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# Voluntary Exercise Induces a BDNF-Mediated Mechanism That Promotes Neuroplasticity

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**Gómez-Pinilla, Fernando, Zhe Ying, Roland R. Roy, Raffaella Molteni, and V. Reggie Edgerton.** Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. *J Neurophysiol* 88: 2187–2195, 2002; 10.1152/jn.00152.2002. We have investigated potential mechanisms by which exercise can promote changes in neuronal plasticity via modulation of neurotrophins. Rats were exposed to voluntary wheel running for 3 or 7 days, and their lumbar spinal cord and soleus muscle were assessed for changes in brain-derived neurotrophic factor (BDNF), its signal transduction receptor (trkB), and downstream effectors for the action of BDNF on synaptic plasticity. Exercise increased the expression of BDNF and its receptor, synapsin I (mRNA and phosphorylated protein), growth-associated protein (GAP-43) mRNA, and cyclic AMP response element-binding (CREB) mRNA in the lumbar spinal cord. Synapsin I, a synaptic mediator for the action of BDNF on neurotransmitter release, increased in proportion to GAP-43 and trkB mRNA levels. CREB mRNA levels increased in proportion to BDNF mRNA levels. In separate experiments, the soleus muscle was paralyzed unilaterally via intramuscular botulinum toxin type A (BTX-A) injection to determine the effects of reducing the neuromechanical output of a single muscle on the neurotrophin response to motor activity. In sedentary BTX-A-treated rats, BDNF and synapsin I mRNAs were reduced below control levels in the spinal cord and soleus muscle. Exercise did not change the BDNF mRNA levels in the spinal cord of BTX-A-treated rats but further reduced the BDNF mRNA levels in the paralyzed soleus relative to the levels in sedentary BTX-A-treated rats. Exercise also restored synapsin I to near control levels in the spinal cord. These results indicate that basal levels of neuromuscular activity are required to maintain normal levels of BDNF in the neuromuscular system and the potential for neuroplasticity.

## INTRODUCTION

The capacity of physical activity to maintain and compensate for deterioration of neural function is becoming increasingly recognized (Kempermann et al. 2000). Physical exercise can preserve cognitive function in elderly populations (Kramer et al. 1999), promote functional recovery after CNS traumatic injury (Jones et al. 1999), and induce neurogenesis in the adult CNS (Kempermann et al. 2000). Physical activity also increases trophic factor production in select regions of the brain (Neeper et al. 1995) and spinal cord (Gomez-Pinilla et al. 2001). How an exercise-induced elevation of select trophic factors can modulate crucial aspects of neural cellular

plasticity, however, remains unknown. Brain-derived neurotrophic factor (BDNF) delivered to the injured spinal cord can promote regenerative growth (Bregman et al. 1997) and can stimulate hindlimb stepping (Jakeman et al. 1998). These observations, together with evidence that BDNF is a powerful modifier of neuronal excitability and synaptic transmission (Causing et al. 1997; Kafitz et al. 1999; Lu and Figurov 1997), suggest that BDNF is a crucial effector of experience-dependent plasticity.

Synapsin I, growth-associated protein 43 (GAP-43), and cyclic AMP response element-binding protein (CREB) may play central roles in the mechanisms by which BDNF affects neuronal and synaptic plasticity. Synapsin I is a well-characterized member of a family of nerve terminal-specific phosphoproteins and is implicated in neurotransmitter release, axonal elongation, and maintenance of synaptic contacts (Brock and O'Callaghan 1987; Wang et al. 1995). BDNF affects the synthesis (Wang et al. 1995) and phosphorylation (Jovanovic et al. 1996) of synapsin I, resulting in elevated neurotransmitter release (Jovanovic et al. 2000). GAP-43 is present in growing axon terminals and has important roles in axonal growth, neurotransmitter release (Oestreicher et al. 1997), and learning and memory (Routtenberg et al. 2000). CREB, one of the best-characterized transcription factors in the brain, is required for various forms of memory including spatial learning (Silva et al. 1998) and appears to play a role in neuronal resistance to insult in conjunction with BDNF (Walton et al. 1999). CREB is an important regulator of gene expression induced by BDNF (Finkbeiner 2000).

The physiological significance of changes in neurotrophin expression is contingent on the availability of appropriate signal transduction receptors. Neurotrophin effects are mediated by a family of specific transmembrane tyrosine kinase receptors (trk), with trkB being the primary signal transduction receptor for BDNF (Barbacid 1994). In the present study, we evaluate the possibility that exercise induces an integrated response of BDNF and its receptor that may result in synaptic modification/adaptation. We have chosen to study the neuromuscular system because of the well-defined location of the motor pools innervating specific hindlimb muscles.

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## METHODS

*Experimental design*

Sprague-Dawley male rats (3 mo old) individually housed in a 12/12 h light/dark vivarium with ad lib access to food and water were assigned randomly to a voluntary exercise group or a sedentary control group. The exercise apparatus included a polyethylene cage (30 × 40 × 20 cm) equipped with a running wheel (Ishihara et al. 1998) (diameter, 31.8 cm; width, 10 cm) with adjustable resistance. The wheel rotates on the shaft whenever the rat moves in either direction, and the number of revolutions is recorded continuously by a Macintosh computer. On the first day of exercise, the rats were exposed to freely moving running wheels (no load) as an adaptation period. The load then was increased to 100 g for all subsequent days. 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 for this study because of its high level of recruitment during running (Roy et al. 1991).

After 3 or 7 days of voluntary running, all rats to be used for biochemical assays ( $n = 5-7$  per group at each time point) were killed around 8 AM, and the entire spinal cord and the soleus muscle were removed. The spinal cord was laid on a cork strip, carefully oriented, and frozen on dry ice. Subsequently, the lumbar enlargement was identified, separated, and used for analysis. We used the entire enlargement to evaluate the effects of exercise on motor and sensory function. The soleus muscle was cleaned of excess fat and connective tissue, wet weighed, pinned on a cork strip and quick frozen in isopentane cooled in liquid nitrogen. All fresh-frozen tissues were stored at  $-70^{\circ}\text{C}$  until processed. Rats to be used for the histological analyses were exercised for 7 days ( $n = 5$  per group). After the last day of running, both exercised and sedentary control rats were anesthetized deeply with pentobarbital sodium (75 mg/kg ip) and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M Sorensen's phosphate buffer and 20% sucrose in PBS. Perfused tissues were stored at  $-70^{\circ}\text{C}$  until sectioned in a cryostat.

*BTX injection*

To evaluate the possibility that muscle activation associated with locomotion is the main stimulus for the induction of neurotrophins, we paralyzed the soleus muscle of the right leg with botulinum toxin type A (BTX-A, Sigma) in a separate group of rats ( $n = 12$ ). The rats were deeply anesthetized with a mixture of ketamine hydrochloride (70 mg/kg body wt) and acepromazine maleate (5 mg/kg body wt) administered intraperitoneally. Under aseptic conditions, a skin incision was made on the lateral aspect of the lower leg. The superficial fascia overlying the lateral gastrocnemius was incised, and the belly of the lateral gastrocnemius was retracted to expose the soleus muscle. Using a Hamilton microsyringe (10  $\mu\text{l}$ ), a single dose of BTX-A (1 ng/kg) was injected at four sites across the midbelly of the muscle, i.e., near the endplate region of the muscle. Care was taken to minimize the amount of leakage from the muscle by injecting the BTX-A slowly and then by using cotton tip applicators to absorb any fluid emanating from the muscle. The incision sites were flushed liberally with a saline solution and then closed in layers, i.e., the fascial layer with 4.0 absorbable gut and the skin with 4.0 Ethicon. The rats were allowed 1 day of recovery in sedentary cages prior to being placed in cages with voluntary running wheels for 7 days.

A series of preliminary experiments was conducted to evaluate a possible effect of mechanical damage related to the intramuscular injection on BDNF mRNA levels. Saline was injected into the right soleus muscle, and the levels of BDNF mRNA were measured in the right and left spinal hemi-cords and soleus muscles. There were no left/right differences in levels of BDNF mRNA in either the spinal cord or the soleus muscles of saline-injected rats. In addition, the

levels of BDNF mRNA in saline-injected rats were similar to those in uninjected rats for each of the tissues examined. Other preliminary experiments were conducted to determine the correct dosage of BTX-A. The BTX-A dosage used in the present study was found to produce a complete silencing of the soleus muscle between 2 and 8 days after injection as determined from the electrical stimulation of the muscle via its nerve in an *in situ* preparation. With the rats anesthetized as described in the preceding text, the muscle did not respond to either a single or multiple pulses ( $\leq 200$  Hz for 300 ms) of stimulation at voltages ranging from 1 to 10 V. In these preliminary experiments, it was determined that some of the surrounding muscles, i.e., particularly the plantaris and lateral gastrocnemius muscles were modestly affected by the BTX-A injections, i.e., in several cases these muscles did not respond to a single pulse but were activated by trains of pulses. For the rats in the present study, we verified a complete block of the soleus muscle in two rats just prior to the removal of the spinal cord and soleus muscles. All animal procedures were approved by the Animal Use Committee at UCLA and followed the American Physiological Society Animal Care Guidelines.

*Taqman RT-PCR*

Total RNA was isolated using the RNA STAT-60 kit (TEL-TEST, Friendswood, TX) as per the manufacturer's protocol. The mRNAs for BDNF, synapsin, GAP-43, and CREB were measured by TaqMan real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer, Applied Biosystems). The technique is based on the ability to directly detect the RT-PCR product with no downstream processing. This is accomplished with the monitoring of the increase in fluorescence of a dye-labeled DNA probe specific for each factor under study plus a probe specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene used as an endogenous control for the assay. Total RNA (100 ng) was converted into cDNA using TaqMan EZ RT-PCR Core reagents (Perkin-Elmer, Branchburg, NJ). The sequences of probes, forward and reverse primers, designed by Integrated DNA Technologies (Coralville, IA) were BDNF: probe, (5'-AGTCATTTGCGCACAACTTTAAAAGTCTGCATT-3'), forward, (5'-GGACATATCCATGACCAGAAAGAAA-3'), reverse, (5'-GCAA-CAAACCACAACATTATCGAG-3'); synapsin I: probe, (5'-CATGGCACGTAATGGAGACTACCGCA-3'), forward, (5'-CCGCCAGCTGCCTTC-3'), reverse, (5'-TGCAGCCCAATGACCAAA-3'); GAP-43: probe, (5'-CTCATAAGGCTGCAACCAAAATTCAGGCT-3'), forward, (5'-GATGGTGTCAAACCGGAGGAT-3'), reverse, (5'-CTTGTATGTGTCCACGGAAGC-3'); CREB: probe, (5'-CATGGCACGTAATGGAGACTACCGCA-3'), forward, (5'-CCGCCAGCATGCCTTC-3'), reverse, (5'-TGCAGCCCAATGACCAAA-3'); trkB: probe, (5'-CCGACTCTTGCCCTTCGAAC-3'), forward, (CCCAATTGTGGTCTGCCG), reverse, (CTTCCCTTCCACCGTG). An oligonucleotide probe specific for the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control to standardize the amount of sample RNA. The RT reaction conditions were 2 min at  $50^{\circ}\text{C}$  as the initial step to activate uracil glycosylase (UNG), followed by 30 min at  $60^{\circ}\text{C}$  as the reverse transcription and completed by a UNG deactivation at  $95^{\circ}\text{C}$  for 5 min. The 40 cycles of the two-step PCR reaction conditions were 20 s at  $94^{\circ}\text{C}$  and 1 min at  $62^{\circ}\text{C}$ .

*Protein immunoassay measurements*

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, 220 TIUs/l Aprotinin, 0.1 mM benzethonium chloride, 1 mM benzamide HC, and 4% triton X-100). Homogenates were centrifugated and supernatants collected. Protein concentrations were estimated with the MicroBCA procedure (Pierce, Rockford, IL) using bovine serum albumin (BSA) as the standard. BDNF protein was quantified using an enzyme-linked im-

munosorbent assay (ELISA, BDNF Emax ImmunoAssay System Kit, Promega, Madison, WI) as per the manufacturer's protocol. Unknown BDNF concentrations were compared with known BDNF concentrations using a calibration curve. Synapsin I and phospho-synapsin I proteins were analyzed by Western blot, quantified by densitometric scanning of the film under linear exposure conditions, and normalized to actin levels. Membranes were incubated with the following primary antibodies: anti-synapsin I (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-synapsin I (1:2000, Santa Cruz Biotechnology), anti-actin (1:2000, Santa Cruz Biotechnology) followed by anti-goat IgG horseradish peroxidase (Santa Cruz Biotechnology). Immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

### Immunohistochemistry

Spinal cord tissues were sliced in the sagittal plane (30  $\mu$ m), collected free floating in PBS and processed for BDNF immunohistochemistry as previously described (Gomez-Pinilla et al. 2001). A 1:1000 dilution was used for the rabbit polyclonal anti-BDNF antisera (Chemicon International, Temecula, CA).

### Statistical analyses

GAPDH and actin were employed as internal standards for real-time RT-PCR and for Western blots, respectively. For quantification of TaqMan RT-PCR results, fluorescent signal intensities were plotted against the number of PCR cycles on a semilogarithmic scale. The amplification cycle at which the first significant increase of fluorescence occurred was designated as the threshold cycle ( $C_T$ ). The  $C_T$  value of each sample then was compared with those of the internal standard. This process is fully automated and carried out with ABI sequence detector software version 1.6.3 (PE Biosystem, Foster City, CA). Taqman EZ RT-PCR values for GAPDH were subtracted from BDNF, trkB, synapsin I, CREB, or GAP-43 values. The resulting corrected values were used to make comparisons across the different experimental groups. The mean mRNA or protein levels were computed for the control and experimental rats for each time point. Linear regression analysis was performed on the individual samples to evaluate the association between variables. An ANOVA with repeated measures and Fisher's test (Statview software, Abacus Concepts) were used to assess the statistical significance among the different groups. Statistical differences were considered significant at  $P < 0.05$ .

The results were expressed as mean percent of sedentary control values for graphic clarity and represent the means  $\pm$  SEM of five to seven independent determinations. For the BTX-A injection experiments, all results are expressed as a percent of measured mRNA values in the spinal hemi-cord or soleus muscle contralateral to the BTX-A injection of sedentary rats.

## RESULTS

The levels of mRNA for BDNF, its trkB receptor, and the synaptic associated protein synapsin I were assessed in the lumbar region of the spinal cord and the soleus muscle of rats that were exposed to voluntary wheel running for 3 or 7 days. We measured the mRNA levels of GAP-43 and CREB on the same tissue used for BDNF and trkB measurements. In another series of experiments, the right soleus muscle was pharmacologically paralyzed to study the effects of reducing the mechanical output during locomotion on the neurotrophin response in the lumbar spinal cord and the soleus muscle.

### Exercise effects on the spinal cord

**BDNF AND TRKB.** BDNF mRNA levels increased to 156% ( $P < 0.05$ ) of the sedentary controls levels after 3 days of voluntary exercise, and these levels remained elevated at 7 days ( $P < 0.05$ ; Fig. 1A). There was a direct relationship between the levels of BDNF mRNA and the distance that the animals ran ( $r = 0.91$ ;  $P < 0.01$ ; Fig. 1B). Levels of BDNF protein, assessed using ELISA, were 123% ( $P = 0.35$ ) by day 3 and 153% ( $P < 0.05$ ) by day 7 of sedentary controls (Fig. 1C). TrkB mRNA levels were 145% ( $P = 0.07$ ) by day 3 and 158% ( $P < 0.05$ ) by day 7, relative to sedentary controls (Fig. 1D). We performed immunohistochemistry for BDNF in rats exercised for 7 days (Fig. 2) to determine the phenotypic expression for the observed increase in BDNF protein (Fig. 1C). BDNF immunoreactivity was localized to ventral horn motoneurons and their axonal processes coursing through the white matter in both sedentary control and exercised rats. Fiber elements in the substantia gelatinosa of the dorsal horn showed BDNF immunostaining. Sparse astrocyte-like cells in the white matter also were BDNF immunopositive. A qualitative in-

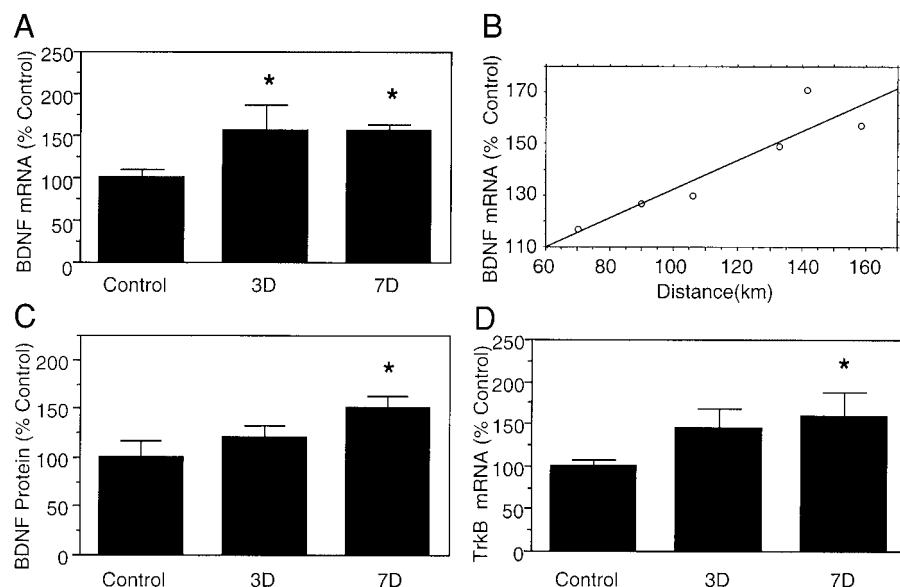


FIG. 1. A: spinal cord lumbar levels of brain-derived neurotrophic factor (BDNF) mRNA were increased after 3 ( $n = 6$ ) and 7 ( $n = 6$ ) consecutive days of voluntary running wheel exercise relative to sedentary controls ( $n = 6$ ). B: increases in BDNF mRNA were positively correlated ( $r = 0.91$ ,  $P < 0.01$ ,  $n = 6$ ) with the distance run. BDNF protein (C,  $n = 7$ ) and transduction receptor (trkB) mRNA (D,  $n = 5$ ) were increased following 7 days of exercise. All measurements were performed in the lumbar region of the spinal cord. Changes in mRNA levels were detected using real time RT-PCR and corrected for equivalent levels of total mRNA using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cRNA probe in the same assay solution. BDNF protein was detected and quantified using an enzyme-linked immunosorbent assay (ELISA). Means  $\pm$  SE are expressed as a percent of control. (\* $P < 0.05$ ; ANOVA, Fisher test).

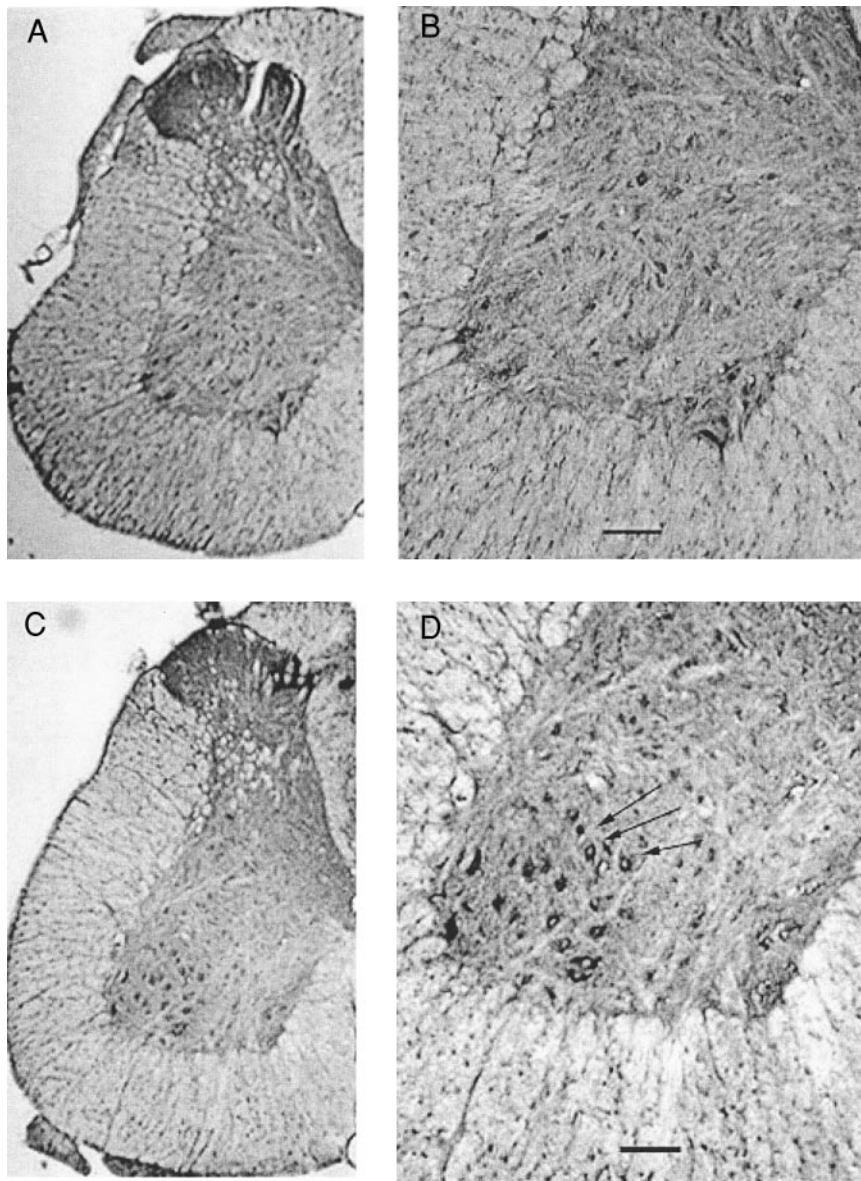


FIG. 2. BDNF immunohistochemistry was performed in coronal sections of the lumbar spinal cord of sedentary and exercised for 7 days rats. Low (A) and high (B) magnifications show typical BDNF immunostaining in a sedentary rat. In sedentary rats, light BDNF immunostaining was observed in motoneuron cell bodies in the ventral horn (arrows) and astrocyte-like cells located in the white matter. Low (C) and high (D) magnifications show typical BDNF immunostaining in an exercised rat. In trained rats, BDNF staining appeared stronger in exercised than sedentary rats, particularly in the cell bodies (arrows) and axons (arrowheads) of motoneurons. Astrocyte-like-cells distributed along BDNF-labeled axons also showed strong BDNF labeling in exercised rats. Calibration is 20  $\mu\text{m}$  (bar shown in C and D,  $n = 5$  per group).

crease in BDNF immunostaining was observed in the motoneurons and axonal elements in the exercised (Fig. 2, C and D) compared with the control (Fig. 2, A and B) rats.

**SYNAPSIN I.** Levels of synapsin I mRNA were measured on the same spinal cord tissue used for BDNF and trkB measurements to evaluate a possible effect of exercise on synaptic formation or function. Synapsin I mRNA levels were significantly higher in exercised ( $P < 0.05$ ; Fig. 3A) than control rats at both day 3 (135%) and day 7 (147%). Synapsin I protein (measured using Western blot analysis) was significantly higher in exercised ( $P < 0.05$ ; 135%; Fig. 3B) than control rats at both time points. In the exercised rats, phosphorylated synapsin I was 131 and 136% of control ( $P < 0.05$ , Fig. 3C) at 3 and 7 days, respectively. Synapsin I mRNA levels were positively correlated with the distance run ( $r = 0.86$ ;  $P < 0.01$ , Fig. 3D). Based on evidence that BDNF affects synapsin I function via an interaction with the trkB receptor complex (Jovanovic et al. 2000), we examined the interaction between trkB and syn-

apsin I mRNA at the 7-day time point. We found a significant positive correlation between the levels of trkB and synapsin I mRNA ( $r = 0.88$ ;  $P < 0.05$ , Fig. 3E).

**GAP-43 AND CREB.** GAP-43 mRNA levels were used as an indicator of axonal growth and synaptic remodeling. GAP-43 mRNA was higher in the exercised than control rats ( $P < 0.05$ ; Fig. 4A) after 3 days (139%) and 7 days (154%) days of exercise. A significant positive correlation between mRNA levels for GAP-43 and synapsin I was observed at day 7 ( $r = 0.80$ ;  $P < 0.05$ ; Fig. 4B). CREB mRNA was measured in the spinal cord because of the involvement of CREB in several events associated with the action of BDNF on neuronal plasticity. We found a trend for an increase in CREB mRNA after 3 days of voluntary running (131%;  $P = 0.12$ ) that reached statistical significance (165%;  $P < 0.05$ ) after 7 days of exercise (Fig. 4C). A positive correlation between the levels of BDNF mRNA and CREB mRNA was observed after 7 days of exercise ( $r = 0.95$ ;  $P < 0.01$ ; Fig. 4D).

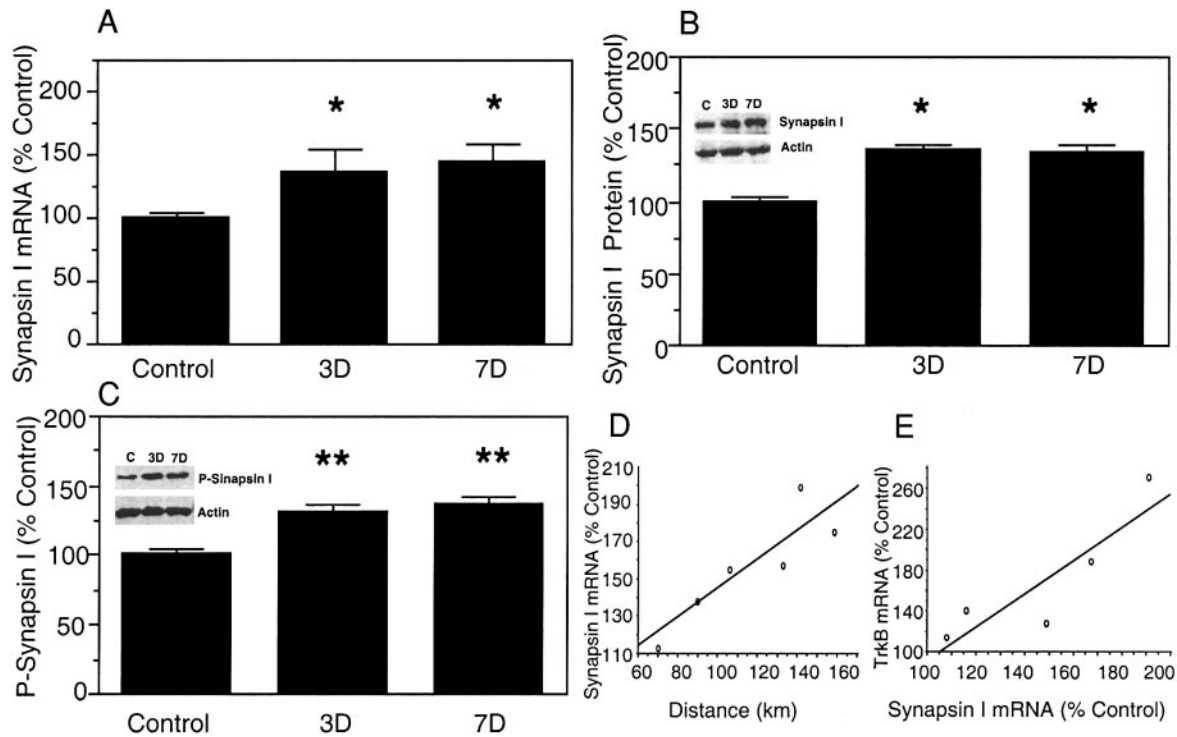


FIG. 3. Relative (% of control) levels of synapsin I mRNA (A), synapsin I protein (B), and phosphorylated synapsin I (C) were measured in the lumbar region of the spinal cord following 3 ( $n = 6$ ) and 7 ( $n = 6$ ) days of running wheel exercise. Levels of synapsin I mRNA were positively correlated with the amount of wheel running (D; shown for 7 days;  $r = 0.86$ ,  $P < 0.01$ ,  $n = 6$ ). Increases in synapsin I mRNA and trkB mRNA were positively correlated (E; shown for 7 days;  $r = 0.88$ ,  $P < 0.05$ ,  $n = 5$ ). mRNA levels were measured using real time RT-PCR and corrected for equivalent levels of total mRNA using a GAPDH cRNA probe in the same assay solution. Synapsin I protein was detected and quantified by Western blots using actin as a standard control. Values are means  $\pm$  SE expressed as a percent of sedentary control. (\* $P < 0.05$ , \*\* $P < 0.01$ ; ANOVA, Fisher test).

*Exercise effects on the soleus muscle*

**BDNF, TRKB, AND SYNAPSIN I.** Compared with sedentary control levels, there was a robust increase in BDNF mRNA ( $P < 0.01$ ) in the soleus muscle after 3 days (391%) and 7 days (236%) of exercise (Fig. 5A). Similarly, BDNF protein levels assessed using ELISA were significantly elevated after 3 days (204%;  $P < 0.01$ ) and 7 days of voluntary running (164%;  $P < 0.05$ ; Fig.

5B). TrkB mRNA levels in the exercised rats were higher (185%,  $P < 0.05$ ; Fig. 5C) than control after 3 days of exercise. Synapsin I mRNA levels also were higher than control after 3 days of exercise (184%;  $P < 0.05$ ; Fig. 5D). The levels of both trkB mRNA and synapsin I mRNA returned to control levels after 7 days of exercise. There were no exercise-associated changes in GAP-43 mRNA in the soleus muscle (data not shown).

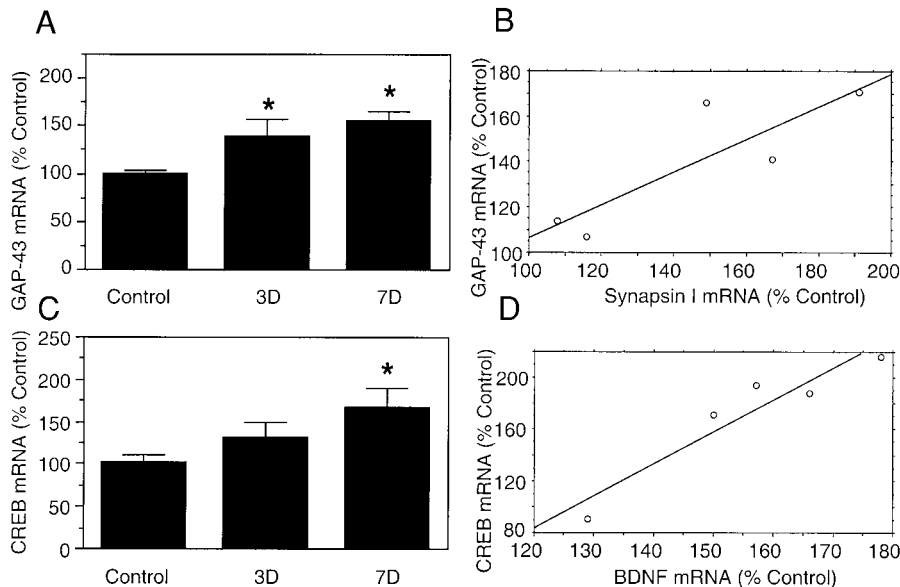


FIG. 4. Relative (% of control) levels of growth-associated protein (GAP-43) mRNA (A;  $n = 6$  for 3 days,  $n = 5$  for 7 days) and its correlation with levels of synapsin I mRNA (B;  $r = 0.80$ ,  $P < 0.05$ ,  $n = 5$ ) are shown for the lumbar region of the spinal cord after running wheel exercise. Relative levels of CREB mRNA (C;  $n = 6$  for 3 days,  $n = 5$  for 7 days) and its correlation with levels of BDNF mRNA (D;  $r = 0.95$ ,  $P < 0.01$ ,  $n = 5$ ) are also shown. mRNA levels were measured using real time RT-PCR and corrected for equivalent levels of total mRNA using a GAPDH cRNA probe in the same assay solution. Values are means  $\pm$  SE expressed as a percent of sedentary control. (\* $P < 0.05$ ; ANOVA, Fisher test).

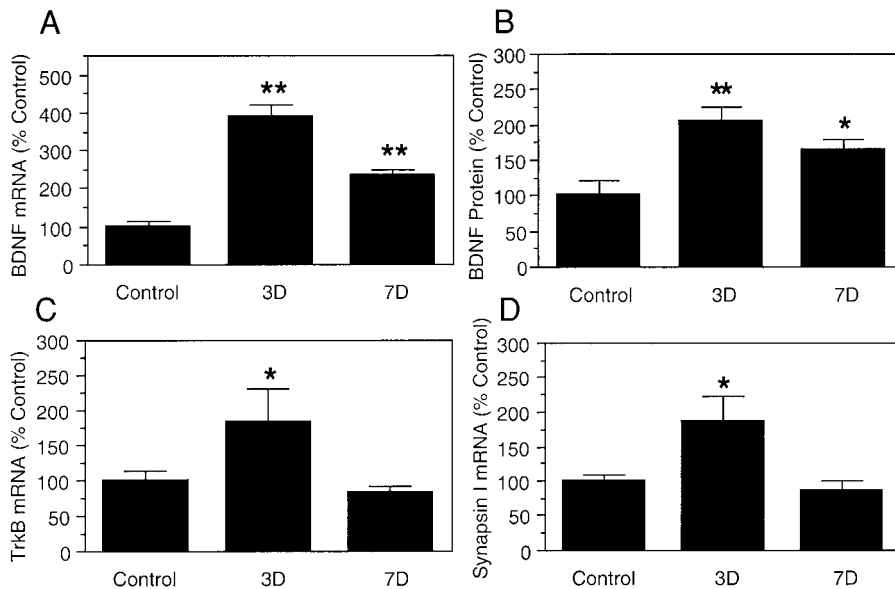


FIG. 5. Relative (% of control) levels of BDNF mRNA (A), BDNF protein (B), trkB mRNA (C), and synapsin I mRNA (D) were measured in the soleus muscle after 3 ( $n = 7$ ) days and 7 ( $n = 7$ ) days of running wheel exercise. mRNA levels were measured using real time RT-PCR and corrected for equivalent levels of total mRNA using a GAPDH cRNA probe in the same assay solution. Values are means  $\pm$  SE expressed as a percent of sedentary control. (\* $P < 0.05$ ; ANOVA, Fisher test).

#### Effects of unilateral BTX-A injection into the soleus muscle

BTX-A was injected into the soleus muscle unilaterally (right side) to determine the effects of eliminating the contractile activity of this slow plantarflexor muscle on the neurotrophin response to 7 days of voluntary activity. The BTX-A-injected soleus muscle showed no visible contractile response to electrical stimulation of its nerve for  $\leq 8$  days after the injection (see METHODS).

**SPINAL CORD—BDNF MRNA.** In sedentary rats, BDNF mRNA levels were reduced to (86%,  $P < 0.05$ ) in the hemi-cord ipsilateral to the BTX-A injection relative to the hemi-cord contralateral to the injection (Fig. 6A). After 7 days of exercise, BDNF mRNA levels were elevated (142%,  $P < 0.05$ ) in the hemi-cord contralateral to the injection and decreased (83%,  $P < 0.05$ ) in the hemi-cord ipsilateral to the injection, relative to BDNF mRNA levels in the hemi-cord contralateral to the injection of sedentary rats.

**SOLEUS MUSCLE—BDNF MRNA.** In sedentary rats, BDNF mRNA values were reduced (77%,  $P < 0.05$ ) in the BTX-A injected relative to the noninjected soleus muscle (Fig. 6B). After 7 days of voluntary wheel running, BDNF mRNA levels were elevated (175%,  $P < 0.01$ ) on the noninjected and reduced (40%,  $P < 0.01$ ) on the injected side compared with the noninjected side of sedentary rats.

**SPINAL CORD—SYNAPSIN I MRNA.** We measured synapsin I mRNA in the spinal cord to evaluate the possible effects of BTX-A treatment on synaptic function or plasticity associated to the action of BDNF. As explained in the preceding text, BDNF affects synapsin I function (Jovanovic et al. 2000). In sedentary rats, synapsin I mRNA levels were reduced (65%,  $P < 0.05$ ) in the hemi-cord ipsilateral to the injection relative to the noninjected side (Fig. 6C). After 7 days of exercise, synapsin I mRNA levels were increased (156%,  $P < 0.01$ ) in the hemi-cord contralateral to the injection compared with the noninjected side of the sedentary rats. In contrast, synapsin I mRNA levels on the injected side of the exercised rats were similar to the noninjected side, but higher ( $P < 0.01$ ) than those for the injected side in sedentary rats.

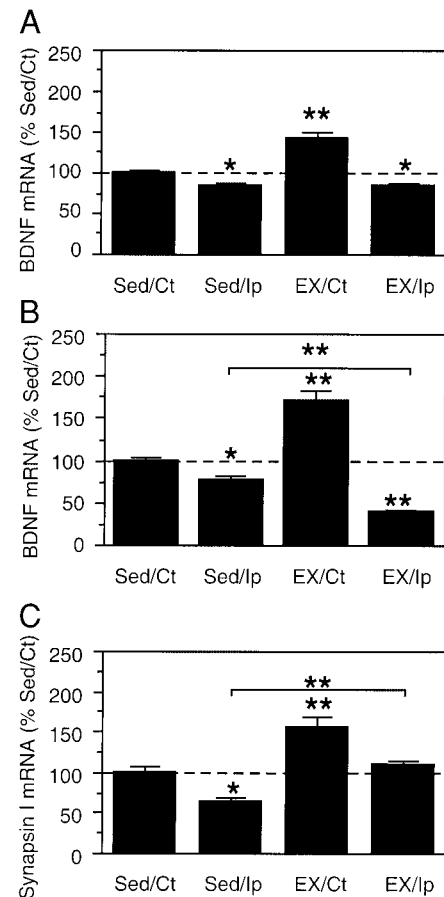


FIG. 6. BTX-A was injected into the soleus muscle unilaterally (right side) to determine the effects of eliminating the contractile activity on the neurotrophin response to 7 days of voluntary running activity. Levels of BDNF mRNA were measured in the lumbar region of the spinal hemi-cord (A) and soleus muscle (B) ipsilateral (Ip) and contralateral (Ct) to the injection. Levels of synapsin I mRNA were measured in the lumbar region of the spinal cord (C). All measurements were performed in sedentary (Sed;  $n = 6$ ) and exercised (EX;  $n = 6$ ) rats. Values are means  $\pm$  SE expressed as a percent of sedentary contralateral (Sed/Ct;  $n = 6$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; ANOVA, Fisher test).

## DISCUSSION

Voluntary exercise increased the expression of several molecules associated with the action of BDNF on synaptic function and neurite outgrowth in the lumbar region of the spinal cord and the soleus muscle. The soleus muscle was paralyzed by BTX-A injection to determine the effects of inactivating this normally highly recruited slow plantarflexor on the neurotrophin response to voluntary locomotor activity in the lumbar spinal cord and in the muscle itself. Following paralysis, BDNF and synapsin I mRNA levels were reduced in both tissues, demonstrating that active muscle contraction plays a role in modulating the levels of BDNF mRNA in both the muscle and the spinal cord. These results indicate that a basal level of neuromuscular activity is important to maintain normal levels of BDNF, and the potential for neuroplasticity, in the neuromuscular system.

*Timing of neurotrophin induction in the spinal cord and soleus muscle*

The time course of the changes in the expression of BDNF and its receptor in the spinal cord was different from that for the skeletal muscle (compare Fig. 1 vs. 5). It is possible that this may be part of a coordinated response between the spinal cord and muscle, as it is known that motoneurons can retrogradely transport BDNF from the skeletal muscle (Koliatsos et al. 1993; Sagot et al. 1998). An elevation in BDNF protein and trkB receptor mRNA occurred between exercise days 3 and 7 in the spinal cord (Fig. 1, C and D), whereas BDNF protein was decreasing in the soleus muscle during the same time period (Fig. 5B). These observations are consistent with the possibility that BDNF produced in the muscle was transported retrogradely into the cell bodies of the associated motoneurons. Further experiments will be necessary to determine whether this indeed is the case.

*Impact of exercise on neuroplasticity*

To evaluate a possible functional role for the increases in the BDNF system, we measured the levels of various molecules that have a recognized interaction with BDNF and are important for synaptic plasticity. The levels of phosphorylated protein, total protein, and mRNA for synapsin I were elevated in the spinal cord of exercised rats. Also, the modulation of synapsin I mRNA was associated closely with changes in the levels of trkB mRNA in the spinal cord. BDNF phosphorylates synapsin I primarily through the trkB receptor to induce the mitogen-activated protein-kinase-signaling pathway that modulates neurotransmitter release (Jovanovic et al. 2000). In a separate set of experiments (Gomez-Pinilla, unpublished observations), we have been able to reduce the increase in synapsin I mRNA associated with exercise in the hippocampus by blocking BDNF action using the tyrosine kinase receptor blocker K252a. Therefore it appears that the elevated expression of BDNF and its receptor after exercise may affect synapsin I at the transcriptional and posttranslational levels. The fact that synapsin I can be modulated by BDNF (Jovanovic et al. 2000) suggests that increases in BDNF and its receptor as a result of exercise can impact synaptic growth and/or function.

A potential action of BDNF on synaptic plasticity associated with exercise would likely involve mechanisms that support

neurite outgrowth. Accordingly, we determined the changes in GAP-43 given its role on input-dependent alterations of synaptic morphology (Oestreicher et al. 1997). Increases in BDNF, its receptor, and synapsin I in the spinal cord were accompanied by increases in GAP-43 mRNA after 3 and 7 days of exercise. Furthermore, the observation that increases in GAP-43 mRNA and synapsin I mRNA were positively correlated suggests a functional interaction between GAP-43 and synapsin I in our paradigm. The roles of GAP-43 on axonal growth, neurotransmitter release, and learning and memory suggest that exercise-related increases in GAP-43 can be associated with maintaining synaptic function.

Exercise also increased CREB mRNA in the spinal cord. CREB is one of the best-characterized transcription factors in the brain and can be modulated by BDNF (Finkbeiner et al. 1997). CREB is phosphorylated by BDNF at the transcription regulatory site, and CREB can feed-back on BDNF by regulating its gene transcription via a calcium-dependent mechanism (Finkbeiner 2000). CREB is required for various forms of memory (Silva et al. 1998) and appears to play a role in neuronal resistance to insult (Walton et al. 1999). Given the preponderant effect of BDNF and associated molecules on synaptic plasticity and function, it is possible that these molecules can participate in the synaptic events associated with locomotion. For example, BDNF can stimulate central pattern generation (fictive locomotion) in adult rats, clearly demonstrating an effect of BDNF on the excitability of spinal locomotor networks (Jakeman et al. 1998). BDNF also can affect muscle excitability. For example, Kleiman et al. (2000) reported that BDNF stimulates the release of acetylcholine at the neuromuscular synapse of cultured myocytes resulting in the potentiation of spontaneous twitching. It is notable that BDNF, synapsin I, CREB, and GAP-43 also have been associated with learning, and motor learning has been described in the spinal cord of spinal transected cats (de Leon et al. 1998). Whether the molecular mechanisms described in the present results are linked within the specific neural pathways that demonstrate the spinal learning remains to be determined.

*Neuromuscular activity regulates BDNF levels and neuroplasticity*

We evoked paralysis of the soleus muscle by blocking neurotransmission using BTX-A in rats maintained under sedentary conditions and in rats that were allowed to run voluntarily. BTX-A injection in sedentary rats resulted in lower levels of BDNF mRNA in the soleus muscle and in the spinal hemi-cord of the injected side. These rats were housed in sedentary cages and performed minimal spontaneous physical activity, suggesting that a basal level of neuromuscular activity is required to maintain normal levels of BDNF in the muscles and spinal cord. These results also raise the issue as to whether the BDNF levels are "subnormal" in sedentary rats and only reach normal levels when the rats are allowed to exercise when they chose to do so. In this case, one would have to consider the possibility that a rat housed in a sedentary cage is not an appropriate control condition for a variety of experiments. On the other hand, the activity that occurs in rats housed in sedentary cages may be analogous to the sedentary life of many humans.

Exercise resulted in an increased expression of BDNF and



synapsin I mRNAs in the spinal cord contralateral to the BTX-A-injected soleus muscle as would be expected from the results in noninjected rats. Exercise subsequent to the BTX-A injection decreased the levels of BDNF mRNA below control values in the spinal cord ipsilateral to the injection. Adaptations in gene expression were not related to differences in voluntary exercise levels as differences in mRNA were observed between the ipsilateral and contralateral sides to the BTX-A injection in the same animals. Some modest and transient paralysis was detected in muscles immediately adjacent to the soleus (see METHODS); that may be the result of some leakage of BTX-A from the injected soleus. This, in turn, could have further reduced the total level of sensory input normally associated with active muscle contractions. It is also possible that less activation of the lumbar circuits from suprasegmental input could be caused indirectly by the muscle paralysis. Although passive stretching of the paralyzed soleus muscle undoubtedly occurred during running, without gamma control it was not sufficient to sustain BDNF transcription in the spinal cord at the level observed without paralysis. BTX-A injection also reduced the levels of synapsin I mRNA in the spinal cord of sedentary rats. Exercise increased the synapsin I mRNA to control levels in the hemi-cord ipsilateral to the BTX-A injection, counteracting the inhibitory effect of BTX-A. These findings suggest some compensatory effect of exercise on paralysis-induced synapsin I transcription.

It is also possible that the soleus muscle may have suffered some "damage" during the initial stages of voluntary wheel running in our model as has been reported for mice (Irintchev and Wernig 1987; Wernig et al. 1991). It is plausible that BDNF may have been involved with these events considering that this "damage" includes fiber type remodeling and enhanced sprouting, which are typical components of a regenerative response within the spectrum of action of trophic factors. The possibility of this effect would be consistent with the lack of an increase in BDNF in the BTX-treated soleus muscle in our study, as the same authors have reported that denervated muscle that is passively stretched during running is not damaged.

Decreases in BDNF and synapsin I mRNAs in the spinal cord after pharmacological inactivation of the soleus muscle demonstrated the crucial role of muscle activity and perhaps neuronal activity in the induction of these genes. The observation that complete paralysis of only one small muscle of the lower limb depressed the BDNF and synapsin I levels in the ipsilateral segment that normally receives sensory input from many muscle and joints of the limb was somewhat of a surprise. However, this substantial depression may reflect the extensive divergence from each sensory receptor projecting back to the spinal cord. For example, each muscle spindle sends 10–15 projections to each motor neuron of the homonymous motor pool and to many of the motor neurons of synergistic motor pools (Nelson and Mendell 1978).

The results clearly demonstrate the capacity of voluntary physical activity to upregulate select genes associated with neuronal plasticity in the spinal cord and skeletal muscle. It appears that BDNF plays a central role in the molecular mechanisms by which exercise translates into changes of neuronal plasticity and function in the neuromuscular system. The results also show the critical impact of physiological levels of

neural activity in maintaining the expression of select genes during homeostatic conditions. It is likely that molecular mechanisms activated by exercise in the neuromuscular system also may be applicable to more complex neural systems and provide molecular substrates for the action of experience on neural function. Further studies are required to determine the exact role of BDNF and exercise on specific functional aspects of neural plasticity. BDNF-exercise interactions appear to be critical factors to manipulate in the design of behavioral therapy for decreasing the neuronal degeneration as occurs with aging and many other neuromotor disorders.

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