of NT-3 is to support muscle spindle afferents conveying sensory function. Indeed, separate studies have shown that exercise increases the levels of NT-3 mRNA in muscle spindle afferent cell bodies in the dorsal root ganglia [32]. We also have previously reported important changes in brain-derived neurotrophic factor (BDNF) mRNA and protein in the soleus muscle as a result of voluntary exercise [11]. Therefore, it appears that voluntary exercise may orchestrate the action of various neurotrophins.

4.3. Consequences of NT-3 up-regulation on repair of injured spinal cord circuits

The changes in NT-3 and TrkC observed in our study may have important consequences for synaptic function or reorganization in the neuromuscular system. It is known that NT-3 is important for maturation and facilitation of synaptic transmission during development of the neuromuscular junction [16,33,35]. NT-3 can modulate the connectivity between sensory afferents and motoneurons during development and adult life [18].

Exogenous NT-3 can reduce atrophy of damaged neurons, and promote axonal regeneration and functional recovery [2,21,24,29,36]. In these procedures, NT-3 has been delivered into the spinal cord using osmotic minipumps, gel foam, or genetically modified fibroblasts. These procedures, however, have the intrinsic limitations associated with adding exogenous NT-3 to the CNS. In addition, proper functioning of added NT-3 requires the existence of active TrkC receptors and co-factors. Voluntary running has the advantage in providing NT-3 by physiological means using the pharmacology that is intrinsic to the spinal cord. It also seems likely that specific patterns or doses of activity can be therapeutically used to modulate specific changes in NT-3 in the normal and injured spinal cord [9].

Interestingly, there is a large volume of evidence demonstrating the beneficial effects of exercise on functional recovery after spinal cord injury [1,6,13,17,34]. For example, treadmill training has been shown to improve the locomotor capability in animals with a complete low-thoracic spinal cord transection [17,25]. We have previously shown that exercise also modulates the expression of BDNF in the spinal cord and soleus muscle [10]. The present results demonstrate that exercise also can increase the expression of NT-3 in the spinal cord. Thus, neurotrophins appear to be logical candidates for mediating the beneficial effects of neuromuscular activity on functional recovery after spinal cord injury.

5. Conclusion

In conclusion, this study demonstrates that physical activity can impact the expression of NT-3 and its signal transduction receptor TrkC in the lumbar spinal cord and the associated skeletal muscles. Since NT-3 has been shown to play a critical role in the development and repair of neural circuits, exercise-induced NT-3 up-regulation may be an important strategy to facilitate functional recovery after spinal cord injury.

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