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Treadmill training stimulates BDNF mRNA expression in motor neurons of the lumbar spinal cord in spinally transected rats

Michael S. Joseph¹, Niranjala J.K. Tillakaratne², and Ray D. de Leon³

¹Department of Biological Science, California State University, Los Angeles

²Department of Integrative Biology and Physiology and Brain Research Institute, University of California, Los Angeles

³School of Kinesiology and Nutritional Science, California State University, Los Angeles

Abstract

Brain derived neurotrophic factor (BDNF) induces plasticity within the lumbar spinal circuits thereby improving locomotor recovery in spinal cord injured animals. We examined whether lumbar spinal cord motor neurons and other ventral horn cells of spinally transected (ST) rats were stimulated to produce BDNF mRNA in response to treadmill training. Rats received complete spinal cord transections as neonates (n=20) and one month later, received 4 weeks of either a low (100 steps/training session; n=10) or high (1000 steps/training session; n=10) amount of robotic-assisted treadmill training. Using combined non-radioactive *in situ* hybridization and immunohistochemical techniques, we found BDNF mRNA expression in HSP27-labeled motor neurons and in non-motor neuron cells was greater after 1000 steps/training session compared to the 100 steps/training session and was similar to BDNF mRNA labeling in untrained intact rats. In addition, there were significantly more motor neurons that contained BDNF mRNA labeling within processes in the ST rats that received the higher amount of treadmill training. These findings suggested that motor neurons and other ventral horn cells in spinally transected rats synthesized BDNF in response to treadmill training. The findings support a mechanism by which postsynaptic release of BDNF from motor neurons contributed to synaptic plasticity.

Keywords

body weight supported treadmill training; plasticity; neurotrophins; locomotion

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Send Correspondence to: Ray D. de Leon, Ph.D., Professor, Department of Kinesiology and Nutritional Science, California State University, Los Angeles, 5151 State University Drive, Los Angeles, CA 90032-8162, ph (323) 343-4855, fx (323) 343-6024, rdeleon@calstatela.edu.

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INTRODUCTION

The neurotrophin, brain derived neurotrophic factor (BDNF), improves the ability of the lumbar spinal cord to generate locomotion in spinal cord injured animals (Boyce et al., 2007a, Ying et al., 2008, Boyce et al., 2012). The mechanisms are unknown, but it is believed that BDNF triggers changes in the spinal circuits by raising the excitability of spinal neurons (Boyce et al., 2012) and strengthening synaptic connections within the circuitry (Ying et al., 2005). The beneficial effects can be produced by the delivery of exogenous BDNF to the injured spinal cord (Boyce et al., 2012) but BDNF levels are also increased by hindlimb exercise (Hutchinson et al., 2004, Ying et al., 2005, Macias et al., 2009, Sandrow-Feinberg et al., 2009, Cote et al., 2011, de Leon et al., 2011). Treadmill training in particular is effective in raising BDNF levels (Hutchinson et al., 2004). Thus, stimulating the production of endogenous BDNF promotes plasticity within the spinal circuits and leads to improved locomotor recovery after spinal cord injury.

Interestingly, the cells that synthesize and release BDNF in spinal cord injured animals are unknown. We and others have shown that treadmill training increased BDNF protein expression in motor neurons of spinally transected (ST) rats (Macias et al., 2009, de Leon et al., 2011). These findings suggest that BDNF was secreted by motor neurons in response to treadmill training. However, it was not clear whether the motor neurons themselves synthesized BDNF or if the BDNF was derived from other cells such as muscle fibers (Gomez-Pinilla et al., 2002). Large increases in BDNF mRNA levels in hindlimb muscles were stimulated by a single bout of hindlimb exercise in ST rats (Dupont-Versteegden et al., 2004), suggesting target hindlimb muscles synthesized BDNF then retrogradely transported BDNF to motor neurons (Koliatsos et al., 1993). In order to understand the possible contribution of spinal neurons to BDNF levels in the lumbar spinal cord, it was necessary to demonstrate that synthesis of BDNF mRNA occurred in spinal neurons. Only one study to date has examined cellular expression of BDNF mRNA expression in spinal cord injured animals (Keeler et al., 2012). In this study, passive cycling of the hindlimbs was sufficient to raise BDNF mRNA levels in motor neurons, raising the possibility that activity during treadmill training would have a similar effect. In addition to motor neurons, other cells in the ventral horn, e.g. interneurons, glial cells, may produce BDNF that influenced enhanced plasticity, but their contribution to the BDNF pool has not yet been examined in exercised spinal cord injured animals.

In the present study, we examined whether treadmill training in ST rats influenced motor neuronal expression of BDNF mRNA. We used *in situ* hybridization and immunohistochemical techniques (Tillakaratne et al., 2002) to study the expression of BDNF mRNA in HSP27-labeled motor neurons and other ventral horn cells in rats that received a complete mid-thoracic spinal cord transection at five days of age. Previous studies have shown that BDNF mRNA is targeted to dendrites of postsynaptic neurons and locally translated into protein (An et al., 2008, Chiaruttini et al., 2009). Thus, we included an analysis of BDNF mRNA in motor neuron processes. A robotic treadmill system was used to train the ST rats to perform either 100 or 1000 steps/training session and the kinematic data from this study have previously been reported (Cha et al., 2007). Here, we hypothesize that imposing a higher amount of treadmill training would result in a greater synthesis of

BDNF by motor neurons and affect its subcellular localization. The findings were consistent with this hypothesis and support a mechanism in which postsynaptic release of BDNF from motor neurons contributed to plasticity within the lumbar spinal circuits controlling locomotion.

METHODS

Experimental design

Twenty female Sprague-Dawley rats received a complete mid-thoracic spinal transection at five days of age. After weaning (21 days old), a robotic device was used to assess the ability of the rats to perform hindlimb stepping on a treadmill. The rats were distributed into two experimental groups that were balanced according to their locomotor performance during the baseline tests. One group (n=10) received daily treadmill training that consisted of 100 steps/session while the other group (n=10) performed 1000 steps/training session and these rats will be referred to as the ST100 and ST1000 rats respectively. Training was performed five days/week for four weeks. The animals were perfused with 4% paraformaldehyde, the spinal cords were removed and processed for the histology, *in situ* hybridization and immunohistochemical experiments. All procedures with rats were carried out in accordance with NIH guidelines and the protocols were approved by the Institutional Animal Care and Use Committee at California State University, Los Angeles.

Spinal cord transection

The spinal cords of the rats were transected at a mid-thoracic level as previously described (Cha et al., 2007). Briefly, the pups were anesthetized using isoflurane (1%). A dorsal mid-line skin incision was made over the mid-thoracic vertebra and the overlying fascia and muscles were retracted to expose the dorsal surface of the vertebrae. A partial laminectomy was performed at the mid-thoracic level to expose the spinal cord. The spinal cord was then lifted with a curved probe and completely transected. Afterwards, the skin incision was closed with sutures. The entire surgical procedure took about 10–15 minutes.

Following surgery, the rats were allowed to recover in a warm incubator. The temperature was maintained at 37°C. The neonatal pups were placed in the incubator until fully alert (10–30 min) and then returned to the mothers. After the rats were weaned (21 days old), the rats were housed in spacious cages, 2–3 rats per cage. The bladders and colons of the rats were checked daily.

Robotic-Assisted Treadmill Training

A commercially-available robotic device (Rodent Robot 3000, Robomedica Inc.) was used to train treadmill stepping in the rats. It consisted of two robotic arms that were attached to the ankles of the rat, a motorized body weight support system and a treadmill (Cha et al., 2007). A thin, padded strip of neoprene was placed around the rat's ankle. A metal clip at end of the robotic arm held the two ends of the neoprene strip together to form a loop around the ankle. A soft vest was placed over the shoulders of the rat and was attached to a mechanical arm, which raised the rat's body above the treadmill and controlled the amount of weight exerted on the hindlimbs. The robotic device was used to count the number of

steps performed by the rats as previously described (Heng and de Leon, 2009). Briefly, a step was detected whenever the robotic arm was displaced by 1 mm in the horizontal direction. A training session was completed when the total number of steps performed by both hindlimbs was 100 or 1000 steps.

Tissue Preparation

Beginning two hours after the last training session, the rats were anesthetized with isoflurane (1%) followed by intracardiac perfusion with 4% paraformaldehyde in Sorensen phosphate buffer (Tillakaratne et al., 2002). The spinal cord was dissected and was post-fixed in 30% sucrose solution for a period of 48–72 hours for cryoprotection and then embedded in Tissue-Tek compound, as spinal cord blocks. Transverse sections (18 μ m thick) of the spinal cords were cut using a cryostat and collected as free-floating sections in PBS. After washing in PBS, adjacent sections were processed for *in situ* hybridization and immunohistochemistry. The tissue sections used to compare the experimental groups were processed simultaneously. To minimize tissue damage that may occur with tissue handling, free-floating sections were processed in net-wells (75 μ m mesh; Costar, Cambridge, MA). Spinal cord sections in net-wells were transferred sequentially to net-well trays containing appropriate solutions. Incubation with c-RNA probes, antibodies, and ribonuclease A (Sigma, St. Louis, MO) and color reactions were performed in 24-well plates.

Non-Radioactive In Situ Hybridization and Immunohistochemistry

In order to localize BDNF mRNA in the spinal cord, *in situ* hybridization (ISH) using Digoxigenin-labeled (DIG) RNA probes was performed. DIG-labeled BDNF riboprobe was prepared from a cDNA template coding for the full-length rat BDNF gene (kindly provided by Dr. Amelia Russo-Neustadt). A pBluescript KS 700 base pair fragment, linearized with *Xba*I, and transcribed with T3 RNA polymerase, generated the anti-sense cRNA. Linearization with *Hind*III and transcription with T7 RNA polymerase generated the sense cRNA. For non-radioactive in situ hybridization, a mix of unlabeled and digoxigenin (DIG) labeled Uracil (Roche Applied Sciences, Indianapolis, IN) was used as described previously (Tillakaratne et al., 2002). The concentration of DIG-labeled ribo-probe was then quantified using known amounts of a control labeled probe provided in the RNA detection kit (Roche Applied Sciences, Indianapolis, IN). Following the quantification of the probe, the proper working concentration was optimized for the spinal cord tissues. The rat hippocampus was used as a positive control.

Five spinal cord sections from each rat representing lumbar segments L2–L5 were selected for ISH. Each hybridization well contained 20 μ g labeled probe/100 μ l hybridization solution consisting of 50% dextran sulfate, 250 μ g/ μ l salmon sperm DNA, 50% Formamide, 5X hybridization salt 1X Denhardt's solution, and DEPC water as described before (Tillakaratne et al., 2002). Hybridization was carried out overnight in a 52°C incubator. Antibody against dig conjugated to a peroxidase (anti-DIG-POD) (Roche Applied Scientific, Indianapolis, IN) was used to detect DIG-labeled hybrids. Next, the Tyramide Signal Amplification (TSA) assay (PerkinElmer, Waltham, Ma) with Cyanin 3 was used to amplify the fluorescent signal in ISH.

Following the BDNF mRNA labeling using non-radioactive ISH, the motor neurons were labeled using an antibody against heat shock protein 27 (HSP27; Santa Cruz Biotech, Santa Cruz, CA). A previous study has shown that HSP27 is expressed in motor neurons and can be reliably used to identify them (Plumier et al., 1997). The IHC process begins with three rounds of washes in PBS and into one hour of blocking with 3% normal donkey serum. The tissue was then transferred into wells containing rabbit anti- HSP27 1:500 diluted in PBS into a 96 well-plate and incubated overnight at 4°C with slow continuous shaking. After 16–18 hrs the spinal cord sections was transferred into net-wells and washed in PBS three times. The sections were then transferred to wells in a 24 well plate containing the secondary antibody (anti rabbit IgG, conjugated with Fluorescein-isothiocyanate FITC, 1:500, (Jackson ImmunoResearch Lab, West Grove, PA) diluted in PBS and incubated for one hour at room temperature. Following the incubation, sections were again washed in PBS three times and mounted on microscope slides and cover-slipped with Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA) for visualization and protection from photo bleaching.

Image analysis

A semi-quantitative analysis was performed to measure BDNF mRNA in motor neurons and other surrounding cells in the ventral horn. Three-five spinal cord sections from each animal were analyzed. Microscopic images were acquired under uniform conditions for all spinal cord sections using C-Imaging software (Compix Inc., PA) under Leica DMLA microscope equipped with a Hamamatsu Digital color camera. A region of interest (ROI) was drawn around the ventral horn using the dorsal edge of the central canal as the vertical border. Objects (cells) with BDNF mRNA label (red) and/or HSP27 label (green) were identified based on intensity values relative to a threshold level. Motor neurons were differentiated from non-motor neuron objects based on size of the object and HSP27 label. After background labeling was subtracted from the images, the mean red (intensity per pixel) value corresponding to BDNF mRNA label was subsequently measured within each identified motor neuron and non-motor neuron object. Motor neurons with processes expressing BDNF mRNA were identified by carefully inspecting the HSP27-positive processes emanating from the soma. A motor neuron process was considered to contain BDNF mRNA only if BDNF mRNA label (red) could be unambiguously detected within the outline of the HSP27-positive (green) process. For each rat, the total number of motor neurons (Fig. 3A), total number of non-motor neuron objects (Fig. 5A) and total number of motor neurons with BDNF mRNA labeled processes was calculated by summing the number of identified objects found across tissue sections. The intensity values for motor neurons (Fig. 3B) and for non-motor neuron objects (Fig 5B) was averaged across tissue sections for each rat.

Statistical Analyses

Group means for the number of motor neurons, number of non-motor neurons and intensity per tissue section were calculated by averaging the values from individual rats in each group. One-way ANOVA with Tukey post-hoc test was used to determine significant differences between the Intact, ST100 and ST1000 group means. Histograms of motor neuron BDNF mRNA label intensity were constructed for each group (Fig 4). The group

distribution of intensity values in motor neurons contained within a spinal cord section was calculated by averaging the within-tissue-section distributions from individual rats. The interquartile range of intensities was calculated by subtracting the intensities corresponding to the upper and lower quartiles. Correlations between BDNF mRNA labeling and stepping performance were analyzed using Pearson's correlation with significance test. For each group, the average intensity of BDNF mRNA label for a rat was plotted against the average area of the step cycle for the rat and the Pearson correlation (r) was calculated. All statistical analyses were performed using SPSS (17.0 for Windows Software).

RESULTS

Cellular expression of BDNF mRNA in the ventral horn

Figure 1 shows an example of BDNF mRNA and HSP27 labeling in the ventral horn of a representative ST rat (see red and green labeling respectively in Fig. 1). HSP27-positive motor neurons expressed BDNF mRNA label (see yellow-colored cells in Fig. 1A) but there were also numerous smaller cells (i.e. non motor neurons) that expressed BDNF mRNA label and they were found primarily in Lamina VII and VIII (see arrows in Fig. 1A). This pattern of HSP27 and BDNF mRNA labeling was observed in the Intact rats as well as the ST rats that received 100 or 1000 steps of training (see Fig. 2). BDNF mRNA labeling filled most of the cell body of motor neurons (see Fig. 1B–D), however, BDNF mRNA labeling was also observed in processes emanating from the motor neuron cell body (see arrowheads in Fig. 1E–G).

Comparison of motor neuronal expression of BDNF mRNA between the Intact, ST100 and ST1000 rats

Figure 2 shows BDNF mRNA labeling in the ventral horn of representative Intact, ST100 and ST1000 rats. HSP27-positive motor neurons were observed in ventral horn of Intact, ST100 and ST1000 rats (see green cells in Fig. 2A–C). BDNF mRNA labeling in HSP27-positive motor neurons was greater in the Intact and ST1000 rats than in the ST100 rats (see yellow-orange cells in Fig. 2D, E, F). The amount of motor neuronal BDNF mRNA labeling in the ST1000 rats was similar to the amount observed in the Intact rats (compare Fig. 2D and F). Semiquantitative analysis of BDNF mRNA label intensity in HSP27-positive motor neurons was performed. On average, the number of motor neurons analyzed in each rat was 94.4 ± 13.4 , 109 ± 19.2 , 111.5 ± 21.1 for the Intact, ST100 and ST1000 groups respectively and there was no significant difference in these values between the groups based on an analysis of variance ($F[2,26] = 0.285$, $p = 0.755$; Fig. 3A). However, there was a significant difference in the intensity of BDNF mRNA label in motor neurons between groups ($F[2,26] = 5.912$, $p = 0.008$; Fig. 3B). Post-hoc comparisons revealed that the intensity of BDNF mRNA label in motor neurons was significantly greater in the ST1000 and Intact rats relative to the ST100 rats (Fig. 3B). No differences between the Intact and ST1000 groups were found.

A comparison of BDNF mRNA label in motor neuronal processes between the groups was next performed. Only motor neurons with HSP27-positive processes were included in these analyses (e.g. see arrows Fig 2). The percentage of motor neurons with HSP27-positive processes was $73.7 \pm 2.5\%$, $77.5 \pm 2.8\%$ and 76.3 ± 3.3 in the Intact, ST100 and ST1000 groups

respectively and these values were not significantly different between the groups ($F[2,26] = 0.451$, $p = 0.642$; Fig. 3C). However, there was a significant difference in the percentage of motor neurons that contained BDNF mRNA label in processes ($F[2,26] = 10.918$, $p = 0.001$; Fig. 3D). Specifically, $78.8 \pm 3.6\%$ and $59.1 \pm 9.9\%$ of motor neurons in Intact and ST1000 rats respectively had BDNF mRNA labeling in processes. In contrast, only $28 \pm 8.1\%$ of motor neurons in ST100 rats had BDNF mRNA labeling in processes and this value was significantly less than Intact and ST1000 values based on post-hoc comparisons (Fig. 3D).

The expression of BDNF mRNA labeling varied in motor neurons found in the same spinal cord section. For example, although most motor neurons expressed BDNF mRNA label, there were some motor neurons that had very low levels of BDNF mRNA labeling (see Fig. 1H–J). Figure 4 shows histograms of the intensity of BDNF mRNA labeling in motor neurons found in the same spinal cord section. There was a range of BDNF mRNA labeling intensities in the Intact, ST100 and ST1000 rats (Fig. 4). The mean interquartile ranges (Intact: 29.2, ST100: 21.0, and ST1000: 28.1) were not significantly different between the groups.

Expression of BDNF mRNA in non-motor neuronal cells

Other cells in the ventral horn besides motor neurons expressed BDNF mRNA (see arrows in Fig. 1A). The intensity of BDNF mRNA labeling in these non-motor neuronal cells was significantly different between groups based on analysis of variance ($F[2,26] = 4.456$, $p = 0.023$). Post-hoc comparisons revealed a significant difference between the Intact and ST100 rats (Fig. 5B). No significant differences in the number of non-motor neuronal cells expressing BDNF mRNA were found between the groups ($F[2,26] = 0.513$, $p = 0.605$; Fig. 5A).

BDNF mRNA in the ventral horn cells was not correlated with stepping ability

The ST1000 rats recovered significantly better stepping than the ST100 rats and these behavioral and kinematic data have been reported elsewhere (Cha et al., 2007). Here, we examined the correlation between BDNF mRNA labeling and step area. In the ST1000 rats, there was a positive correlation between the BDNF mRNA label in motor neurons and step area but this correlation was not statistically significant (Fig. 6A; $p = 0.353$). In contrast, BDNF mRNA label in motor neurons of the ST100 rats was negatively correlated with step area, but this correlation was also not statistically significant (Fig. 6B; $p = 0.359$). For non-motor neurons, BDNF mRNA labeling was positively correlated with step area in the ST1000 (Fig. 6C) rats but negatively correlated with step area in the ST100 rats (Fig. 6D). These correlations were also not statistically significant ($p = 0.611$ for ST1000 and $p = 0.378$ for ST100).

DISCUSSION

In summary, we found that the expression of BDNF mRNA in the motor neurons and non-motor neurons in the ventral horn of the lumbar spinal cord was greater in ST rats that received a high amount of treadmill training relative to a low amount and resembled the normal expression. BDNF mRNA labeling was present in motor neuron processes, but the

high amount of training resulted in a greater number of motor neurons containing BDNF mRNA label in processes than the low amount of training. BDNF mRNA expression was not correlated with stepping performance in the ST rats.

BDNF synthesis by motor neurons was enhanced by treadmill training following spinal cord injury

Previous studies have shown that motor neurons in the lumbar spinal cord expressed BDNF mRNA (Buck et al., 2000) and that treadmill training increased motor neuronal expression of BDNF mRNA in intact rats (Macias et al., 2007). The present results expand on these findings by demonstrating that the effect of treadmill training on BDNF mRNA expression occurred even in animals that had a complete spinal cord injury. Our findings were consistent with a recent report that hindlimb cycling exercise in ST rats increased BDNF mRNA expression in motor neurons (Keeler et al., 2012). Whether changes in BDNF protein expression were coincident with the observed BDNF mRNA expression cannot be determined from the present study. However, we and others have shown that BDNF protein in motor neurons was increased by treadmill training in ST rats (Macias et al., 2009, de Leon et al., 2011). Taken together, these findings suggested that motor neurons in the lumbar spinal cord were stimulated by treadmill exercise to synthesize BDNF. In addition to muscle-derived BDNF, BDNF synthesized by motor neurons may provide another source of BDNF used to enhance the plasticity after spinal cord injury.

Treadmill training in spinally transected animals improved hindlimb function in part by influencing synaptic inputs to motor neurons (Tillakaratne et al., 2002, Cote et al., 2003, Cote and Gossard, 2004, Macias et al., 2009, Cote et al., 2011, de Leon et al., 2011, Ichiyama et al., 2011). In other systems, activity-dependent release of BDNF from the dendrites of postsynaptic neurons influenced synaptic plasticity (for review see (Kuczewski et al., 2012). A similar phenomenon may be occurring with synapses onto motor neurons. Under this scenario, BDNF secreted by motor neuron dendrites affected synaptic inputs onto the motor neurons themselves or perhaps modulated other, nearby synapses. In the present study, BDNF mRNA labeling was observed in motor neuron processes (presumably, some of these were dendrites) and the expression in these processes was augmented by training. Other studies have reported BDNF protein to cluster within motor neuron dendrites following treadmill training (Skup et al., 2002, Macias et al., 2005). These findings are consistent with targeting of BDNF mRNA to the dendritic compartment and local translation into BDNF protein (An et al., 2008, Chiaruttini et al., 2009).

Recent findings suggested that another neurotrophin, NT-3, enhanced motor neuronal inputs within the lumbar spinal circuitry. Viral-delivery of NT-3 resulted in enhanced excitatory synaptic potentials recorded from ankle extensor motor neurons in ST rats (Boyce et al., 2012) and a similar result was reported in intact rats (Petruska et al., 2010). Interestingly, delivery of BDNF did not affect excitatory synaptic potentials. Based on electrophysiological data and c-fos labeling in the spinal cord, the effect of BDNF was to raise the level of excitation in motor neuron and interneurons, some of which may have been involved in central pattern generation (Boyce et al., 2012). Endogenous BDNF derived from

motor neurons may have a similar effect on neuronal excitability but clearly further studies are necessary to examine the role of exercise-induced BDNF on spinal plasticity.

One important question was how much treadmill exercise was necessary for stimulating BDNF synthesis by spinal neurons? Based on the present findings, a higher amount of treadmill training (1000 steps/session) was better than the lower amount (100 steps/session). Compared to previous studies of BDNF mRNA, the exercise regimen in the present study can be considered low intensity. Intact rats in other studies ran on the treadmill for 30–80 minutes/session (Gomez-Pinilla et al., 2001, Macias et al., 2007). In the present study, the treadmill speed was kept low (i.e. walking speed) to facilitate stepping in the ST rat and the rats were trained for shorter durations (approximately 5 and 20 minutes for the 100 and 1000 rats respectively). The number of treadmill training sessions was another factor determining BDNF mRNA expression. In intact rats, a single treadmill training session was not sufficient for increasing BDNF mRNA (Gomez-Pinilla et al., 2001). Only when rats underwent five or more treadmill training sessions was there an increase in BDNF mRNA (Gomez-Pinilla et al., 2001). The rats in the present study underwent a total of 20 training sessions. Taken together, these findings suggested that imposing low intensity treadmill training may be a sufficient stimulus for BDNF synthesis if it is continued over a long period of time. This finding has implications for body weight supported treadmill training which is typically carried out at slower treadmill speeds for individuals with spinal cord injury.

We chose to use a group that received a low amount of exercise rather than an untrained ST control as has been commonly used in previous studies. Our rationale was that BDNF mRNA levels are influenced by non-specific factors related to the robotic training protocol, in particular restraint of the rats during training and robotic linkage stimulating the ankle. Hippocampal BDNF mRNA levels were influenced by restraint alone (Smith et al., 1995). Since the ST100 group received robotic training, effects due to restraint of the rats or stimulation from the robotic linkages on BDNF mRNA were taken into account. It is possible that imposing a low amount of training was sufficient to stimulate BDNF mRNA expression but this cannot be determined in the present study. If BDNF synthesis was activity-dependent, then one would expect that the low amount of training would result in greater BDNF mRNA expression than in the absence of training. We previously showed that training ST rats to perform 100 step/training session did not significantly raise BDNF protein expression in motor neurons suggesting the low amount of training did not generate enough activity to stimulate BDNF protein (de Leon et al., 2011). This finding suggested that there may be a threshold level of activity that was necessary to induce BDNF protein and perhaps mRNA expression.

Cellular expression of BDNF mRNA was not associated with improved locomotor function

The positive but insignificant correlation between BDNF mRNA expression in motor neurons and the kinematic variable, step area suggested that the increased BDNF synthesis by motor neurons was not associated with improved locomotor recovery. We previously showed that BDNF protein expression in the ventral horn was raised by treadmill training in ST rats there was a significant correlation between the overall expression of BDNF protein in the ventral horn region and locomotor recovery (de Leon et al., 2011). other studies have

reported that the overall levels of BDNF protein and mRNA in the lumbar spinal cord were significantly correlated with stepping recovery in exercised spinal cord injured rats (Ying et al., 2005).

One explanation for the lack of correlation to locomotor recovery may be related to the variability in BDNF mRNA expression that was observed in the ventral horn. BDNF mRNA expression in motor neurons ranged from low to high in the ST and Intact rats (Fig. 4). This conclusion was based on the intensity of labeling observed in motor neurons found *within* the same tissue section thus, the variability was not due to other factors that potentially influenced intensity values across different tissue sections. The repetitive use of specific spinal pathways during treadmill training would be expected to result in BDNF synthesis by specific motor pools. We performed a broad analysis of motor neurons and this may have reduced the strength of correlations to locomotor recovery particularly if localized motor pools were recruited during training. The idea that treadmill training differentially modulated hindlimb motor pools is supported by previous findings (Tillakaratne et al., 2002, Khristy et al., 2009). For example, treadmill training in ST rats increased in GABA receptor expression in ankle extensor motor pools but decreased receptor expression in flexor motor pools (Khristy et al., 2009). Further studies are necessary to explore the differential cellular expression of BDNF expression by integrating retrograde labeling of motor pools.

Other cells in the ventral horn, particularly in Lamina VII and VIII that were not motor neurons also expressed BDNF mRNA. The expression of BDNF mRNA in these cells was affected by training and similar to motor neurons, the expression of BDNF mRNA in these cells was not significantly correlated with stepping recovery. Unfortunately, other markers for neurons and glial cells were not used in the present study, thus, the identity of these cells is unknown. Some of these cells may have been interneurons that were involved in the generation of stepping and like motor neurons, training may have induced activity in these interneurons thereby stimulating them to synthesize BDNF. Glial cells cannot be ruled out though since microglia have been shown to express BDNF in spinal cord injured rats (Dougherty et al., 2000). In any case, the present findings suggested that in addition to motor neurons, other ventral horn cells may contribute to the pool of BDNF within the spinal cord circuitry.

Clinical implications

Body weight supported treadmill training has been shown to be effective in improving locomotor function in spinal cord injured patients (Behrman et al., 2005). Understanding BDNF's role in spinal plasticity may lead to new interventions that enhance the effectiveness of this form of therapy. Past studies have successfully delivered BDNF and NT3 to the lumbar spinal cord to improve locomotor recovery in spinal cord injured animals (Boyce et al., 2007b, Boyce et al., 2012). While more studies are necessary, the present findings indicated that spinal plasticity may be under the control of endogenously-produced neurotrophins such as BDNF. BDNF from spinal cord cells and muscle fibers may trigger plasticity within the locomotor-generating circuitry of the spinal cord. If this hypothesis is true, the implications are that individuals with spinal cord injury may benefit from activity-based therapies that optimally stimulate the synthesis and release of endogenous BDNF.

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ABBREVIATIONS

mRNA	messenger ribonucleic acid
BDNF	brain derived neurotrophic factor
HSP27	heat shock protein 27
ST	spinally transected
ST1000	spinally transected + treadmill training for 1000 steps/session
ST100	spinally transected + treadmill training for 100 steps/session

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HIGHLIGHTS

- Spinally transected rats received robotic treadmill training
- Training increased BDNF mRNA expression in motor neuron soma and processes
- These findings suggested BDNF from motor neurons contributed to spinal plasticity

Figure 1.

Expression of BDNF mRNA in ventral horn cells of a representative ST rat. (A) BDNF mRNA labeling (red) in HSP27-positive motor neurons (green) and other ventral horn cells. Overlap of BDNF mRNA and HSP27 label (yellow-orange) indicates HSP27 positive motor neurons expressing BDNF mRNA. Arrows indicate non-motor neuron ventral horn cells that express BDNF mRNA. A dashed line indicates the edge of the ventral horn. One motor neuron (see lower box in A) is shown in (B–D) to demonstrate BDNF mRNA labeling in the soma. A motor neuron with a long process is shown in (E–G) to demonstrate BDNF mRNA labeling in the process (see arrow heads). Two motor neurons (see upper box in A) are shown in (H–J) to demonstrate high and low intensities of BDNF mRNA labeling. Scale bar in (A) and (J) are 100 and 50 μ m respectively.

Figure 2.

Expression of BDNF mRNA in the ventral horn. HSP27-labeled motor neurons (green) are shown in the ventral horns of transverse spinal cord sections of representative Intact (A), ST100 (B) and ST 1000 (C) rats. The same sections are shown in (D–F) with both BDNF mRNA labeling (red) and HSP-27 label (green). Colocalization of BDNF mRNA label and HSP27 label (yellow-orange) are motor neurons expressing BDNF. Arrows indicate motor neurons with processes that contain BDNF mRNA label. The scale bar is 100 μm in (C).

Figure 3.

Plots summarizing analyses of BDNF mRNA label in motor neurons. Average number of HSP27 positive motor neurons per rat (A), average intensity of BDNF mRNA labeling in motor neurons (B), average percentage of motor neurons with processes (C) and average percentage of motor neurons with processes that contained BDNF mRNA staining (D) in the Intact, ST100 and ST1000 groups is shown. Both the mean intensity of BDNF mRNA in motor neurons and % of motor neurons with BDNF mRNA labeling in ST100 are significantly lower than Intact and ST1000 groups (3B and D). * and ** indicate significant difference at $p < 0.05$ and $p > 0.01$ levels respectively. Average values $\pm$ standard errors are shown for $n = 10$ per each group.

Figure 4.

Histograms of BDNF mRNA label intensity in motor neurons found within the same spinal cord section for Intact (A), ST100 (B) and ST1000 (C). The frequency was calculated as a percentage of total motor neurons within the spinal cord section. The data are the averages $\pm$ standard errors from $n=10$ for each group. See methods for details.

Figure 5.

Plots summarizing analyses of BDNF mRNA label in non-motor neurons cells in the ventral horn. Average number of BDNF mRNA labeled objects per rat (A) and average intensity of BDNF mRNA staining in non-motor neurons (B) in the Intact, ST100 and ST1000 groups is shown. Mean intensity of BDNF mRNA in non-motor neuron cells in ST100 is significantly lower ($p < 0.05$) than the Intact group (B). Average values are shown $\pm$ standard errors for $n=10$ per each group.

Figure 6.

Correlation of BDNF mRNA labeling and area of step cycle trajectory. Step area was correlated with BDNF mRNA labeling in the motor neurons (A, B) and non-motor neurons (C, D). White squares and black triangles are data from ST1000 and ST100 rats, respectively. The line is the linear regression. The Pearson correlation coefficient (r) is shown.