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Afferent Input Modulates Neurotrophins and Synaptic Plasticity in the Spinal Cord

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Gómez-Pinilla, Fernando, Zhe Ying, Roland R. Roy, John Hodgson, and V. Reggie Edgerton. Afferent input modulates neurotrophins and synaptic plasticity in the spinal cord. *J Neurophysiol* 92: 3423–3432, 2004; doi:10.1152/jn.00432.2004. The effects of eliminating or decreasing neuromuscular activity on the expression of neurotrophins and associated molecules in the spinal cord and subsequent effects on spinal cord plasticity were determined. Spinal cord isolation (SI), which eliminates any supraspinal and peripheral monosynaptic input to the lumbar region but maintains the motoneuron-muscle connectivity, decreased the levels of brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) mRNA and protein in the isolated segments. Synapsin I, an important mediator for the effects of BDNF on synaptic plasticity, also was lower in the lumbar region of SI rats. In contrast, the levels of BDNF, synapsin, and growth-associated protein (GAP-43) were increased in the cervical spinal cord enlargement rostral to the isolated region, most likely reflecting an increased use of the forelimbs in the SI rats. GAP-43 levels were also increased in the lumbar spinal cord region, probably associated with compensatory mechanisms related to the deafferentation. In a separate set of experiments, the soleus muscle was paralyzed unilaterally via intramuscular botulinum toxin type A (BTX-A) injection to determine the effects of reducing the propioceptive input, of this normally highly active muscle on neurotrophin expression in the spinal cord. BDNF and synapsin I mRNAs were lower and NT-3 levels were higher in the lumbar hemicord ipsilateral to the BTX-A injection. Combined, these results indicate that the level of supraspinal and muscle afferent input plays an important role in modulating the levels of BDNF and NT-3 in the spinal cord.

INTRODUCTION

The ability of neural activity to modulate neurite outgrowth, synaptic remodeling, and neuronal survival in the developing and adult CNS is well documented (Kalb and Hockfield 1992). In particular, varying degrees of afferent activity seem to play an important role in shaping the function of the spinal cord (Edgerton et al. 2001; Kalb and Hockfield 1992). It is now recognized that supplementary neuromuscular activity, such as exercise, is beneficial to neural function by modulating the expression of molecules that are associated with neuroplasticity (Gomez-Pinilla et al. 2002; Molteni et al. 2002). It remains to be determined whether a decrease or a lack of afferent input simply has the opposite effects of increased input and whether this adaptive response involves distinctive molecular systems.

Recently neurotrophins have emerged as important molecular translators for the effects of activity on neural function. Neurotrophins such as brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) are powerful modifiers of neuronal excitability and synaptic transmission (Kafitz et al. 1999; Mendell et al. 1999; Poo 2001). The finding that the level of neural activity can modulate BDNF and NT-3 provided the first indication that these molecules could mediate the effects of experience on neural function. In particular, recent evidence indicates that physical activity elevates BDNF (Gomez-Pinilla et al. 2002) and NT-3 (Ying et al. 2003) in the spinal motor system with subsequent effects on synaptic plasticity. Synapsin I and GAP-43 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 stimulates 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 an important role in axonal growth, neurotransmitter release (Benowitz and Routtenberg 1997; Oestreicher et al. 1997), and learning and memory (Routtenberg et al. 2000).

In the present study, using two well-defined paradigms we have examined the relative importance of different sources of modulation of afferent input to the spinal cord that may control BDNF, NT-3, and their downstream effectors. In spinal cord isolation (SI), the lumbar region of the spinal cord is deprived of any supraspinal and peripheral monosynaptic input, but the connectivity between the motoneurons and muscle is left intact (Fig. 1*A*). Therefore the target muscles are normally innervated but essentially inactive due to the electrically silenced motoneurons (Grossman et al. 1998; Haddad et al. 2003; Roy et al. 2000). In another set of experiments, we have produced inactivity in the highly active soleus muscle using botulinum toxin type A (BTX-A; Fig. 1*B*). BTX-A treatment blocks cholinergic transmission at the intrafusal motor end plates and thus significantly reduces spindle afferent input into the spinal cord from the muscles that are blocked (Filippi et al. 1993; Manni et al. 1989; Rosales et al. 1996). In addition, blocking the neuromuscular synapse can change motoneuron electrophysiological properties via retrograde signaling mechanisms (Nick and Ribera 2000). The present experiments are designed to determine the differential effects of the levels and sources of activation of select neuronal systems in modulating the expres-

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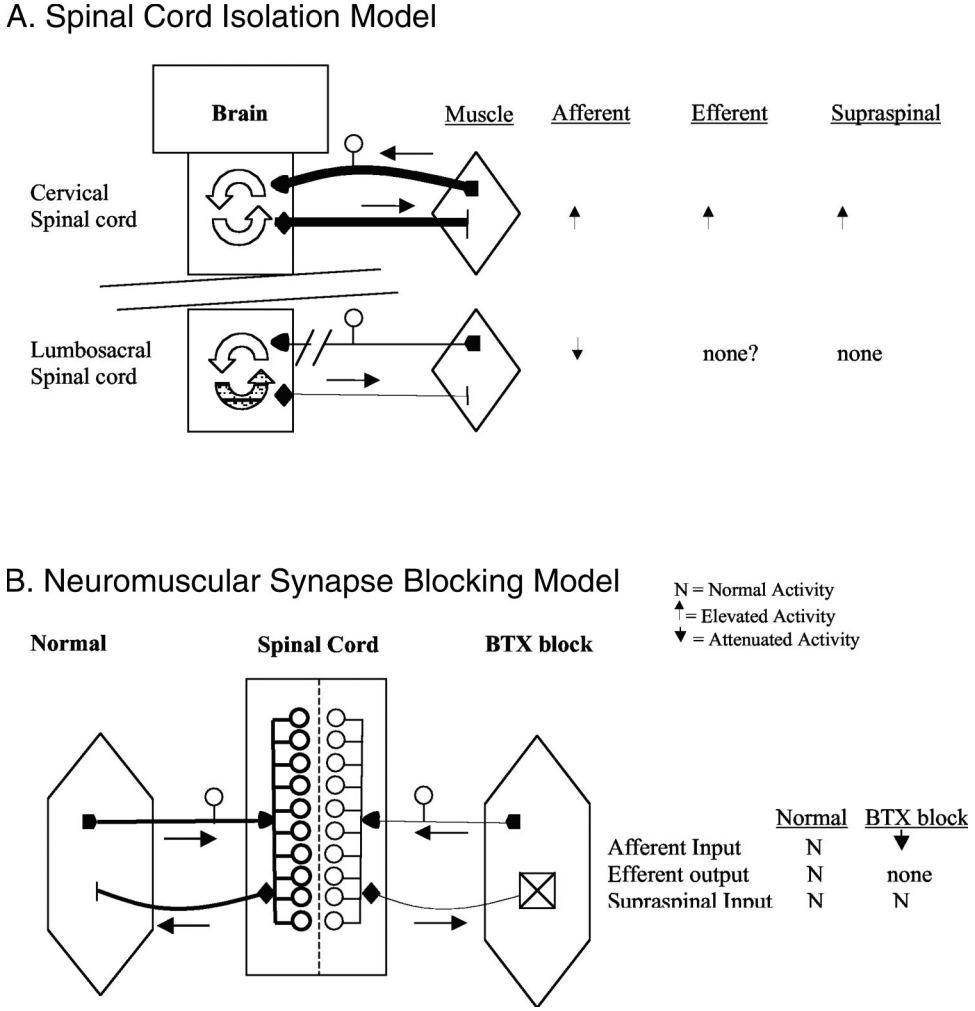

FIG. 1. Schematic modeling for the involvement of afferent input, efferent output, and supraspinal input in the spinal cord isolation (SI) paradigm (*A*) and the unilateral neuromuscular synapse blocking of the soleus muscle with botulinum toxin type A (BTX-A) injection (*B*). In the SI paradigm (*A*), the levels of both the afferent input and efferent output of the cervical region of the spinal cord, i.e., rostral to the spinal cord transection at a mid-thoracic level, are elevated above normal (thick lines between the spinal cord and muscles) due to the compensatory functions of the forelimbs. In contrast, both the afferent input and efferent output are virtually eliminated in the lumbosacral region of the spinal cord (thin lines between the spinal cord and muscles). Note that both supraspinal and peripheral input are eliminated in the lumbosacral region of the spinal cord via a complete spinal cord transection and bilateral dorsal rhizotomies, respectively. In the unilateral neuromuscular synapse blocking model (*B*), the afferent input to the spinal cord from the BTX-A-injected muscle is eliminated on the ipsilateral but not the contralateral side. This is likely to have a severe effect on the efferent output. The neurons that are impacted are illustrated as circles, with the realization that each spindle afferent diverges and innervates many motoneurons monosynaptically (homonymous and synergistic) and many Ia interneurons which project inhibitory signals to antagonist motoneurons. Although this figure emphasizes the role of "activity" as an important variable in these experimental paradigms, this does not preclude the possibility that other factors may be involved with their biological effects.

sion of BDNF and NT-3 and their downstream effectors in shaping their role on the synaptic plasticity of the spinal cord.

METHODS

Animals and surgical procedures

Adult female Sprague–Dawley rats [200 ± 10 (SD) g body wt] were assigned randomly to a control or a SI group. The SI procedures (Fig. 1*A*) are a modification of the original protocols of Tower (1937), and these procedures have been used routinely in our laboratory (Roy et al. 1992). Briefly, the rats were anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine (8 mg/kg) administered intraperitoneally. Supplemental doses (30% of the initial dose, ip) were given as needed. Under aseptic conditions, a longitudinal dorsal midline skin incision was made over the spinal column from a high thoracic to a low sacral vertebral level, and the muscles overlying the vertebral column were reflected. A partial laminectomy was performed approximately between vertebral levels T_7 and S_3. After opening the dura mater, the dorsal roots were cut intradurally bilaterally from the mid-thoracic spinal cord level to a mid-sacral level. Lidocaine hydrochloride (1%; 2 or 3 drops) was applied to the transection sites. The spinal cord was lifted gently with a curved probe or fine forceps and completely transected at both a mid-thoracic and a high sacral spinal cord level using microdissection scissors. The cut ends of the cord were lifted gently using forceps to verify a complete transection. Gelfoam was packed between the cut ends of the spinal cord at each transection site. A strip of gelfilm then was placed along the length of the exposed spinal cord. The paravertebral muscles and fascia surrounding the spinal column were sutured using 4-0 chromic gut, and the skin incision was sutured using 4-0 Ethilon. The rats were allowed to fully recover from anesthesia in an incubator (27°C) and were given lactated Ringer solution (5 ml sc). PolyFlex (G. C. Hanford Manufacturing, Syracuse, NY), a general antibiotic, was administered (100 mg/kg sc, twice daily) during the first 3 day of recovery. The rats were housed in polycarbonate cages (10.25 ft × 18.75 ft × 8 ft) individually and the room was maintained at 26 ± 1°C, with 40% humidity and a 12:12 h light:dark cycle. Postsurgical care involved manual expression of the bladder three times daily for the first 3 days and twice daily thereafter. On a daily basis, cage bedding was changed to prevent skin infections, animals were assessed for health (e.g., body weight, urination, defecation, and hydration), the hindlimbs were manipulated passively once through a full range of movement to maintain joint flexibility, and reflexes in the hindlimbs were assessed (i.e., withdrawal reflex and toe spread response). Throughout the study there was no response to reflex testing or toe pinching, and the hindlimbs remained completely flaccid. Experimental and control rats were supplied with rat chow and water ad libitum. The studies were approved by the UCLA Chancellor's Animal Research Committee and followed the American Physiological Society Animal Care Guidelines.

All rats to be used for biochemical assays ($n = 6$ or 8 per group at each time point) were killed by decapitation between 8:00 and 10:00 AM, the entire spinal cord was removed, laid on a cork strip, carefully oriented, and frozen on dry ice. Subsequently, the lumbar enlargement was identified, separated, and used for analysis. All fresh-frozen

tissues were stored at −70°C until processed. Rats used for immunohistochemical analyses were anesthetized deeply with sodium pentobarbital (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°C until sectioned in a cryostat.

BTX-A injection

To evaluate the role of sensory input from a highly active antigravity muscle on the expression of neurotrophins, we paralyzed the soleus muscle of the right leg with BTX-A (Sigma) in a separate group of rats ($n = 6$; Fig. 1*B*). The rats were anesthetized deeply with a mixture of ketamine hydrochloride (70 mg/kg body wt ip) and acepromazine maleate (5 mg/kg body wt ip). 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 this muscle was retracted to expose the soleus muscle. Using a Hamilton microsyringe (10 μl), a single dose of BTX-A (1 ng/kg) was injected at four sites across the mid-belly 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 Ethilon.

As reported previously (Gomez-Pinilla et al. 2002), initial preliminary experiments were conducted to determine the correct dosage of BTX-A. Using electrical stimulation of the muscle via its nerve (single or multiple pulses ≤200 Hz for 300 ms at 1–10 V) in an in situ preparation, we showed that the BTX-A dosage used in the present study completely silenced the soleus muscle between 2 and 8 days after injection. For the rats in the present study, we verified a complete block of the soleus muscle in two rats just prior to death. We also verified that there was no effect of saline injection on the levels of BDNF mRNA in the spinal cord or the soleus muscles eliminating any possible effect of mechanical damage related to the intramuscular injection on BDNF mRNA levels.

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, and GAP-43 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^{I}-AGTCATTTGCGCACAACTTT AAAA GTCTGCATT-3^{I}), forward, (5^{I}-GGACATATCCATGACCAGAAAGAAA-3^{I}), reverse, (5^{I}-GCAACAAACCACAACATTATCGAG-3^{I}); NT-3: probe, 5′-TGACCGACAAGTCCTCAG CCATTGAC-3′, forward, 5′-TGTGACAGTGAGAGCCTGTGG-3′, reverse, 5′-TGTAACC TGGTGTCCCCGAA-3′; synapsin I: probe, (5^{I}-CATGGCACGTAATGGAGACTACC GCA-3^{I}), forward, (5^{I}-CCGCCAGCTGCCTTC-3^{I}), reverse, (5^{I}-TGCAGCCCAATGACCAAA-3^{I}); and GAP-43: probe, (5^{I}-CTCATAAGGCTGCAACCAAAATTCAGGCT-3^{I}), forward, (5^{I}-GAT GGTGTCAAACCGGAGGAT-3^{I}), reverse, (5^{I}-CTTGTTATGTGTCCACGGAAGC-3^{I}). An oligonucleotide probe specific for the rat GAPDH gene was used as an endogenous control to standardize the amount of sample RNA. The RT-reaction conditions were 2 min at 50°C as the initial step to activate uracil glycosylase (UNG), followed by 30 min at 60°C as the reverse transcription and completed by an UNG deactivation at 95°C for 5 min. The 40 cycles of the two-step PCR-reaction conditions were 20 s at 94°C and 1 min at 62°C.

Protein immunoassay measurements

Lumbar spinal cord samples were homogenized in 3 volumes of homogenization buffer [(in mM) 50 Tris-HCl pH 8.0, 600 NaCl, 0.1 phenylmethylsulfonyl fluoride (PMSF), 0.1 benzethonium chloride, and 1 benzamidine HC plus 1% bovine serum albumin (BSA), 220 TIUs/l Aprotinin, and 4% triton X-100]. Homogenates were centrifugated and supernatants collected. Protein concentrations were estimated with the MicroBCA procedure (Pierce, Rockford, IL) using BSA as the standard. BDNF and NT-3 were quantified using an enzyme-linked immunosorbent assay (Emax ImmunoAssay System Kit, Promega, Madison, WI) as per the manufacturer's protocol. Synapsin I protein was 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-GAP-43 (1:500, Santa Cruz Biotechnology), anti-actin (1:2000, Santa Cruz Biotechnology) followed by anti-goat IgG horseradish peroxidase conjugate. 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 μm), collected free floating in PBS, and processed for BDNF immunohistochemistry as previously described (Gomez-Pinilla et al. 2002). A 1:1,000 dilution was used for the rabbit polyclonal anti-BDNF antisera (Chemicon Internationa, Temecula, CA) and goat polyclonal anti-synapsin I (Santa Cruz Biotechnology).

Statistical analyses of biochemical measurements

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, NT-3, synapsin I, 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. An ANOVA and Fisher's test (Statview software, Abacus Concepts, CA) 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 ± SE. For the BTX-A injection experiments, all results are expressed as a percent of measured mRNA values in the spinal hemi-cord contralateral to the BTX-A injection. The contralateral side was used as control based on results showing that levels of BDNF in the contralateral side did not differ from intact controls (intact 100 ± 5% vs. contralateral side 97 ± 6%, $n = 6$/group).

Electromyographic recordings

The electromyographic (EMG) studies were done on a separate group of rats ($n = 4$) using procedures performed routinely in our laboratory (Roy et al. 1991). A skin incision was made along the saggital suture of the skull. Three screws were anchored firmly to the skull and a nine-pin (gold-plated) amphenol connector was cemented (dental cement) to the skull and screws. Eight multistranded Teflon-insulated stainless-steel wires (15 strands, 50-μm gauge; Cooner Electronics) were led subcutaneously from the connector to the hindlimb (see following text). A ground wire was embedded in the middle back region and served as a common ground.

All EMG recordings were performed using the same cages in which the animals were normally housed. A nine-conductor swivel (Alice King Chatham Medical Arts, Inglewood, CA) was mounted on the top of the cage, allowing the animals to move freely during the recordings. Signals were amplified (1,000 times, custom-built portable amplifiers) on exiting the swivel and then recorded digitally at 2 kHz using custom acquisition software. Recordings began between 8:00 and 10:00 AM and concluded 24 h later. All normal animal care activities were maintained during the recording period (i.e., expression of the bladder and daily health checks). Recordings were performed 4 and 7 days prior to and 7 and 30 days after SI surgery.

The EMG data were analyzed using in-house software developed and LabVIEW (National Instruments, Houston, TX). The methods have been reported in previous publications (Hodgson et al., 2001). Briefly, all EMG data were first reviewed by displaying the recorded data on a computer monitor at selectable time resolutions ranging from fractions of a second to several minutes. Segments of data containing interference were identified and excluded from further analysis. The remaining data were digitally high pass filtered at 10 Hz and rectified. Integrated EMG (IEMG) and the duration of EMG activity above a threshold level were calculated. The threshold level was set at the highest value required to exclude 95% of the baseline data. Data were corrected to properly represent each hour of activity in those instances where interference excluded some data from our analysis. The post-SI data were treated slightly differently because the data indicated that the muscles were inactive the majority of the time. In this case, considerable time was saved by analyzing only records containing EMG activity and adding the remaining time in each hour to the zero-amplitude bins of the amplitude distributions. The SI data (means ± SE) are presented as a percent of pre-SI values (mean of the 2 pre-SI recordings).

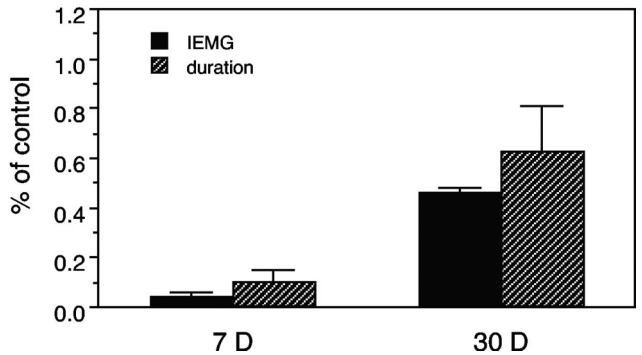

FIG. 2. Daily integrated electromyograph (IEMG = EMG amplitude × EMG duration) and EMG duration (amount of time showing some activity) of the soleus muscle for an entire 24-h period was recorded before and 7 and 30 days (*D*) after spinal cord isolation (SI). Data are expressed as a percentage of the pre-SI values and demonstrate that the spinal cord lesion reduced both the IEMG and EMG duration after 7 and 30 days to <1% compared with pre-SI values. Values are means ± SE for 4 rats.

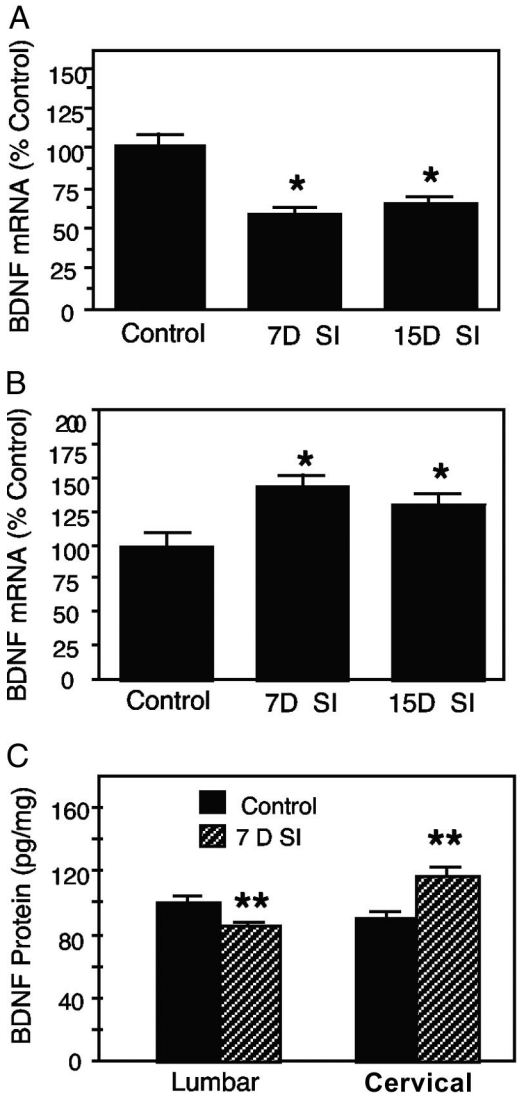

FIG. 3. Relative levels of brain-derived neurotrophic factor (BDNF) mRNA were measured in the lumbar (*A*) and cervical (*B*) regions of the spinal cord in control and SI rats. Compared with control, BDNF mRNA levels were lower in the lumbar but higher in the cervical regions after 7 and 15 days of SI. BDNF mRNA levels were measured using real-time RT PCR and corrected for equivalent levels of total mRNA using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA probe in the same assay solution. Levels of BDNF protein were measured in the lumbar and cervical spinal cord regions of control and SI rats (*C*). Compared with control, the BDNF levels were lower in the lumbar and higher in the cervical region after 7 days of inactivity. Values are means ± SE for mRNA ($n = 8$) and protein ($n = 6$) measurements at each time point. * and **, significantly different from control at $P < 0.05$ and $P < 0.01$, respectively; ANOVA, Fisher's test.

RESULTS

SI nearly eliminates the electrophysiological activity in the spinal cord. EMG activity is an excellent predictor of spinal cord electrophysiological activity. Therefore EMG was recorded from the soleus muscle before and after 7 and 30 days of SI to evaluate how a complete elimination of supraspinal and peripheral input to the cord would affect intrinsic spinal cord activity. The soleus muscle was chosen for recordings because of its normally very high activity levels during routine function (Roy et al. 1991). SI surgery resulted in immediate

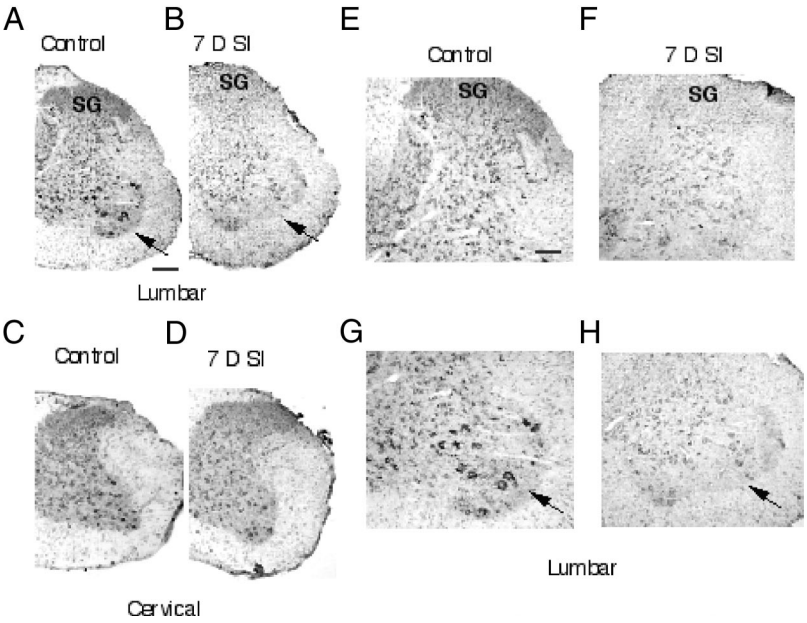

FIG. 4. BDNF immunohistochemistry was performed in coronal sections of the lumbar (*A* and *B*) and cervical (*C* and *D*) spinal cord of control (*A* and *C*) and 7-day SI rats (*B* and *D*) to localize the changes in BDNF protein within the spinal cord. A higher magnification of the lumbar region is shown for controls (*E* and *G*) and SI (*F* and *H*) rats. Generally, changes in BDNF related to SI were localized to the substantia gelatinosa (SG) of the dorsal horn (*E* and *F*) and motoneurons (←) in the ventral horn (*G* and *H*).

paralysis of the hindlimbs and this was reflected in very low levels and durations of EMG activity (Fig. 2). Both the mean daily IEMG and duration of activity at 7 and 30 days post-SI were <1% of pre-SI levels.

BDNF levels after SI

After 7 and 15 days, the levels of BDNF mRNA in the lumbar region of the spinal cord of SI rats were 58 and 64% of intact control values, respectively (Fig. 3*A*). Based on ELISA analyses, BDNF protein levels in the lumbar region were 85% of control values in the 7-day SI group (Fig. 3*C*). We performed immunohistochemistry to document the cellular distribution of BDNF after SI (Fig. 4). Microscopic inspection showed qualitatively lower BDNF immunostaining in motoneurons in the ventral horn (Fig. 4, *B* and *H*) and in neurons in the superficial layers in the dorsal horn (Fig. 4, *B* and *F*) of the lumbar spinal cord in SI than control rats (Fig. 4, *A, E,* and *G*). In the cervical spinal cord, the levels of BDNF mRNA were 43 and 30% higher in SI than control rats at 7 and 15 days, respectively (Fig. 3*B*). The levels of BDNF protein in the cervical spinal cord were higher in SI than control rats at day 7 (Fig. 3*C*). BDNF immunohistochemistry showed that higher BDNF levels appeared to be localized in ventral horn motoneurons and dorsal axonal elements of the spinal cord (Fig. 4, *C* and *D*).

Synapsin I levels after SI

The levels of synapsin I mRNA were measured, according to the role of BDNF on synapsin I phosphorylation (Jovanovic et al. 1996) and mRNA production (Vaynman et al. 2003). Synapsin I mRNA levels in the lumbar spinal cord region of SI rats were lower than control at 7 (44% of control) and 15 (47% of control) days after surgery (Fig. 5*A*). Based on Western blot analysis, the 7-day SI rats had a lower (54% of control) synapsin I protein level than control rats (Fig. 5*C*). In contrast, levels of synapsin I mRNA in the cervical spinal cord region of SI rats were 20 and 43% higher than control at 7 and 15 days postsurgery, respectively (Fig. 5*B*). In addition, the synapsin I protein levels in SI rats were 33% higher than control 7 days post-SI in the cervical spinal cord (Fig. 5*D*).

GAP-43 levels after SI

The levels of GAP-43 mRNA in the lumbar region of the spinal cord were 23 and 33% higher in SI than control rats after 7 and 15 days of SI (Fig. 6*A*). Similar increases were observed in the cervical region of the spinal cord: GAP-43 mRNA levels were 22 and 31% higher in SI than control rats after 7 and 15 days of SI (Fig. 6*B*). After 7 days of SI, GAP-43 protein was 24% ($P > 0.05$) and 74% higher in SI than control rats in the lumbar (Fig. 6*C*) and cervical regions (Fig. 6*D*), respectively.

NT-3 mRNA levels after SI

NT-3 mRNA levels in the lumbar region of the spinal cord of SI rats were 43 and 68% of control at 7 and 15 days post-SI, respectively (Fig. 7*A*). No effects of SI on the levels of NT-3 mRNA were observed in the cervical spinal cord region (Fig. 7*B*). After 7 days of SI, NT-3 protein content was lower in the lumbar region but unchanged in the cervical region compared with control levels (Fig. 7*C*). The levels of NT-3 protein were more than threefold higher in the lumbar than cervical region of the spinal cords of control rats (Fig. 7*C*). After 7 days of SI, NT-3 protein values were lower than control in the lumbar region and reached values similar to those observed in the cervical region.

Effects of muscle inactivation on neurotrophin levels in the spinal cord

BTX-A was injected into the soleus muscle unilaterally (right side) to determine the effects of eliminating its contractile activity on neurotrophin expression in the spinal cord lumbar enlargement. There was no visible contractile response in the BTX-A-injected muscle to electrical stimulation of its

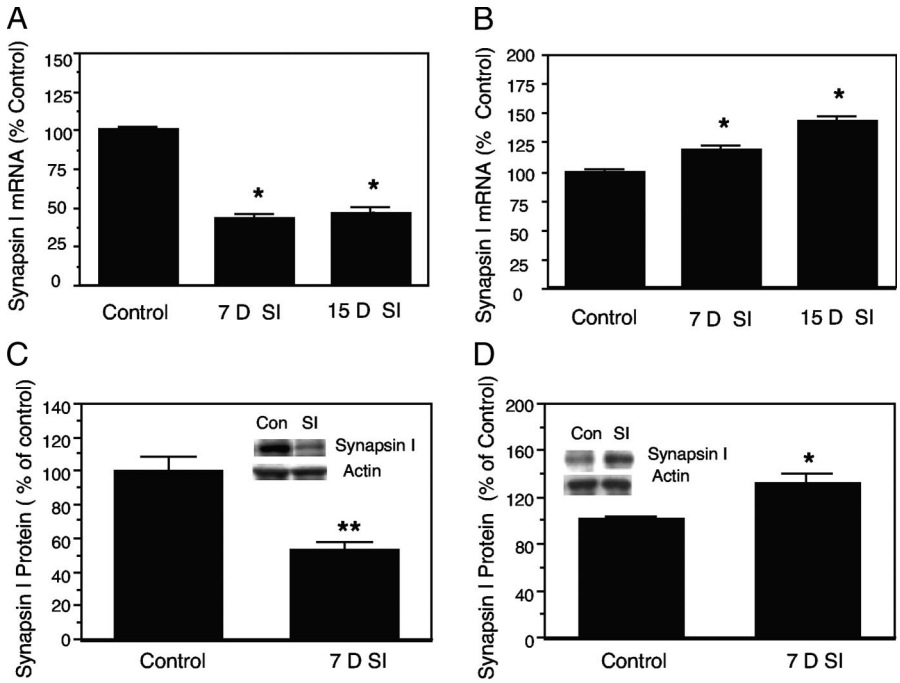

FIG. 5. Relative levels of synapsin I mRNA (*A* and *B*) and protein (*C* and *D*) were measured in the lumbar (*A* and *C*) and cervical (*B* and *D*) regions of the spinal cord in control and SI rats. Compared with control, synapsin I mRNA levels were lower in the lumbar (*A*) but higher in the cervical (*B*) region 7 and 15 days post-SI. Synapsin I mRNA levels were measured using real-time RT PCR and corrected for equivalent levels of total mRNA using a GAPDH mRNA probe in the same assay solution. Synapsin I protein was lower in the lumbar (*C*) but higher in the cervical (*D*) regions of SI than control rats. Synapsin I protein was quantified by Western blots using actin as a standard control. Values are means ± SE for rats used for mRNA (n = 8/group) or protein (n = 6/group) analyses at each time point and are expressed as a percent of control values. * and **, significantly different from control at $P < 0.05$ and $P < 0.01$, respectively; ANOVA, Fisher's test.

nerve for ≤8 days after the injection. After BTX-A injection, BDNF mRNA levels were 14% lower on the ipsilateral (same side as the BTX-A injection) hemi-cord, than the contralateral noninjected side (Fig. 8*A*). Furthermore, BDNF protein content was 18% lower on the injected than noninjected side (Fig. 8*B*). Because BDNF affects synapsin I activity (Jovanovic et al. 2000), we measured synapsin I mRNA in the spinal cord to evaluate the possible effects of BTX-A treatment on a molecular system important for synaptic function dependent on BDNF. After BTX-A injection, synapsin I mRNA levels were 35% lower in the hemi-cord ipsilateral to the injection than on the noninjected side (Fig. 9*A*). In contrast with that observed after SI, the NT-3 mRNA and protein levels after BTX-A injection were 50 and 30% higher, respectively, in the injected than the noninjected hemi-cord (Fig. 8, *C* and *D*). GAP-43 mRNA in the lumbar region of the spinal cord was unaffected in the BTX-A-treated rats (Fig. 9*B*).

DISCUSSION

We have examined how an alteration in neuromuscular activity can influence the modulation of select neurotrophins in the spinal cord. Complete elimination of supraspinal and peripheral input to the spinal cord in the SI model (Fig. 1*A*) resulted in near elimination of EMG activity in the soleus muscle, presumably reflecting low levels of synaptic input to the motor pools innervating the hindlimb musculature. The mRNA and protein levels of BDNF and its downstream effectors on synaptic plasticity, synapsin I, were lower than control values in the lumbar spinal cord segments that were deprived

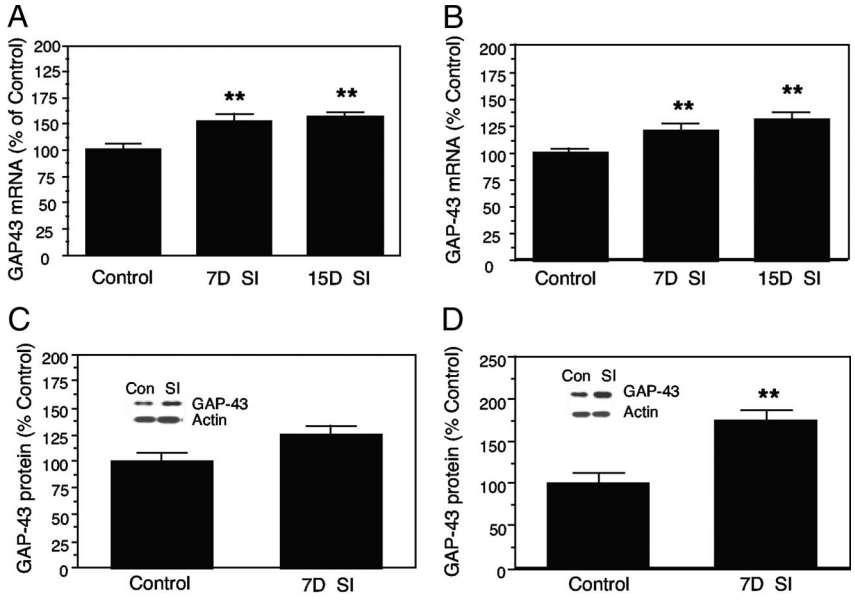

FIG. 6. Relative levels of growth-associated protein (GAP-43) mRNA (*A* and *B*) and protein (*C* and *D*) were measured in the lumbar (*A* and *C*) and cervical (*B* and *D*) regions of the spinal cord in control and SI rats. Compared with control, GAP-43 levels were higher in both the lumbar (*A*) and cervical (*B*) regions after 7 and 15 days of SI. GAP-43 mRNA levels were measured using real-time RT PCR and corrected for equivalent levels of total mRNA using a GAPDH mRNA probe in the same assay solution. GAP-43 protein levels in 7-day SI rats were higher than control in the cervical (*D*) but not the lumbar (*C*) region of the spinal cord. GAP-43 protein was quantified using Western blotting analysis. Values are means ± SE for rats used for mRNA (n = 8/group) and protein (n = 6/group) at each time point and are expressed as a percent of control values. * and **, significantly different from control at $P < 0.05$ and $P < 0.01$, respectively; ANOVA, Fisher's test.

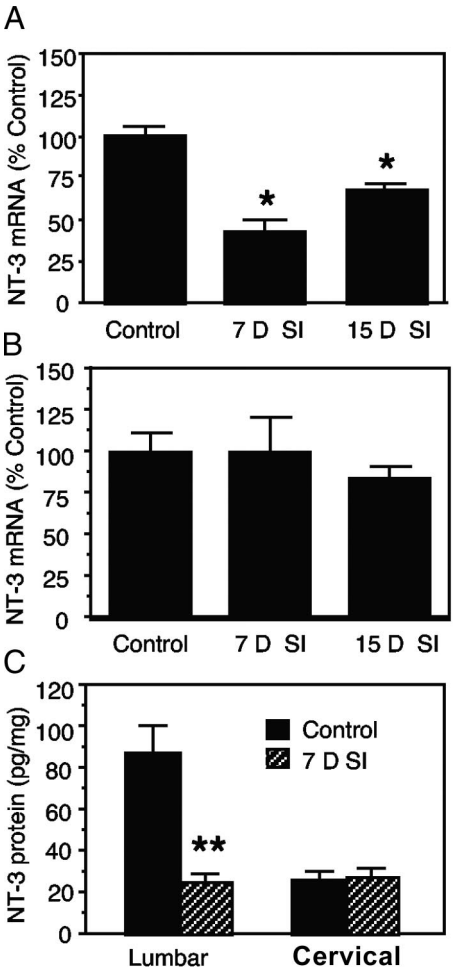

FIG. 7. Relative levels of neurotrophin 3 (NT-3) mRNA (A, B) were measured in the lumbar (*A*) and cervical (*B*) regions of the spinal cord in control and SI rats. Compared with control, NT-3 mRNA levels were lower in the lumbar (*A*), but higher in the cervical (*B*) region after 7 and 15 days of SI. NT-3 mRNA levels were measured using Real Time RT PCR and corrected for equivalent levels of total mRNA using a GAPDH mRNA probe in the same assay solution. The amount on NT-3 protein was dramatically lower in the lumbar, but unaffected in the cervical regions, of the 7-day SI compared with control rats (*C*). NT-3 protein was quantified using ELISA. Values are means ± SE for 8 rats used for mRNA and 6 rats used for protein analyses at each time point. * and **significantly different from control at $P < 0.05$ and $P < 0.01$, respectively; ANOVA, Fisher test.

of any afferent input. In contrast, the levels of the same molecules in the cervical spinal cord region lying rostral to the isolation were higher than control, presumably reflecting elevated afferent input and efferent output in the spinal circuits due to the higher use of the forelimbs to compensate for the deficits in hindlimb activity.

To evaluate the contribution of peripheral afferent input on neurotrophin regulation, neuromuscular function in the soleus muscle was blocked unilaterally using BTX-A (Fig. 1*B*). Neuromuscular synapse inactivation can affect the spinal cord by blocking retrograde signal transmission from the muscle and by reducing afferent activity subsequent to muscle paralysis. Given that the BTX-A injection did not have an effect in BDNF levels in the contralateral lumbar spinal cord, we used this side as a control for all the biochemical measurements. Similar to SI, BTX-A injection significantly decreased BDNF and synapsin I levels in the ipsilateral lumbar spinal cord region. Interestingly, NT-3 was modulated differentially in these two models of muscle inactivity: NT-3 was lower in SI rats but higher in BTX-A-treated rats than control rats. Based on these results it is apparent that the normal expression of BDNF and NT-3 is linked to spinal cord neuronal activity. It also emerges that differences in the intensity and source of afferent activity can distinctively modulate BDNF and NT-3. Recall that NT-3 was elevated whereas BDNF was depressed in the ipsilateral hemi-cord after BTX injection into the soleus muscle. Although neural activity is the main candidate for neurotrophin modulation, it is possible that other factors such as hormones and inflammatory signals may have contributed to the observed changes in neurotrophins and synaptic plasticity. For example, insulin-like growth factor-1 (IGF-1) is an important candidate to modulate BDNF expression as IGF-1 has been shown to be increased by locomotor activity (Carro et al. 2001).

Effects of SI on the expression of neurotrophins in the spinal cord

We have used the SI model in the rat to determine whether basal levels of neuronal input from the brain and peripheral afferents have a role in modulating the expression of neurotrophins in the spinal cord. Although the SI model eliminates neuromuscular activity, it preserves the connectivity between the motoneurons and innervated muscles and thus maintains the integrity of neuromuscular biochemical processes. The present results indicate that the total amount and duration of daily EMG activity after 7 or 30 day of SI was <1% of the pre-SI levels. The levels of all the molecular systems examined in the spinal cord segments affected by the isolation, except for GAP-43, were downregulated by the imposed inactivity. These results strongly imply that the levels of supraspinal and afferent input modulate the effects of specific neurotrophins in the maintenance of normal functionality and neuroplasticity in the spinal cord. Indeed, evidence indicates that voluntary exercise can influence the basic biophysical properties of motoneurons, making neurons more excitable (Beaumont and Gardiner 2002). Further evidence for this activity-dependent modulation is the observed upregulation in BDNF and synapsin I in the cervical enlargement of the spinal cord, presumably as a result of enhanced activity of the forelimbs as a consequence of hindlimb paralysis. This scenario is consistent with our previous observations of exercise-induced increases in BDNF and synapsin I expression in the spinal cord (Gomez-Pinilla et al. 2002). In addition, this is also consistent with increases in BDNF and NT-3 levels in the C_3–C_6 segments after cervical dorsal rhizotomy, a model that decreases tonic inhibition to the respiratory system and thus enhances activity of selected neural populations in the spinal cord cervical region (Johnson et al. 2000).

Effects of neuromuscular synapse blocking

We blocked neuromuscular transmission to a single muscle, i.e., the soleus, using BTX-A, and one striking result was an increase in NT-3 expression in the spinal cord segments that normally receive afferent projections from the paralyzed muscle. It has been shown previously in *Xenopus* neuron/myocyte co-cultures that activity-dependent postsynaptic signals origi-

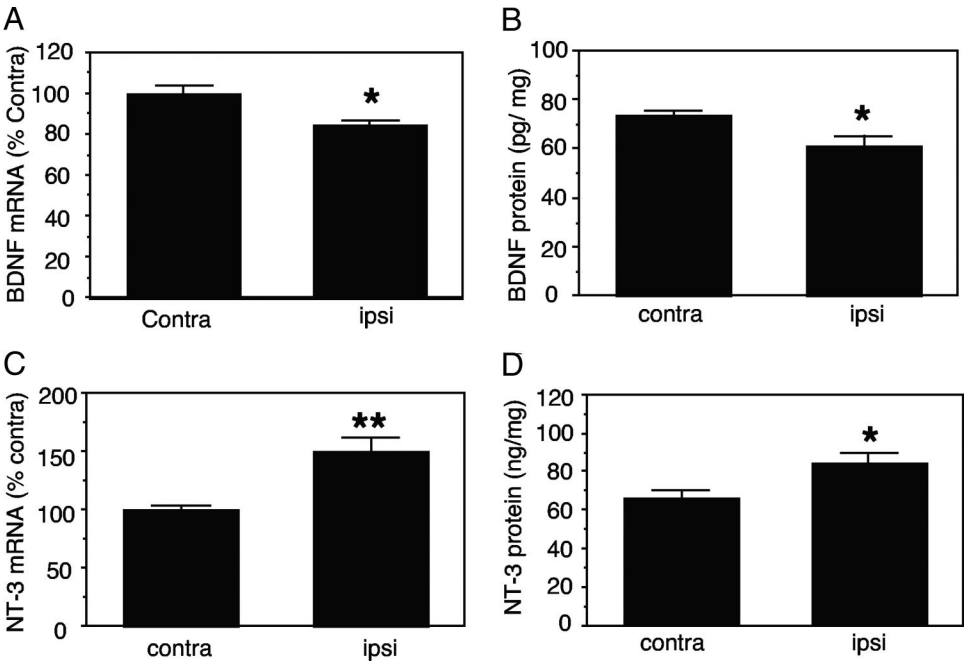

FIG. 8. BTX-A was injected into the soleus muscle unilaterally (ipsi, ipsilateral) to determine the effects of blocking its activation on the basal expression of BDNF and NT-3. BDNF mRNA (*A*) and protein (*B*) levels were lower in the lumbar region of the ipsilateral (ipsi) than contralateral (contra) hemi-cord 7 days after BTX-A treatment. mRNA levels for the ipsi side are expressed as a percent of contra control values. NT-3 mRNA (*C*) and protein (*D*) levels were higher in the ipsi than contra hemi-cord of the same rats. Protein levels were determined using ELISA. Values are means ± SE for 6 rats per group used for mRNA and protein analyses. * and **significantly different from contra control at $P < 0.05$ and $P < 0.01$, respectively; ANOVA, Fisher test.

nating from muscle cells can modulate presynaptic excitability via a mechanism involving NT-3 (Nick and Ribera 2000). NT-3, but not BDNF, application reversed these effects, suggesting that NT-3 can act as an activity-dependent retrograde signal. Therefore in the present studies, the upregulation of NT-3 expression after blocking the soleus neuromuscular junctions with BTX-A may be linked in some specific way with a NT-3-mediated interdependence of motoneurons and their target muscles. It is known that habitual activity influences biophysical properties of the neuromuscular transmission (Desaulniers et al. 2001), therefore the lack of activity likely modifies the biophysical properties of the interdependence between motoneurons and muscle. Alternatively, elevated NT-3 may have been due to a compensatory increase in supraspinal input rather than a loss of proprioceptive feedback from the soleus muscle (Canedo 1997). In this case, it would be apparent that such supraspinal input would have different modulatory effects on BDNF and NT-3 in our paradigm.

Similar to SI, paralysis of the soleus muscle resulted in a downregulation of BDNF and synapsin I in the lumbar spinal cord region. It is particularly striking that the paralysis of one small muscle from the entire hindlimb could result in such a significant reduction in BDNF in the ipsilateral spinal cord. The soleus muscle, however, does have a higher density of muscle spindles than most other hindlimb muscles (Peck et al. 1984; Swett and Eldred 1960). In addition, there is an extensive divergence of the Ia afferents among the motor pools, such that silencing a single Ia afferent could impact many motoneurons and Ia interneurons (Nelson and Mendell 1978) (Fig. 1*B*). However, it is not known whether silencing all of the muscle spindles within the soleus would be sufficient to silence the motoneurons they project to because these motoneurons receive input from many other sources. The overlapping sensory input from other muscles may attenuate the effects of this extensive divergence across several segments. The level of divergence of other peripheral afferent fibers that could have played a role in the present results is also substantial, but there has been less quantitative assessment of their extent.

BDNF versus NT-3 modulation

Interestingly, the use of the SI and neuromuscular synaptic block models to depress neural input to the spinal cord was instrumental in identifying a differential regulation of NT-3, i.e., a downregulation in the lumbar region after SI but an upregulation after BTX-A injection. Another intriguing finding was that NT-3 mRNA levels were unaffected in the cervical region (rostral to the isolated portion of the spinal cord) after either intervention in spite of elevated levels of BDNF and the previous reports showing that exercise upregulates NT-3 in the

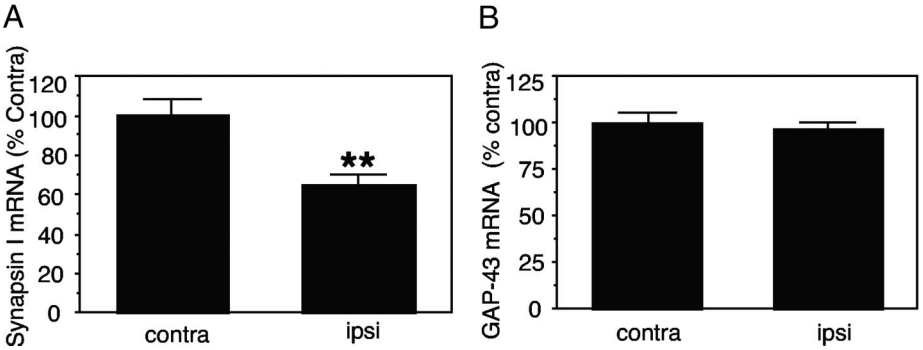

FIG. 9. Relative levels of synapsin I mRNA (*A*) and GAP-43 mRNA (*B*) were measured in the lumbar region of the spinal cord in control and BTX-A treated rats. After 7 days, the levels of synapsin I were higher and GAP-43 similar in the ipsilateral (ipsi) compared with the contralateral (contra) hemi-cord. Values are means ± SE for 6 rats per group and are expressed as a percent of contra values. **, significantly different from control at $P < 0.01$; ANOVA, Fisher test.

spinal cord (Ying et al. 2003). One possible explanation for this apparent discrepancy is that the increase in the amount of work performed by the forelimbs may have not been sufficient to elevate the levels of NT-3. An alternative possibility may be related to the nature of the modulation of NT-3 by neural activity. According to the literature, neural activity has the opposite effects on BDNF and NT-3 expression, and, in turn, BDNF and NT-3 elicit different electrophysiological responses when applied to neuronal preparations in vitro. For example, seizure activity results in increased BDNF but decreased NT-3 levels in the hippocampus (Gall and Lauterborn 1992). Although NT-3 can depolarize central neurons, its effects are much less robust than those elicited by BDNF (Kafitz et al. 1999). However, it is well known that NT-3 plays an important role in the regulation of the excitability of spinal cord cells and motoneuron spindle physiology (Mendell et al. 1999). Therefore although NT-3 appears to play an important role in spinal cord activity-dependent plasticity, its regulation by neuromuscular activity levels seems to be more complex than that for BDNF. Differential regulation of NT-3 and BDNF in the spinal cord has also been reported after cervical dorsal rhizotomy (Johnson et al. 2000).

Possible roles of activity-regulated neurotrophins in spinal cord plasticity

To evaluate the possible functional implications for changes in the neurotrophin system resulting from decreased neuromuscular activity, we measured the levels of various molecules that have a recognized interaction with BDNF and NT-3 and are important for synaptic plasticity. Similar to BDNF, the levels of synapsin I mRNA and protein were downregulated in the isolated portion (lumbar) of the spinal cord of SI rats and upregulated in a spinal cord region rostral (cervical) to the isolated segments. The coordinated response between BDNF and synapsin I is not surprising given the established association between these two molecular systems. BDNF phosphorylates synapsin I to induce the mitogen-activated protein kinase signaling pathway, which, in turn, modulates neurotransmitter release (Jovanovic et al. 2000). In addition, we have been able to reduce the exercise-induced increase in synapsin I mRNA in the hippocampus by blocking BDNF action using the tyrosine kinase receptor blocker K252A (Vaynman et al. 2003). Therefore the change in synapsin I after SI in the present study is a likely indicator of modified synaptic plasticity and function associated to the function of BDNF.

It is interesting that SI resulted in an upregulation in GAP-43 mRNA in the lumbar region of the spinal cord. The involvement of GAP-43 in axonal growth (Benowitz and Routtenberg 1997) suggests that the increase in GAP-43 may be associated with synaptic reorganization of the spinal circuitry in response to the loss of both supraspinal and afferent input. The noninjured neurons intrinsic to the spinal cord may generate additional synaptic projections on cell surfaces that have been vacated by the supraspinal and afferent projections. Indeed, there are previous accounts that denervation upregulates GAP-43 and induces a sprouting response in motoneurons (Harding et al. 1999). This sprouting response would be consistent with an increase in BDNF and GAP-43 mRNAs in the cervical region as it is known that BDNF promotes axonal sprouting in the spinal cord (Jakeman et al. 1998). An alternative explanation for the reductions of neurotrophins and synaptic associated proteins in the isolated spinal cord region could be related to a surgically induced loss of neurons or their projections that supply neurotrophins. The application of BTX to a single muscle (soleus) is highly unlikely to have induced a loss of neurons or their projections within the ipsilateral spinal cord.

The present results are consistent with the viewpoint that basal levels of neural activity play a critical role in maintaining the expression of select molecular systems in the spinal cord. These results also suggest that the differential modulation of BDNF and NT-3 in the spinal cord may depend on the relative activation levels of specific neural circuits. It appears that the sensory input from muscles that project to the spinal cord is a major source of modulation of BDNF and NT-3. Therefore neuromuscular activity seems to be an important element in the modulation of these neurotrophins and of the molecular events associated to synaptic plasticity in the spinal cord.

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GRANTS

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