Role of Spared Pathways in Locomotor Recovery after Body-Weight-Supported Treadmill Training in Contused Rats

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Abstract

Body-weight-supported treadmill training (BWSTT)-related locomotor recovery has been shown in spinalized animals. Only a few animal studies have demonstrated locomotor recovery after BWSTT in an incomplete spinal cord injury (SCI) model, such as contusion injury. The contribution of spared descending pathways after BWSTT to behavioral recovery is unclear. Our goal was to evaluate locomotor recovery in contused rats after BWSTT, and to study the role of spared pathways in spinal plasticity after BWSTT. Forty-eight rats received a contusion, a transection, or a contusion followed at 9 weeks by a second transection injury. Half of the animals in the three injury groups were given BWSTT for up to 8 weeks. Kinematics and the Basso-Beattie-Bresnahan (BBB) test assessed behavioral improvements. Changes in Hoffmann-reflex (H-reflex) rate depression property, soleus muscle mass, and sprouting of primary afferent fibers were also evaluated. BWSTT-contused animals showed accelerated locomotor recovery, improved H-reflex properties, reduced muscle atrophy, and decreased sprouting of small caliber afferent fibers. BBB scores were not improved by BWSTT. Untrained contused rats that received a transection exhibited a decrease in kinematic parameters immediately after the transection; in contrast, trained contused rats did not show an immediate decrease in kinematic parameters after transection. This suggests that BWSTT with spared descending pathways leads to neuroplasticity at the lumbar spinal level that is capable of maintaining locomotor activity. Discontinuing training after the transection in the trained contused rats abolished the improved kinematics within 2 weeks and led to a reversal of the improved H-reflex response, increased muscle atrophy, and an increase in primary afferent fiber sprouting. Thus continued training may be required for maintenance of the recovery. Transected animals had no effect of BWSTT, indicating that in the absence of spared pathways this training paradigm did not improve function.

Key words: body-weight-supported treadmill training; contusion; monosynaptic reflex

Introduction

THE CLINICAL APPLICATION of locomotor training on a treadmill with partial body weight support (BWS) and manual assistance was preceded by studies in spinalized cats that reported improvement in the locomotor abilities after step training on treadmills with partial support (Barbeau and Rossignol, 1987; Edgerton et al., 1997; Lovely et al., 1986). The improved stepping response was attributed to afferent input associated with locomotion on a treadmill, which was integrated by the lumbar spinal cord neural circuitry (the central pattern generation, CPG) to generate a rhythmic, alternating hindlimb stepping pattern (Barbeau and Rossignol, 1987; de Leon et al., 1998a, 1998b, 2002; Edgerton et al., 1997; Hodgson et al., 1994). In the absence of supraspinal input, the impor-

tance of the CPG in the mechanism of recovery with body-weight-supported treadmill training (BWSTT) was evident. Whether or not a similar mechanism exists after incomplete spinal cord injury (SCI), in which some descending pathways are spared, remains unclear. Since most SCIs in humans are incomplete, and BWSTT benefits these individuals, further animal studies using incomplete SCI models are required to better understand the mechanisms of locomotor recovery after BWSTT as observed in the clinical setting.

Animal studies have shown that spared descending pathways play a significant role in spontaneous functional recovery after incomplete SCI. In a mouse hemisection model, Courtine and associates (2008) reported that animals attained stepping abilities within 10 weeks post-hemisection. When a second hemisection was performed on the contralateral side

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at the same level, a complete hindlimb paralysis resulted with no recovery in stepping over 4 weeks, indicating that the spared descending pathways were involved in locomotor recovery. Basso and colleagues (1995) also reported that eliminating the spared axons a few weeks after incomplete SCI led to loss of spontaneous behavioral recovery. These observations were based on animals that received no rehabilitation training after incomplete SCI, so the contribution of activity-dependent neuroplasticity at the lumbosacral spinal cord, or interactions between the spared descending pathways and plasticity within the caudal spinal cord could not be evaluated. Only one study (Barrière et al., 2008) examined the involvement of both supraspinal and spinal mechanisms in locomotor recovery after treadmill training in hemisected cats. After a hemisection, the cat's ipsilateral hindlimb was paralyzed, but over time with or without training, the animals regained some stepping abilities. When a complete transection was performed on these animals, only the trained hemisected cats exhibited bipedal walking within hours after complete transection (Barrière et al., 2008). Thus, training modified the spinal cord during the period of locomotor recovery through an interaction between the spinal sensorimotor mechanisms and remaining descending pathways. This study in cats provides insight into the mechanism of recovery after training in an incomplete lesion model. No such studies have been performed in the rat, the most commonly used model for investigating treatment strategies after SCI. Replications of experimental paradigms in rats that are comparable to those used in cats will help us understand reorganizations that are independent of species-specific spinal plasticity. Finally, since the type and severity of the injury influences the behavioral outcome, there is a need to replicate this study in a more clinically relevant incomplete SCI model such as contusion injury. Our goal was to examine the contribution of spared supraspinal input to the lumbar spinal cord in locomotor recovery after BWSTT in a moderate contusion injury model in adult rats.

It is important to identify the physiological and anatomical changes that occur after incomplete SCI to identify the deficits in motor function. Alterations in the Hoffmann-reflex (H-reflex) rate depression properties have shown changes in the motoneuron excitability after SCI. A direct relationship between the restoration of this rate-depression property and locomotor recovery after rehabilitation training has been described (Pearson et al., 2001; Thompson et al., 1992). Sprouting of primary afferents, which might be involved in the formation of new connections in the injured SC, or maladaptive changes such as neurogenic pain or spasticity, has been investigated in animal studies (Goldberger et al., 1993; Helgren and Goldberger, 1993; Krassioukov and Weaver, 1995; Mitsui et al., 2005; Murray and Goldberger, 1974; Osborn et al., 1990). In this study we focused on changes in locomotor (Basso-Beattie-Bresnahan [BBB] test and kinematics), physiological (H-reflex), and anatomical (sprouting and muscle mass) measures in a clinically relevant SCI animal model (contusion) that received BWSTT, and the role of spared pathways in spinal plasticity after BWSTT.

Methods

Animal groups

In all, 48 female Sprague-Dawley rats (225–250 g; Taconic, Germantown, PA) were studied. The animals were randomly

divided into seven groups: group 1: control (Cntl), group 2: contusion injury with 8 weeks of BWSTT (Ctsn + BWSTT), group 3: contusion injury with no BWSTT (Ctsn), group 4: contusion injury with 8 weeks of BWSTT followed by a transection injury at week 9 (Ctsn + BWSTT/Tx), group 5: contusion injury with no BWSTT followed by a transection injury at week 9 (Ctsn/Tx), group 6: transection injury with 8 weeks of BWSTT (Tx + BWSTT), and group 7: transection injury with no BWSTT (Tx). An outline of the injury and intervention applied to each group is shown in Figure 1. Details of the assessments performed and sample size of each of the seven groups is provided in Table 1.

Surgical procedures

Contusion injury. Rats were anesthetized with a mixture of ketamine (76 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA), xylazine (7.6 mg/kg; Ben Venue Laboratories, Bedford, OH), and acepromazine maleate (0.6 mg/kg; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) given intraperitoneally (IP). The back of the animal was shaved and cleaned with povidone-iodine. A laminectomy at spinal level T9/T10 exposed the dura. All animals received a moderate contusion injury using the New York University (NYU) impactor device, in which a 10-g, 2.0-mm-diameter rod impactor head was dropped from a height of 25 mm onto the exposed spinal cord and immediately removed. Following contusion, subcutaneous fat was placed on the exposed dura to prevent adhesions, the back muscles were sutured, and the skin was closed with surgical wound clips. The animals were kept on a heating pad (36.5°C) overnight and then returned to their home cages.

Transection injury. Rats were deeply anesthetized with isoflurane. The adequacy of anesthesia was confirmed by the absence of corneal reflexes and response to paw pinch. Under aseptic conditions, the spinal cord was exposed by laminectomy of the eighth thoracic vertebra (T8). The meningeal membranes were opened longitudinally, and a 2-mm length of T7 spinal cord was removed by gentle aspiration. The complete transection lesion cavity was filled with gelfoam to achieve hemostasis. The gelfoam was removed, the dura was closed with 10-0 sutures, and the overlying muscles and fascia were closed in layers with sutures. Finally the skin incision was closed with wound clips.

Postoperative care. Beginning immediately following surgery and for the first 2 weeks postoperatively, saline (3–5 mL) and ampicillin (0.3 cc, 22.7 mg/mL SC bid) were administered to minimize dehydration and urinary tract infections. The bladders of all animals were expressed twice daily until the reflex voiding returned, typically by 10 days post-injury. Special absorbent bedding was provided to prevent pressure ulcerations. All procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine, following the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Treadmill training

BWSTT was performed using a commercially available robotic device (Rodent Robot 3000; Robomedica Inc.,

FIG. 1. Study design. Electrophysiological testing and histological analyses were performed at the conclusion of the experiments (Cntl, control; Ctsn, contusion; Tx, transection; IHC, immunohistochemistry; CGRP, calcitonin gene-related peptide; BWSTT, body-weight-supported treadmill training).

Mission Viejo, CA; Fig. 2). The animals were trained in a bipedal position using a previously described training protocol (de Leon et al., 2002, 2006; Timoszyk et al., 2005). Briefly, the animals were secured to a BWS arm via a soft cloth vest with hook-and-loop fasteners to control the amount of weight bearing on the hindlimbs. Robotic arms were attached to both hindlimbs using neoprene cuffs around the ankles. Pre-injury, all animals were trained on the treadmill with 75% BWS at a speed of 7 cm/sec until they performed 15 consecutive steps. Post-injury, the animals in the training groups received BWSTT for 500 steps/day, 5 days/week for 8 weeks (based on previous studies; Timoszyk et al., 2005) with 75% BWS at 7 cm/sec. During treadmill training, the robotic arms moved the ankles in a semi-circular step cycle trajectory. The shape of the trajec-

tory allowed toe clearance during swing and plantar surface weight bearing during stance. If necessary, the trainer manipulated the paws with cotton swab applicators to ensure toe clearance during swing and plantar placement of the paw during stance.

BBB test

Hindlimb motor function was assessed using the open-field Basso-Beattie-Bresnahan (BBB; Basso et al., 1995) scoring system. Hindlimb function was scored from 0 (no observable hindlimb movements) to 21 (normal locomotion). Individual rats were placed in an elliptical enclosure, allowed to acclimate to the enclosure, and then observed for 4 min by two trained observers. The tests were scored by observers who

TABLE 1. NUMBER OF ANIMALS AND ASSESSMENTS FOR EACH GROUP

Group	Sample size (n)	Intervention	Injury type		Assessment
			First surgery (week 0)	Second surgery (week 9)	
Group 1: Cntl	4	None	No injury		Weekly BBB and kinematics at weeks 5 and 9; H-reflex, muscle mass, and IHC at week 9
Group 2: Ctsn + BWSTT	8	8 weeks of BWSTT	Contusion		
Group 3: Ctsn	8	No BWSTT	Contusion		Weekly BBB and kinematics at weeks 5, 9, 10, and 11; H-reflex, muscle mass, and IHC at week 11
Group 4: Ctsn + BWSTT/Tx	8	8 weeks of BWSTT	Contusion	Transection	
Group 5: Ctsn/Tx	8	No BWSTT	Contusion	Transection	
Group 6: Tx + BWSTT	6	8 weeks of BWSTT	Transection		Weekly BBB and kinematics at weeks 5 and 9; H-reflex, muscle mass, and IHC at week 9
Group 7: Tx	6	No BWSTT	Transection		

Cntl, control; Ctsn, contusion; Tx, transection; IHC, immunohistochemistry; CGRP, calcitonin gene-related peptide; BWSTT, body-weight-supported treadmill training; BBB, Basso-Beattie-Bresnahan open field test.

FIG. 2. Robotic device used for body-weight-supported treadmill training (BWSTT) and for locomotor assessment. The animals were trained and tested on the treadmill with 75% body weight support (BWS) at a speed of 7 cm/sec. The robotic arm was attached to the ankle using a neoprene cuff as shown in the photo. These arms moved the ankles in a semi-circular step cycle trajectory during training, and recorded the ankle trajectories during testing.

had inter-rater reliability scores greater than 95%. Observers were unaware of the group identity of the animals.

Kinematic analysis

Data acquisition. Kinematic testing was performed for all the groups pre-injury, and at weeks 5 and 9 post-injury. Groups 4 and 5 were also tested at weeks 10 and 11 (weeks 1 and 2 post Tx injury). The animals were allowed to perform independently on the treadmill at 7 cm/sec with 75% BWS in a bipedal position for 20 sec while no forces were applied by the robotic arm. The same settings (75% BWS and 7 cm/sec) were used during all testing sessions. The duration of testing did not exceed 1 min per rat to minimize training effects during testing. The vertical and horizontal movements of the ankles were recorded by the attached robotic arms for subsequent analysis and reconstruction of the two-dimensional trajectory pattern of the ankles. The hindlimbs were filmed during testing to provide a video record of stepping.

Data analysis. Details of data analyses have been described previously (Timoszyk et al., 2002, 2005). Briefly, a step detection analysis was performed, which consists of identifying the beginning (toe off: TO) and end (paw contact: PC) events for each step cycle based on the horizontal and vertical velocities recorded by the robotic arms. Peaks in horizontal velocity that exceeded a threshold value of 125 Hz were used to identify TO events. The same threshold value was used for the step detection analyses across all experimental groups. PC was identified based on changes in the direction of vertical velocity recorded by the robotic arms following the detected TO event. Once TO and PC were identified, measurements of the kinematic characteristics (i.e., the length and height of the step) and duration of the swing phase were calculated to evaluate changes in the gait.

Electrophysiological measures (H-reflex rate depression property measure)

Surgical procedure. Animals were deeply anaesthetized by IP injection of xylazine (7.6 mg/kg) and ketamine (76 mg/

kg), which minimally depresses the spinal monosynaptic reflex and does not alter the time course of presynaptic inhibition (Ho and Waite, 2002; Lodge and Anis, 1984; Tang and Schroeder, 1973). The tibial nerve of the right hindlimb was exposed below the popliteal fossa and mounted on a stimulating bipolar hook electrode. Exposed tissues were covered with warm mineral oil to prevent drying. Bipolar electromyography (EMG) electrodes were inserted subcutaneously in the digital interosseous muscles between the fourth and fifth metatarsals. The ground electrode was located in the skin of the leg.

Data acquisition. The reflex was first tested with single pulses (100 μ sec duration) to determine the threshold and maximal response level for both the M-wave (direct motor response) and the H-wave. The level of stimulation for a maximum H-wave response was used for 2 series of 16 consecutive stimulations, one at 0.3 Hz and the other at 10 Hz, with a 10-min rest period between runs. Stimuli were delivered via an isolated pulse stimulator (A-M Systems Model 2100; A-M Systems Inc., Carlsborg WA). EMG responses were recorded with a differential AC amplifier (gain $\times 1000$; bandpass 10–5 kHz; A-M Systems Model 1700). Data acquisition (sampled at 10 kHz) was controlled by customized software (Labview; National Instruments Corp., Austin, TX) running on a personal computer.

Data analysis. Averages of the last 11 H-wave amplitudes were calculated for both stimulus frequency trains (10 Hz and 0.3 Hz). Changes in the H-wave at 10 Hz were calculated as a percentage of the response at 0.3 Hz in order to determine the stimulation depression of the H-wave ($H_{10\text{Hz}}/H_{0.3\text{Hz}} \times 100$), a method of analysis proposed by Skinner and associates (Reese et al., 2006; Skinner et al., 1996).

Muscle atrophy

After H-reflex recording, the rats were injected IP with sodium pentobarbital (100 mg/kg; Abbot Laboratories, North Chicago, IL) and the left soleus (S) muscle was excised and weighed. Wet muscle weight (MW) was expressed as a percent of total body weight (BW) (Nothias et al., 2005).

Tissue preparation and histology

After the muscle was harvested, deeply-sedated animals were transcardially perfused with 0.9% saline followed by 500 mL of ice-cold 4% paraformaldehyde fixative in phosphate buffer. The spinal cord was removed and post-fixed for 24 h at 4°C, followed by cryoprotection in phosphate-buffered 30% sucrose solution for 3–5 days. Tissues were embedded in mounting compound and stored at -80°C .

An 11-mm segment of thoracic spinal cord containing the lesion was sectioned transversally at 20 μm . Every fifth section was stained for Nissl-myelin in order to determine the extent of lesion and spared tissue. NIH ImageJ software was used to make the measurements as follows. The scale bar in the image was used to calibrate the image with the Analyze tool, and the total area of the spinal cord and the spared white matter were delineated. These measurements were used to determine the percentage of spared white matter.

The L4–L6 region of the spinal cord was sectioned transversely at 30 μm for immunocytochemical (ICC) analysis. ICC was performed for the detection of anatomical plasticity using antibody to calcitonin gene-related peptide (CGRP). Five

sections at 200- μ m intervals from each animal were rinsed in phosphate-buffered saline (PBS), and then permeabilized with 10% goat serum in 0.3% Triton X-100/PBS for 2 h. The sections were treated overnight at 4°C with CGRP antibody (1:6000; Peninsula Laboratories, Belmont, CA) diluted in 5% normal goat serum in PBS containing 0.3% Triton X-100. The next day, after several rinses in 1×PBS, the sections were incubated for 2 h in goat anti-rabbit IgG conjugated to rhodamine (1:200; Jackson ImmunoResearch, West Grove, PA), and then placed on the slides at room temperature, followed by cover-slipping with Vectashield (Vector Laboratories, San Diego, CA). Computer-assisted image analysis was used for densitometric measurement of the distribution of immunolabeling for CGRP within the entire dorsal horn. All five sections from each animal were digitized, made binary, contrast enhanced, and assigned a range of optical densities to correspond to positively-labeled structures. A mean density for immunolabeled structures in the dorsal horn was obtained for each animal. A mean of all means from all animals in each group was used for statistical analysis.

Statistical analysis

For behavioral and kinematic data we used repeated-measures analysis of variance (ANOVA) between groups and time, with time taken as a repeated measure beginning at 1 week after training. *Post-hoc* analysis was performed using Tukey's test for parametric data or the Mann-Whitney *U* test for nonparametric data (such as BBB scores). H-reflex and ICC data were analyzed using one-way ANOVA between groups, followed by Tukey's *post-hoc* test when appropriate. All values are expressed as mean $\pm$ standard error of mean (SEM). All statistical analyses were performed using SPSS 11.5 for Windows software, and significance was set at $p < 0.05$.

Results

Open-field locomotor test (BBB)

BBB scores decreased immediately following either contusion or transection injury, and then partially recovered to weight-support levels (BBB score > 9) in the contusion-only groups (groups 2 and 3) by week 2 (Fig. 3). Mean BBB scores for the transected animals remained below 3 throughout the study. No effect of BWSTT was observed for either injury group, and no difference in BBB scores was found between trained and untrained animals with similar injuries. Trained and untrained animals in groups 4 and 5 showed a decrease ($p < 0.05$ by Mann-Whitney *U* test) in BBB scores after transection injury performed at 9 weeks post-contusion, compared to controls and their performance at weeks 1–8.

Kinematic performance

Kinematic data obtained from animals in groups 2 and 4 (BWSTT) and groups 3 and 5 (no BWSTT) were pooled before the second injury. Data obtained at weeks 10 and 11 are from animals in groups 4 and 5 only. At weeks 10 and 11, all 8 BWSTT-contused animals (group 4) were able to step after transection injury; however, only 5/8 animals at week 10 and 4/8 animals at week 11 of the untrained contused animals (group 5) were able to step after transection injury. In transection animals (groups 6 and 7), no effect of BWSTT was observed in either group, as all animals failed to perform any steps.

At week 5, all untrained contused animals exhibited a decrease in the swing duration (Fig. 4A), step height (Fig. 4B), and step length (Fig. 4C), when compared to control animals ($p < 0.05$ by ANOVA). Trained contused animals did not show a similar deficit. By week 9, the untrained group recovered all kinematic measures so that there was no difference between the trained and

FIG. 3. Open-field locomotion by Basso-Beattie-Bresnahan (BBB) testing. Transected animals had significantly lower BBB scores than contused rats, and no effect of BWSTT was observed for either injury group (Cntl, control; Ctsn, contusion; Tx, transection; BWSTT, body-weight-supported treadmill training).

FIG. 4. Mean $\pm$ standard error of mean of swing duration (**A**), step height (**B**), and step length (**C**; * = significant difference from controls; # = significant difference between the two groups [$p < 0.05$]; ^ = significant difference from week 9). Body-weight-supported treadmill training (BWSTT) resulted in a significant difference in the trained versus untrained contused animals at week 5, but not at week 9. After Tx injury, at week 10 trained animals retained their locomotor performance and were significantly different from untrained animals, but not at week 11, except for step height.

untrained contused animals. At week 10 (week 1 post-Tx), an effect of BWSTT was observed such that only the untrained animals had a decrease in the swing duration ($p = 0.001$ by ANOVA), step height ($p = 0.005$ by ANOVA), and step length ($p = 0.026$ by ANOVA) compared to controls. The trained animals had greater swing duration ($p = 0.002$ by ANOVA), step height ($p = 0.029$ by ANOVA), and step length ($p = 0.001$ by ANOVA) than untrained animals at this time point. This effect was transient, and by week 11 (week 2 post-Tx), trained animals had a decrease in both swing duration (week 9: $p = 0.007$, controls: $p = 0.000$ by ANOVA), and step length (week 9: $p = 0.017$, controls: $p = 0.016$ by ANOVA), compared to their week 9 performance and to controls, and these measures did not differ from those seen in untrained animals. Step height in the trained animals remained higher ($p = 0.001$ by ANOVA) than in untrained animals, indicating a more persistent improvement in this measure.

Thus, BWSTT led to an accelerated recovery in contused SCI animals. No effect of training was observed in transected animals. Also, trained contused animals retained improved kinematic parameters immediately after removal of supraspinal input, but the absence of further training resulted in a loss of this improvement.

Changes in H-reflex

H-reflex is an electrical analog of the spinal stretch reflex that provides information on the functional properties of Ia

afferents and homonymous alpha-motoneurons. A reproducible H-reflex response was present in 4/4 in the uninjured control animals (group 1), 6/8 in the BWSTT contused only animals (group 2), 5/8 in the untrained contused only animals (group 3), 6/8 in the BWSTT contused and transected animals (group 4), 4/8 in untrained contused and transected animals (group 5), 4/6 in trained transected only animals (group 6), and 4/6 in untrained transected only animals (group 7). The remaining animals exhibited an erratic H-reflex response and were excluded from the analysis.

To determine the stimulation depression of the H-reflex, changes in the H-wave response at 10 Hz were calculated as a percentage of the response at 0.3 Hz ($H_{10\text{Hz}}/H_{0.3\text{Hz}} \times 100$). A lower ratio indicates greater rate depression. Uninjured control animals ($n = 4$) showed 100% rate depression at 10 Hz (ratio = 0). After SCI, this rate depression property of the H-reflex was less sensitive to stimulus frequency in all injury groups (Figs. 5 and 6). However, in the BWSTT contusion (group 2), and BWSTT transection (group 6) animals, the rate depression was higher ($p < 0.05$ by ANOVA) compared to the untrained animals with similar injury (Fig. 5; groups 3 and 7). Trained contused rats lost this improvement after a second transection injury (group 4) and did not differ from untrained rats (group 5). Thus, BWSTT improved the rate depression property of the H-reflex in both complete and incomplete SCI animals and returned H-reflex response towards normal. Removal of supraspinal

FIG. 5. Mean $\pm$ standard error of mean of the ratio of H-reflex ($H_{10\text{Hz}}/H_{0.3\text{Hz}} \times 100$; # = significant difference from untrained animals with similar injury, $p < 0.05$ by analysis of variance). Body-weight-supported treadmill training (BWSTT) led to a significant improvement in the rate depression property of the H-reflex in animals with only contusion or transection injury (Cntl, control; Ctsn, contusion; Tx, transection).

input after training in contused rats led to loss of this improvement by 2 weeks post-transection.

Muscle weight

A protective effect of BWSTT on the soleus muscle weight was observed in trained animals with contusion or transection or both injuries with a significantly higher MW:BW ratio in trained than untrained animals with similar injuries ($p < 0.01$ by ANOVA; Fig. 7). Additionally, the soleus muscle mass of trained contused animals (group 2) and unoperated controls (group 1) did not differ from each other. Thus, BWSTT diminished atrophy of the soleus muscle.

Lesion size

There was no difference in the volume of spared white matter tissue in either the trained (group 2, $22.23 \pm 1.31 \text{ mm}^3$) or untrained (group 3, $21.18 \pm 2.8 \text{ mm}^3$) contused animals. The lesion was complete in transected rats.

CGRP-positive fibers

In unoperated control animals, CGRP immunoreactive (IR) axons were localized to laminae I and II of the dorsal horn. CGRP-IR was denser in all injured groups compared to controls ($p < 0.05$ by ANOVA), consistent with

FIG. 6. Superimposed averages of stimulation frequencies of 0.3 Hz and 10 Hz for M-wave and H-wave. One representative animal from each of the seven groups is shown. Uninjured control animals showed 100% rate depression of the H-wave at 10 Hz (no H peak was observed). Injured animals exhibited decreased sensitivity to this rate depression property. Body-weight-supported treadmill training (BWSTT) helped restore this property in trained animals with contusion or transection injury (Cntl, control; Ctsn, contusion; Tx, transection).

FIG. 7. Mean $\pm$ standard error of mean of the soleus muscle weight/body weight (*=significant difference from control, $p < 0.05$ by analysis of variance; #=significant differences between groups with similar injuries; Cntl, control; Ctsn, contusion; Tx, transection; BWSTT, body-weight-supported treadmill training).

sprouting of primary afferent fibers, but the density of the reaction varied with training in contused animals (Fig. 8). BWSTT contused animals (group 2) had lower ($p < 0.05$ by ANOVA) density of CGRP staining than the untrained contused animals (group 3). No difference was observed in trained and untrained animals in other groups with similar injury (Fig.9). Thus, BWSTT in animals that received contusion injury appeared to moderately diminish CGRP sprouting.

Discussion

Recovery after BWSTT in contused rats

Our results show an accelerated recovery of kinematic parameters, recovery in physiological properties of mono-synaptic reflex pathways, reduced muscle atrophy, and primary afferent sprouting in contused animals that received BWSTT. These findings are similar to those reported previously in both animal and human studies in which locomotor

FIG. 8. Calcitonin gene-related peptide (CGRP) immunostaining in the dorsal horn of the L4–L6 lumbar spinal cord. CGRP-immunoreactive (IR) axons were localized to laminae I and II of the dorsal horn in control animals. In all injured animals, the CGRP-IR was significantly denser ($p < 0.05$ by analysis of variance [ANOVA]) compared to controls. However, body-weight-supported treadmill training (BWSTT) in contused animals (Ctsn + BWSTT) appeared to diminish CGRP-IR ($p < 0.05$ by ANOVA) when compared to untrained animals with similar injuries (Ctsn; Cntl, control; Tx, transection).

FIG. 9. Mean $\pm$ standard error of mean of the calcitonin gene-related peptide (CGRP) density (mean gray value) in the dorsal horn of the L4–L6 spinal cord (#=significant difference between groups with similar spinal cord injuries, $p < 0.05$; Cntl, control; Ctsn, contusion; Tx, transection; BWSTT, body-weight-supported treadmill training).

training after a partial spinal lesion facilitated overall functional recovery (Barbeau and Rossignol, 1987; Barbeau and Visintin, 2003; Barrière et al., 2008; Bélanger et al., 1988, 1996; Dietz et al., 1994; de Leon et al., 1998a, 1998b; Fouad et al., 2000; Harkema, 2001; Heng and de Leon, 2009; Lovely et al., 1986, 1990; Plummer et al., 2007; Rossignol et al., 2006; Sullivan et al., 2002; Van de Crommert et al., 1998; Wernig and Muller, 1992). In our study, kinematic analyses showed no difference in the locomotor abilities of the trained and untrained animals at 9 weeks post injury. The contusion injury model used in the current study preserves most of the ventral and ventral-lateral descending pathways, including the reticulospinal and vestibulospinal tracts that are mainly responsible for maintaining locomotion in rats with partial SCI (Fouad and Pearson, 2004). Thus, sparing these pathways led to greater locomotor recovery 9 weeks post-injury in both trained and untrained animals. Also, since we performed kinematic testing only with 75% BWS at a speed of 7 cm/sec, we did not determine if the trained animals could sustain higher body weight while locomoting and/or perform at faster speeds. Future studies are needed to determine whether BWSTT contused rats could sustain greater functional abilities by varying the testing settings of BWS and speed.

Role of spared descending pathways in locomotor recovery

To determine the role of spared descending pathways we used two approaches. We transected the previously contused spinal cord of trained and untrained rats to determine if locomotor abilities were altered after the removal of supraspinal input. We also studied the effects of BWSTT in adult transected rats that did not receive any other injury to determine if sparing of some supraspinal fibers was the key to locomotor recovery after BWSTT.

Trained contused rats showed preservation of kinematic parameters at 1 week after transection. Untrained contused rats exhibited a significant loss of locomotor recovery immediately after transection. Similar results have been reported in hemisected cats that received intensive treadmill training and demonstrated bilateral hindlimb locomotion within hours of the complete lesion (Barrière et al., 2008). In untrained cats, asymmetrical hindlimb locomotion developed, and the limb on the side of the partial lesion developed locomotor patterns well before the other hindlimb. Thus, neuroplastic changes in the spinal central pattern generator (CPG) occur after BWSTT in the incomplete SCI model (Barrière et al., 2008). Furthermore, in the cat studies, intensive training after transection in both trained and untrained cats with partial lesions led to a significant locomotor recovery. In our study, we discontinued the training after the transection to determine if the improvement observed in kinematics persisted. Two weeks post-transection, the improvements in the trained rats were abolished and the motor outcomes were similar to those of untrained rats. In a previous study, a significant reduction in stepping capacity in trained spinalized cats also was observed with discontinued training (de Leon et al., 1999). Thus, continued training is required for maintenance of the locomotor recovery seen after treadmill training.

In the transected rats, no effect of BWSTT was observed at any time point. This finding is different from spinalized cat studies, that have reported a significant improvement in locomotion after treadmill training. In rats, studies showing a beneficial effect of BWSTT in transected rats are reported in animals that received injury as neonates (Nessler et al., 2005; Timoszyk et al., 2005). In adult transected rats, beneficial effects of BWSTT are observed only when a combinational approach using pharmacological or electrical stimulation is applied with treadmill training (de Leon and Acosta, 2006; Lavrov et al., 2008). Thus, there are discrepancies in the

BWSTT outcomes observed in transected cats and rats, and BWSTT alone in rats does not result in locomotor recovery unless some descending pathways are spared or combinational approaches are employed.

Changes in the spinal reflex pathway

When activated the H-reflex that recruits synergistic muscles and inhibits antagonists (Kandel et al., 2000) is a commonly used clinical tool yielding information about altered properties, especially rate sensitivity, of the monosynaptic reflex pathways after SCI. After SCI, a decrease in rate sensitivity of the H-wave is observed (Calancie et al., 1993; Thompson et al., 1992). In agreement with these previously reported studies, a significant loss of rate depression property was observed in the contused and transected rats. However, after BWSTT, an improvement in the H-reflex response was observed. Studies have shown the importance of transmission of cutaneous inputs provided during treadmill training in the reorganization of reflex responses in spinal rats (Cowley and Schmidt, 1997). We postulate a similar mechanism to be involved after BWSTT in both the trained contused and transected rats. In transected rats that were previously contused and trained, a loss of the improved rate depression property was observed. This loss could be attributed to the loss of both the supraspinal input and the locomotor function observed at week 2 post-transection due to discontinued training, since previous studies have shown that the spared white matter at the injury site and extent of hindlimb function after contusion SCI are each strongly correlated with changes in the reflexes (Lee et al., 2005). Since training the animals after complete SCI conditions the caudal spinal circuitry to normalize spinal reflexes (Ollivier-Lanvin et al., 2009), we postulate that continued training of previously contused and trained rats would help maintain the recovered H-reflex, properties even in the absence of supraspinal control, but this is yet to be tested.

Muscle atrophy

Reductions in neural input and mechanical loading following SCI leads to muscle atrophy in the affected skeletal muscles (Giangregorio and Blimkie, 2002; Monroe et al., 1998; Stein and Wade, 2005; Vandenborne et al., 1998; Wilmet et al., 1995). Reduced muscle atrophy with exercise is widely reported in the literature (Beaumont et al., 2004; Dupont-Versteegden et al., 2000, 2004; Giangregorio et al., 2005; Nothias et al., 2005; Wolpaw 1997). We observed similar results with a significantly higher soleus muscle mass in trained animals than untrained animals with similar injury. Thus, BWSTT helped in restoration of muscle mass and reduced the extent of muscle atrophy in SCI animals. Also, the soleus muscle mass in the trained contused rat was higher than in the transected rat that was previously contused and trained. We attribute this rapid decrease in the muscle mass to a decrease in hindlimb function due to the loss of supraspinal input after transection injury and discontinued training.

Lesion size and CGRP

We observed no effect of training on the lesion size in the contused rats. Thus, BWSTT did not result in any neuroprotection, which might have been expected to increase sparing of white matter tissue at the lesion site. These results are

similar to those reported in contused rats with swim training, for which no increase in the sparing of the white matter tissue at the lesion site was observed (Smith et al., 2009). In the current study, changes in sprouting of afferent fibers in the lumbar cord were also studied using CGRP staining. Previous studies have reported sprouting of small diameter dorsal root axons in animals after SCI (Krenz and Weaver, 1998). We observed a similar increase in the density of CGRP fibers in injured animals; however, a decrease in this sprouting was observed in BWSTT contused rats compared to untrained contused rats. Thus, spared descending pathways and locomotor training helped decrease the sprouting of afferent pain fibers, and removal of these pathways led to increased sprouting, even in previously trained animals.

This study models a common clinical SCI and the outcomes are comparable to those observed in clinical situations, where a beneficial outcome of BWSTT is observed after incomplete SCI, and not with complete SCI. Also, although the beneficial effects of treadmill training are evident at various levels of recovery, including behavioral, physiological, and anatomical, the outcome depends in part on the spared descending pathways. Future treatments should consider the role of these spared pathways to enhance the outcome of treatment.

Conclusion

Our results showed significantly accelerated locomotor recovery in the BWSTT contused animals, along with improvement of H-reflex properties, reduced muscle atrophy, and decreased sprouting of small caliber afferent fibers.

When the supraspinal input was removed from previously contused rats, no decrease in locomotor abilities in the trained rats occurred immediately after transection, while untrained previously contused rats exhibited a significant decrease in kinematic parameters.

Discontinued training in the trained contused and then transected rats abolished the improved kinematics within 2 weeks, and led to a reversal of improved H-reflex response and muscle mass restoration, and an increase in CGRP sprouting, suggesting that continued training might be required for maintenance of the observed recovery.

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Author Disclosure Statement

No competing financial interests exist.

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