Forced Exercise as a Rehabilitation Strategy after Unilateral Cervical Spinal Cord Contusion Injury

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Abstract

Evaluation of locomotor training after spinal cord injury (SCI) has primarily focused on hind limb recovery, with evidence of functional and molecular changes in response to exercise. Since trauma at a cervical (C) level is common in human SCI, we used a unilateral C4 contusion injury model in rats to determine whether forced exercise (Ex) would affect spinal cord biochemistry, anatomy, and recovery of fore and hind limb function. SCI was created with the Infinite Horizon spinal cord impactor device at C4 with a force of 200 Kdynes and a mean displacement of 1600–1800 μm in adult female Sprague-Dawley rats that had been acclimated to a motorized exercise wheel apparatus. Five days post-operatively, the treated group began Ex on the wheel for 20 min per day, 5 days per week for 8 weeks. Wheel speed was increased daily according to the abilities of each animal up to 14 m/min. Control rats were handled daily but were not exposed to Ex. In one set of animals experiencing 5 days of Ex, there was a moderate increase in brain-derived neurotrophic factor (BDNF) and heat shock protein-27 (HSP-27) levels in the lesion epicenter and surrounding tissue. Long-term (8 weeks) survival groups were exposed to weekly behavioral tests to assess qualitative aspects of fore limb and hind limb locomotion (fore limb scale, FLS and BBB [Basso, Beattie, and Bresnahan locomotor rating scale]), as well as sensorimotor (grid) and motor (grip) skills. Biweekly assessment of performance during wheel walking examined gross and fine motor skills. The FLS indicated a significant benefit of Ex during weeks 2–4. The BBB test showed no change with Ex at the end of the 8-week period, however hind limb grid performance was improved during weeks 2–4. Lesion size was not affected by Ex, but the presence of phagocytic and reactive glial cells was reduced with Ex as an intervention. These results suggest that Ex alone can influence the evolution of the injury and transiently improve fore and hind limb function during weeks 2–4 following a cervical SCI.

Key words: cervical contusion; forced exercise; forelimb behavior; spinal cord injury

Introduction

INITIAL INJURY TO THE SPINAL CORD damages neurons and glia and disrupts ascending and descending spinal pathways. This is followed by a progressive, secondary wave of degeneration that is more pronounced after a contusion lesion compared to a laceration injury. Implementing treatments to minimize the spread of damage is of great interest since a spinal cord contusion is the most common form of spinal cord injury (SCI). There are many factors that play a role in the formation and evolution of the lesion and limiting the extent of some of these factors may improve long-term prospects for recovery. In the present study, we examined whether forced exercise (Ex) promotes changes in the expression of potentially beneficial proteins within spinal cord tissue following a unilateral cervical contusion injury and whether this Ex

regimen could be effective as a therapeutic strategy to improve fore and hind limb function.

Evaluation of the effects of Ex after SCI has focused primarily on the recovery of hind limb function. Previous studies have demonstrated that treadmill training, a forced activity rehabilitative strategy, promotes functional and molecular changes in hind limb muscle and spinal cord tissue following thoracic SCI. Introducing treadmill Ex to adult or neonatal rats and adult cats displayed increased functional recovery compared to unexercised groups (Lovely et al., 1986; Stevens et al., 2006; Edgerton, 2004). Lovely et al. (1986) showed that spinal transected cats can perform weight-supported stepping after 2 months of treadmill training, possibly due to activated spinal cord circuitry during the initiation of repetitive stepping motions on the treadmill (Edgerton et al., 2001). After injury to the spinal cord, intrinsic inhibitory signals are no

longer regulated by descending pathways, thereby altering the excitability of neurons caudal to the lesion. Another experiment applying Ex after spinal transection in rats gave insight into the molecular changes within the injured spinal cord by demonstrating the attenuation of an inhibitory neurotransmitter enzyme, glutamate decarboxylase (GAD), caudal to the lesion site (Tillakaratne et al., 2000b). This decrease of GAD would likely modify the excitability of motoneurons. In addition, hind limb muscles innervated by motoneurons caudal to a spinal transection were protected from atrophy after bicycling Ex (Dupont-Versteegden et al., 1998; Houle et al., 1999; Liu et al., 2008).

Mechanisms by which Ex may facilitate recovery have focused on changes in the levels of neurotrophic factors (NTF) in the spinal cord and muscle. Increasing levels of NTF after Ex might enhance neuroprotection and/or aid in the regeneration of injured axons (Ying et al. 2005; Gomez-Pinilla et al., 2002; Dupont-Versteegden et al., 2004). Upregulation of BDNF following exercise may contribute to regulating the expression of synapsin I, which plays a role in synapse activity and plasticity (Vaynman et al., 2003). Studies also have addressed the specific change in transcription and translation of c-fos and heat shock protein (HSP) (Dupont-Versteegden et al., 2004). The immediate early gene product, c-fos, aids in regulating the expression of BDNF (Vaynman and Gomez-Pinilla, 2005). Stress response proteins such as HSP protect cells from degeneration and support neuronal survival (Brown, 2007).

The C4 segment of the spinal cord contains motoneuron pools which innervate musculature of the shoulder (acromiotrapius; levator claviculae; spinodeltoideus), upper fore limb (biceps) and lower fore limb (extensor carpi radialis longus; extensor carpi radialis brevis) (McKenna et al., 2000). Disruption of motor neuron control after a unilateral C4 contusion produces temporary paralysis followed by partial recovery, although there are permanent deficits seen in gross and skilled forelimb behaviors (Gensel et al., 2006; Sandrow et al., 2008).

After demonstrating that BDNF and HSP-27 levels increased after 5 days of forced Ex in the spinal cord at and around the injury site (indicating a potential neuroprotective effect), we performed a long-term study to determine whether this noninvasive intervention would produce behavioral changes during early and late stages of recovery post-injury. Forced wheel walking led to a transient improvement in behavioral activity and a long-term effect on the density and distribution of reactive astrocytes and phagocytic cells caudal to the injury epicenter.

Methods

Spinal cord injury

Adult (225–250 g) female Sprague-Dawley rats were anesthetized with a ketamine (60 mg/kg) and xylazine (6 mg/kg) mixture. A unilateral C4 contusion was completed as previously described (Sandrow et al., 2008). A partial C4 laminectomy exposed the right side of the spinal cord and forceps fixed to the platform base of an Infinite Horizon Impact Device (Precision Systems and Instrumentation, LLC, Fairfax Station, VA) were used to stabilize the cord by clamping the C2 and C5 vertebral bodies. Animals were secured on the device platform, and the 1.6-mm stainless steel impact tip was positioned over the right side of the C4 spinal cord midway between the medial dorsal vein and the lateral

edge of the spinal cord. The impactor tip was lowered to 3–4 mm above the dorsal surface of the spinal cord, and the field was flooded with sterile saline up to the impactor tip. A unilateral contusion injury was created using an impact force of 200 Kdynes (± 20 Kdynes) with tissue displacement to a depth of 1600–1800 μm . After injury, animals were released from the clamping forceps and overlying muscles closed with 4-0 silk sutures. The skin incision was closed with wound clips. Animals were given fluids to remain hydrated (3 mL of Ringers solution, subcutaneous injection). The antibiotic ampicillin (100 mg/kg) and the analgesic buprenorphine (0.05 mg/kg) were given for 3 days post-operatively. Animals were housed in threes and maintained on food and water *ad lib* with a 12-h light-dark cycle (lights on at 07:00 a.m.). No respiratory complications occurred with this model, and the mortality rate was low ($<10\%$). All procedures were performed in accordance with protocols approved by the Drexel University College of Medicine's Institutional Animal Care and Use Committee, and followed National Institutes of Health guidelines for the care and use of laboratory animals.

Forced exercise paradigm

One week prior to SCI, animals in the Ex rehabilitation group were acclimated to the forced exercise wheel walking system (Lafayette Inst., Lafayette, IN) used to increase fore and hind limb activity. Rungs of the wheel were covered with a cloth material that created a relatively smooth yet graspable surface. The smooth surface reduced the possibility of additional injury from limbs falling through the rungs during the acute injury treatment period. Animals were placed in the wheels for 10 min before Ex began, with a subsequent running period of 20 min per day for 5 days per week. Rats began Ex 5 days after injury with a beginning speed of 5 m/min, with wheel speed increased daily according to the fore limb capabilities of individual animals to a maximum speed of 14.0 m/min (which is comparable to a treadmill training speed). The number of wheel cycles at different speeds were not counted rather the time spent in the wheels was consistent. Most animals reached the maximum speed by week 4.

Short-term survival: Immediate effects of exercise

We examined two groups of animals that were sacrificed 10 days post-SCI: injury alone ($n = 3$) and injury with 5 days of forced exercise ($n = 3$). These animals were tested behaviorally 2 days before SCI, 3 days after contusion, and prior to the last bout of exercise. Because of severe functional impairments at 1 week post-SCI, we determined that the only behavioral test the animals were capable of performing was open field locomotion. Therefore, the grip and grid tests were not implemented in these short survival groups.

Protein analysis and quantification. Western blot analysis of spinal cord tissue extract following contusion with or without 5 days of wheel Ex was carried out at 10 days after injury. Rats were overdosed with Euthasol (390 mg/kg sodium pentobarbital and 50 mg/kg phenytoin, ip) and cervical spinal cord samples of approximately 2 mm each in length from the lesion epicenter (C4) and rostral (C3) and caudal (C5) segments were harvested, weighed and immediately placed in ice cold extraction buffer (100 mM Tris buffer, pH 7.4,

750 mM NaCl, 2 mM EDTA, 1% BSA, 2% Triton) in the presence of protease inhibitors (Roche) and 10 mM phenyl-methyl sulphonyl-fluoride (PMSF). Samples were sonicated and spun at 14K for 30 min at 4°C. Supernatants were collected and stored at -80°C for Western blot analyses. Samples were boiled in standard Laemmli sample buffer for 2 min. Equal amounts of total protein were resolved onto 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membrane (BioRad). Each nitrocellulose replica was subjected to incubation with primary antibody followed by incubation with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody. Primary rabbit polyclonal anti-HSP-70 (Cell Signaling, 1:1000), mouse monoclonal anti-HSP-27/25 (StressGene, 1:1000), rabbit polyclonal anti-BDNF (Santa Cruz, 1:1000) antibodies, and HRP-conjugated secondary antibodies were used to detect corresponding proteins on the same membrane. To avoid overlapping patterns of immunoreactivity, blots were stripped between incubations and the order in which different primary antibodies using buffer containing 65 mM Tris, pH 6.8, 1% SDS and 1% β -mercaptoethanol for 30 min, were probed was constant for all blots. As a final step, membranes were probed with mouse monoclonal anti-actin antibody (AC15, Sigma, 1:5000) to confirm comparable protein loading for each lane. Immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL, Amersham Biosciences). The optical densities of immunopositive bands were determined using GeneTool Analysis software (Syngene Scientific), and values for each sample were normalized to actin. Our initial analysis separating out the biochemical results by region (epicenter, caudal and rostral to the lesion) found no differences in protein levels so the final results were calculated by combining values for three areas for each animal. Optical density values were combined for each group and the mean from the control SCI-only group was assigned an arbitrary unit of one. Results of the Ex group were compared to their baseline value of the control group.

Long-term survival: Functional recovery with exercise

Behavioral testing. Animals were randomly assigned to one of two groups: contusion only ($n=6$) or contusion + exercise ($n=8$). All behavioral testing was started at least 30 min following the Ex session and completed between 08:00 and 11:00 a.m. After acclimation to the testing apparatus over a 1-week period, pre-injury baseline scores for fore and hind limb open field locomotion, as well as grid walking and grip strength performance were obtained for each animal. Two days later, animals were subjected to a unilateral C4 contusion injury. Open field fore and hind limb evaluation began at 3 days post-operatively, but grip and grid evaluation could not begin until 1 week post-injury, because most animals had difficulty performing the grid and grip tasks at 3 days post-operatively, as was observed previously in the short-term experiment. Animals were evaluated weekly for 8 weeks.

Open field locomotion. Fore and hind limb function were evaluated in an open field measuring 2.5×3 feet. Rats were observed for 4 min by two individuals blinded to the treatment condition. The fore limb locomotor scale (FLS), devised in our behavioral core facility from observation of recovery patterns in cervical level injured rats, is an 18-point scale that

defines deficits based on range of motion, level of weight support, and whether the paw is placed parallel to the body (Cao et al., 2007; Sandrow et al., 2008). The FLS is similar in assessment style to the hind limb Basso, Beattie, and Bresnahan locomotor rating scale (BBB) (Basso et al., 1995) rating scale, except that the individual fore limbs were scored, but not averaged due to the unilateral nature of the injury. Each animal was scored during direct observation in the open field for 4 min and videotaped for later reference if necessary.

Grid-walk (sensorimotor) test. Paw placement for the fore and hind limb of the affected side was assessed as the animals walked on an elevated plastic coated wire mesh grid (36 cm×38 cm with 3-cm² openings). Animals were placed on the grid and allowed to walk freely across the platform. Each limb was scored for the total number of steps over a 2-min period, and the percentage of correct steps and missteps was calculated. A misstep occurred when the entire foot fell through the grid. Data are presented as a percentage of correct steps over the total number of correct steps and missteps. The grid-walk test has been validated as an assessment of sensorimotor function (Grill et al., 1997).

Grip strength (motor) test. The grip test assesses motor function by recording the amount of force (in Newtons) exerted by the affected fore limb prior to releasing the grasp of the grip device (SDI Grip Strength System model DFM-10, San Diego Instruments, San Diego, CA) (Anderson et al., 2005). The animal is placed on the platform of the device and encouraged to grip the metal bars attached to the force transducer with the affected forepaw. Once the grip is secured the animal is slowly pulled horizontally from the bar. The transducer records the force at the point of grip release. Each animal performed the test weekly, and six trials were completed during each session. Data are presented as a percentage of the weekly grip force value divided by the animal's pre-injury baseline.

Wheel walking assessment. This assessment originated in our laboratory to record the performance within the wheel after injury. Each animal was evaluated in a wheel without the cloth material in order to observe three components of stepping in a qualitative manner: weight support, fine motor skill or the ability to grip an individual rung and successful steps where the foot did not fall through the rungs. Animals were observed over 20 consecutive right fore limb steps and five consecutive steps during the middle of the 20 steps were qualitatively evaluated. Each component was analyzed on a separate point scale. A successful step was given 1 point (maximum score = 5), weight support was given a score of 1–4 (1, no weight support; 2, 50% or less of weight-supported steps; 3, 75% of the steps weight bearing; and 4, full weight support) and the ability to grip was given a score based on a three-point scale (1, no grip; 2, partial grip; and 3, full grip). After evaluating five steps, the total score for each cycle was obtained (the maximum (baseline) score per cycle for five steps: 5, for all successful steps +4, for all weight-supported steps +3, for correct gripping = 12).

Statistical analysis

Behavioral data were analyzed by two-way ANOVA between group (injury alone and injury with Ex) and time, with

time taken as a repeated measure. Post-hoc analysis for the grid and grip tests was performed using the Bonferroni test. The FLS and BBB scores covered a sufficient range over time to approximate a normal distribution and therefore could be analyzed in this manner. However, post hoc analysis for the FLS and BBB data did not meet conditions of normality and therefore were analyzed with the Mann-Whitney *U*-test. Post hoc analysis for the grid and grip tests was performed using the Bonferroni test. All statistical analyses were performed using Stat View software (SAS Institute Inc., Cary, NC).

Immunocytochemistry and image analysis

At 8 weeks post-injury, animals were overdosed with Euthasol and perfused transcardially with 4% paraformaldehyde in 0.1M Sorenson's phosphate buffer. C3-C6 spinal cord was removed, post-fixed in paraformaldehyde at 4°C for 4 h, and then immersed in 30% sucrose for 36 h at 4°C. Four series of alternating sections at 25- μ m thickness were prepared with a cryostat in a transverse plane ($n = 4$ from control and $n = 6$ from Ex group) through the rostral to caudal extent of the lesion or in a horizontal plane ($n = 2$ per group) through the dorsal to ventral extent of the lesion. Sections from a single series were mounted on a glass slide and stained for Nissl-myelin to measure lesion size and a second and third series prepared for immunocytochemical detection of reactive astrocytes (glial scar) or phagocytic cells.

For immunocytochemical labeling, all of the spinal cords were cut and their free-floating sections were washed with 0.1M Sorenson's phosphate buffer and methanol solution (50:50), permeabilized in Triton phosphate-buffered saline (T-PBS) for 15 min and blocked for non-specific reactivity with an appropriate normal serum (goat [NGS] or rabbit [NRS])/T-PBS, 1:20 for 15 min. Primary antibody against glial fibrillary acidic protein (GFAP) to detect astrocytes (1:500, Dako, Carpinteria, CA) or the lysosomal membrane of phagocytic cells (ED1 clone, 1:1000, Chemicon International, Temecula, CA) was applied to free floating sections and incubated overnight at room temperature. Sections were washed, incubated with appropriate secondary antibody (either RAGIgG or GARIGG) for 90 min at room temperature, washed, and incubated in appropriate peroxidase-antiperoxidase (PAP) complex for 60 min at room temperature. After another set of washes with T-PBS and incubation in diaminobenzidine (DAB) solution (0.67 mg/kg, Sigma-Aldrich, St. Louis, MO) for 10 min until the desired color intensity was reached, sections were washed in 0.1M phosphate buffer, mounted out of ddH₂O, dried, and cover slipped with Permount (Sigma-Aldrich, St. Louis, MO). Sections were examined under bright field with a Zeiss Axioskop microscope and quantified with Image J imaging software (U.S. National Institutes of Health, 1997–2007; <http://rsb.info.nih.gov/ij/>).

To determine the amount of spared tissue 8 weeks after SCI, the contralateral white and gray matter of the spinal cord and spared gray and white matter on the ipsilateral side were measured separately. Every section that encompassed the lesion cavity was included in these measurements. For densitometric analysis three representative sections each from the lesion epicenter, rostral and caudal areas were evaluated for each animal. The average area fraction was calculated by indicating the region of interest of the cord ipsilateral to the lesion. The immunochemically reactive area fraction was

determined by dividing the area of stained tissue by the area of the entire ipsilateral side. The area fractions of reactive tissue were then averaged together and combined with their representative group. Statistical significance was determined at the 0.05 level by calculating Student *t*-test using Stat View software (SAS Institute Inc., Cary, NC).

Results

Biochemical changes after 5 days of forced exercise

Extracts of fresh spinal cord tissue from the lesion site and surrounding rostral and caudal areas were used to evaluate the expression of BDNF and two HSPs, HSP-27 and HSP-70, using semi-quantitative (optical density) Western blot analyses. Representative images of immunopositive bands in tissues from two animals per group are shown in Figure 1A. Trends of increased expression of HSP-27 and BDNF were evident in all areas of the spinal cord adjacent to the lesion site with Ex when compared to the control group (Fig. 1B), but there was no obvious change in the pattern of HSP-70 protein expression within the spinal cord in or around the lesion site.

Functional recovery after 8 weeks of forced exercise

Forced Ex wheel training began five days following the C4 unilateral contusion injury to allow sufficient time for recovery from anesthesia and initial injury deficits. During the 20 minute sessions of wheel running the speed was adjusted to challenge each animal to perform at its highest level. Speeds ranged from 3.5 m/min to 7.5 m/min during the first week of training. The maximum speed of 14.0 m/min forced a brisk walk by an uninjured rat, similar to walking on a treadmill and this speed was attained 3 to 4 weeks after SCI in the Ex trained group.

Over ground locomotion

FLS (fore limb). ANOVA displayed an effect of Ex [$F(13, 8) = 4.7, p < 0.05$] along with an effect of time [$F(8, 8) = 48.7, p < 0.001$] indicating a significant deficit followed by recovery. The fore limb locomotor scale (FLS) revealed similar deficits in both Ex and non-Ex groups 3 days after SCI, demonstrating consistency of the injury model (Fig. 2A). The mean score of ~ 2.5 indicated extensive movement of one joint and slight movement of another joint of the fore limb. At week 3 after SCI the Ex group showed significant improvement with a mean FLS score of 12.88 ± 0.44 ($p < 0.05$) corresponding to continuous plantar placement with paw position parallel either at initial contact, lift off or both. The control group exhibited continuous plantar stepping at week 3 (mean $\pm$ SEM: 11.67 ± 0.67) which was significantly less than the Ex group performance. The control group did not regain further functional activity at week 4 while the Ex group showed steady improvement, up to 13.5 ± 0.33 ($p < 0.05$). Half of the rats in the Ex group were able to plantar place consistently with paw rotation at initial contact and/or at lift off and showed occasional toe clearance (=FLS of 14). During the remaining weeks the groups had comparable scores indicating spontaneous recovery without intervention and the absence of further improvement with Ex. Previous studies characterized the recovery of function following a unilateral cervical contusion recognizing stages of early recovery, late recovery and plateau (Gensel et al., 2006). Our SCI only group followed the

FIG. 1. Western blot analysis of spinal cord tissue extracts following contusion injury and 5 days of forced exercise (Ex). (A) Representative images of nitrocellulose replicas probed with antibodies to heat shock protein-70 (HSP-70), HSP-27, and brain-derived neurotrophic factor (BDNF) from two groups of animals, spinal cord injury (SCI) alone (animals 1 and 2), and SCI + Ex (animals 1 and 2). (B) Quantitative analysis of immunoblots. The optical densities of immunoreactive bands were normalized to actin, and expressed as a ratio to the animal group with injury only (arbitrary units [AU]). Each bar represents combined values from three areas: epicenter (EPI), caudal (C) and rostral (R) to the injury for each animal (mean $\pm$ SD).

same pattern of recovery; an early phase (weeks 2–4) where animals showed improved function followed by a late phase (weeks 5–8) during which the improvement slowed and eventually plateaued. We first determined whether there was significance with an ANOVA over the 8 week period. This was then followed by post-hoc analysis which examined changes within the early and late phases since both Ex and non-Ex groups had the same initial deficits prior to any training and the plateau phase was reached at similar times post injury in each group. When data was separated into early (2–4 weeks) and late (5–8 weeks) recovery phases an acceleration of recovery with Ex in the early phases of treatment ($p < 0.05$; Fig. 2B) was revealed. The unaffected left limb also was monitored in this study to insure the unilateral effect of the injury. The FLS scores for the left fore limb were unchanged (17.0 ± 0.0) throughout the 8 week period.

BBB (hind limb). The ipsilateral hind limb BBB scores (Fig. 2c) showed a consistent deficit 3 days post-injury in both

groups, with frequent weight support but inability to coordinate steps with the injured fore limb (SCI 11.33 ± 0.29 , SCI + Ex 11.5 ± 0.43). At 1 week post-SCI the ipsilateral hind limb in animals of each group consistently provided weight support and frequent coordination with the affected right fore limb. BBB scores from each group began to plateau at week 4 with BBB near a score of 14, indicating stepping with consistent weight support, consistent fore to hind limb coordination and paw rotation. For the contralateral hind limb there was a deficit in toe clearance at 3 days post-injury, but by 1 week the contralateral limb improved to near normal values (mean 20.3 ± 0.7), followed by normal scores of 21 in all subsequent weeks.

Grip strength test

Measurements of grip strength were expressed as a percentage of the baseline grip strength and showed no improvement with forced Ex (Fig. 3A). An initial deficit was demonstrated by both groups 1 week post-SCI with grip

FIG. 2. Locomotor assessment of fore limbs and hind limbs after forced exercise (Ex). (A) Fore limb locomotor score (FLS) over the 8 week period. Both ipsilateral (spinal cord injury [SCI] alone, square; SCI + Ex, diamond) and contralateral fore limbs (triangle) were evaluated (mean $\pm$ SEM). Testing began 3 days post-SCI, and the effects of Ex were evident by week 3. A significant difference between SCI and SCI + Ex occurred during weeks 3 and 4 (* p < 0.05). Week 5 data displays a slight decrease in the mean FLS performance that was due to the inactivity of a few animals for that week only. (B) Recovery was grouped into two phases: early (weeks 2–4) and late (weeks 5–8). The FLS during the early phase of recovery was significantly higher with Ex (* p < 0.05) compared to SCI alone (mean $\pm$ SEM). (C) Basso, Beattie, and Bresnahan locomotor rating scale (BBB) assessment over the 8-week time period (mean $\pm$ SEM). Injured hind limbs from each group SCI alone (square) and SCI + Ex (diamond) along with the contralateral uninjured hind limb (triangle) were examined. No significant difference in hind limb function was found when animals were subjected to force Ex.

strengths of 65–70% of baseline. Over the remaining weeks both groups recovered to about 85% of their baseline grip strength, indicating a high degree of spontaneous recovery without intervention. As with FLS and BBB scoring, the data

FIG. 3. Grip strength measurements. (A) Percentage of baseline grip strength measurements of spinal cord injury (SCI) alone (square), and SCI + forced exercise (Ex; diamond) display a decrease in grip strength immediately after injury, followed by an increase in force over the remaining 8 weeks (mean $\pm$ SEM). Grip strength measurements are from the ipsilateral fore limb only. (B) Separating recovery into early (weeks 2–4) and late (weeks 5–8) phases showed no significant improvement with exercise.

was grouped into early (weeks 2–4) and late (weeks 5–8) recovery phases (Fig. 3B). There was no significant difference between groups during early or late recovery periods.

Grid walk

Right fore limb. Animals were placed on the grid beginning 1 week post-operatively to examine the percentage of correct steps made by the injured right fore limb and right hind limb. One week after injury the percentage of correct foot placements decreased to about 75% in both groups followed by a small increase in correct placements by week 8 (Fig. 4A). The percentage of correct steps completed by the affected fore limb was not significantly improved with forced Ex. There was no difference between either group performance over the 8 week period, again indicating a high level of spontaneous recovery.

Right hind limb. ANOVA showed a overall significant effect of time [$F(7,7) = 6.2$, $p < 0.001$] but not an effect of treatment between 70–80%. The initial deficits at week 1 were comparable between groups with an average percentage around 75% of the baseline for correct foot placements

FIG. 4. Grid walk performance of the affected fore limb and ipsilateral hind limb. **(A)** Average percentage of correct foot placements on the grid by the right fore limb (mean $\pm$ SEM). The 8-week period displays an initial deficit during 1 week after spinal cord injury (SCI) in both groups to $\sim 75\%$ of baseline. Throughout the remaining weeks both groups perform in a similar manner reaching $\sim 80\%$ by 8 weeks. **(B)** The percentage of correct foot placements completed by the ipsilateral hind limb. SCI alone (square) made significantly fewer correct placements compared to the SCI + forced exercise (Ex; diamond) over the 8-week period (mean $\pm$ SEM). The Ex group made significantly fewer mis-steps during weeks 3, 4, 6, and 7 ($*p < 0.05$). The largest separation between groups occurred at week 3 implying accelerated spontaneous recovery with Ex. Week 5 data displays a decrease but the variability is made evident by the large SEM indicating percentage of placements were not much different. **(C)** Mean percentage of correct foot placements made by the ipsilateral hind limb when split in to two phases of recovery, with the Ex group performing significantly more correct foot placements early after injury (mean $\pm$ SEM).

(Fig. 4B). The Ex group had a significantly higher percentage of correct foot placements ($p < 0.05$) at week 3 (SCI: $69\% \pm 4.2$; SCI + Ex: $95.6\% \pm 1.88$) and week 4 (SCI: $82.8\% \pm 6.19$ and SCI + Ex: $95.2\% \pm 2.19$, respectively) compared to the control group. A decrease in performance at week 5 removed the significant effect of Ex but weeks 6 and 7 followed the same pattern as weeks 3 and 4 with the SCI + Ex group performing significantly better, making fewer mis-steps on the grid walk compared to injury alone (week 6: SCI $88\% \pm 3.44$, SCI + Ex 98.14 ± 1.85 ; week 7: SCI $80.27\% \pm 5.31$, SCI + Ex $93.95\% \pm 2.89$; $p < 0.05$). When combining the weeks into early (weeks 2–4) and late (weeks 5–8) recovery phases (Fig. 4C), the forced Ex intervention group exhibited accelerated recovery in the early phase that was significantly different compared to the injury alone group (SCI: 76.11 ± 6.0 ; SCI + Ex $89.63\% \pm 4.55$; $p < 0.05$). Both groups displayed similar functional recovery on the grid walk test during the late phase of recovery because of poor performance at week 5.

Wheel walking assessment

Biweekly evaluation of the locomotor performance within the wheel revealed an initial deficit 1 week following injury in both groups followed by near full recovery by week 3 (Fig. 5). The initial deficit was significant compared to the baseline assessment in both Ex and non-Ex groups ($p < 0.05$) due to a decline of all three components of the rating scale. Scores increased in both groups and plateaued by week 3 indicating no effect of Ex. Spontaneous recovery in wheel walking after contusion injury did not allow for a lasting severe deficit to display a separation among Ex and non-Ex groups. Although this assessment did not display an effect of treatment, it was useful in characterizing a fore limb deficit similar to the FLS. Examining the rating scale we found that there was no difference between weighting each parameter equally or in assigning each property with a point system as per the current scale. Further testing is necessary to fully validate this assessment as a measure of fore limb recovery.

Histological and immunocytochemical evaluation after 8 weeks of exercise

A majority of the animals in the Ex group displayed sparing of the lateral rim of the dorsal and ventral funiculi with increasing distance from the epicenter however examination of the lesion site for the extent of spared tissue after 8 weeks of Ex showed no difference in the amount of spared gray or white matter compared to SCI alone (Fig. 6A). There was some variation in the extent of spared ventral medial white matter in animals from both Ex and non-Ex groups, but the overall amount of spared tissue was similar among both Ex and non-Ex groups.

Eight weeks after SCI, the presence of reactive astrocytes rostral, at the epicenter and caudal to the injury, was evaluated with GFAP immunostaining (Fig. 6B). In the Ex group, there was a trend towards a decrease in the presence of reactive astrocytes in the rostral spinal cord compared to the epicenter area and there was a significant decrease in the density of reactive astrocytes ipsilateral and caudal to the injury site (SCI: $93\% \pm 4.0$; SCI + Ex: $88\% \pm 10.0$; $p < 0.05$) in the Ex group. Figure 6C displays the percentage of tissue caudal to the injury site which was stained for endogenous microglia and invading macrophages. Animals that received forced Ex

FIG. 5. Wheel assessment. Biweekly evaluation of wheel walking performance. Three parameters were observed: successful steps, fine motor skill (grip ability) and foot placement. A significant difference was found in both groups 1 week after spinal cord injury (SCI) compared to baseline performance (* $p < 0.05$). A significant difference was not found between groups, but there was a trend towards accelerated recovery with forced exercise (Ex) at week 1. Beginning at week 3 and continuing for the duration of the study, both groups displayed similar performance in wheel walking (mean $\pm$ SEM).

had a trend towards decreased phagocytic cell presence caudal to the injury (SCI, mean $25.0\% \pm 7.2$; SCI + Ex, mean $16.2\% \pm 7.0$; $p = 0.076$), which was particularly evident in the dorsal and lateral funiculi. This trend towards decreased inflammatory cell invasion was evident at 1 week after injury (data not shown), although ED1-positive cells were more prominent in injured gray matter at this early time point compared to the clustering in white matter observed at 8 weeks (Fig. 6C). No significant difference in the density of ED1 positive cells in rostral or epicenter areas occurred with Ex.

Discussion

Significant benefits of forced Ex after a cervical unilateral contusion were observed in the behavioral and anatomical measures of this study with additional evidence of changes in protein levels after just 5 days of Ex. Early recovery (2–4 weeks post-injury) of motor and sensorimotor capabilities after SCI was accelerated in the affected fore and hind limbs of animals trained in the exercise wheel compared to non-exercised animals. These results indicate that implementing forced Ex shortly after SCI is advantageous up to 4–5 weeks post-injury, after which no further benefits to these outcome measures were apparent. In the Ex group, tissue caudal to the injury site demonstrated a significant decrease in the presence of reactive astrogliosis and a trend towards decreased presence of phagocytic cells, which in combination may have an impact on reducing secondary tissue damage. Biochemical results after 5 days of Ex indicated an increase in BDNF and HSP-27 within and around the lesion site suggesting an early neuroprotective role for forced Ex. These observations correspond to an increase in mRNA for both BDNF and HSP-27 with short term hind limb Ex following a complete transection injury (Dupont-Versteegden et al., 2004; Ying et al., 2005). Based upon these results, forced wheel-walking is an effective adjunct for the early phase of recovery but to improve the late

phase of recovery some additional treatment interventions appear to be necessary.

Several groups have evaluated cervical contusion models with varying degrees of behavioral and anatomical defects (Schrimsher and Reier, 1992; Soblosky et al., 2001; Pearse et al., 2005; Gensel et al., 2006). Regardless of the cervical level or the unilateral or bilateral nature of the injury, significant spontaneous recovery occurred similar to what we found with our non-exercised control group. In all cases, there was a lasting deficit in skilled fore limb function and further these studies reported ipsilateral hind limb deficits, consistent with what we found in our animals. Gensel et al. (2006) commented on the temporal progression of spontaneous recovery after a C5 unilateral contusion injury, and we applied this information to our C4 injury model. We observed an initial paralysis of the affected fore limb and ipsilateral hind limb lasting for a period of 3–5 days. An early spontaneous recovery phase over the next week resulted in increased range of motion exhibited by the shoulder and the ability of the hind limb to bear weight, resulting in significant improvement in motor scores. Spontaneous recovery continued through the 4th week after injury where animals gradually regained the ability to make plantar placements with their affected fore limb and hind limb. Lasting permanent deficits of the fore limb consisted primarily of fine movements of the digits likely due to rubrospinal tract damage and dorsal column damage which has been shown to alter tactile sensation (Whishaw, et al., 1998; McKenna and Whishaw, 1999; Ballerman et al., 2001; Webb and Muir, 2005; Anderson et al., 2007). Deficits of paw placement at lift off and/or initial contact corresponding to dorsal corticospinal tract disruption and absence of coordinated stepping with the ipsilateral hind paw suggest reticulospinal, vestibulospinal, and long propriospinal tracts also were disrupted (Anderson et al., 2005). Persisting impairments were indicative of a third or plateau phase of recovery. Histological examination at 8 weeks after injury found sparing of the medial portion of the dorsal and ventral funiculi along with the lateral edge of the lateral funiculus. Interruption of the descending spinal pathways mentioned can explain most of the deficits seen after injury and these represent targets for approaches to promote regeneration.

Because forced fore limb exercise had not been evaluated in a rodent model of cervical SCI, it was important to evaluate the performance of animals during wheel walking. We assessed three parameters: the number of weight-supported steps during five consecutive step cycles, the ability to grasp the rung, and the percent of correct (plantar) foot placements. The training conditions varied from the testing conditions in that material was woven within the rungs to create a flatter surface to prevent additional fore limb injury during exercise. With the use of this material, the wheel training was similar to treadmill training except that the animals walked on a slight incline in the wheel. Altering a free access wheel by eliminating the open rungs to create a flat surface was previously shown to improve open field locomotion and grid-walk scores in mice after thoracic contusion injury (Engesser-Cesar et al., 2005). When evaluating fore limb placement on the wheel, the woven material was removed, exposing the wheel rungs to assess skilled motor capabilities of the affected fore limb. Detection of a significant initial deficit following injury demonstrates the potential of this assessment as a measure of fore limb use during locomotion.

FIG. 6. Anatomical and histological evaluation of lesion site after 8 weeks of forced exercise (Ex). (A) Images from the epicenter with a Nissl-myelin stain. Amount of spared white and gray matter were comparable with or without Ex. (B) Representative images caudal to the lesion site labeled with glial fibrillary acidic protein (GFAP). A graph representing the area of reactive astrogliosis located rostral, at the epicenter and caudal to the lesion site (mean $\pm$ SD). A significant decrease in the density of reactive astrogliosis caudal to the lesion site is shown. (C) Representative images caudal to the lesion site with positive labeling of macrophages. The graph displays the area of tissue positive for macrophages caudal to the lesion site, which shows a trend towards decreased phagocytic presence after 8 weeks of Ex (mean $\pm$ SD).

The present study also extends the observation of exercise-induced expression of NTFs to a cervical contusion injury model. Neurotrophins are proteins which are important for neuron survival, differentiation and growth of the immature nervous system and for synaptic stability in the mature nervous system. In our model, the expression of BDNF in and around the lesion site increased substantially during 5 days of forced Ex, and several other studies have shown that Ex increases levels of neurotrophic factors and their receptors in exercised muscle and spinal cord (Dupont-Versteegden et al., 2004; Ying et al., 2003; Gomez-Pinilla et al., 2002; Chytrava et al., 2008; Ying et al., 2008). This increase in expression may directly affect neuroplasticity and/or regeneration of injured axons. A recent study examining axonal sprouting in mice voluntarily exercising after SCI (Goldshmit et al., 2008) found accelerated axonal sprouting and enhanced performance by the Ex group in both the open field locomotion and grid walk test. Because our model involves a unilateral injury, collateral sprouting of axons from the uninjured side of the cord or from axons that were spared from the initial contusion injury may

be available to compensate for disrupted connections after SCI, as described in a recent study examining sprouting after a unilateral corticospinal tract lesion and constraint training (Maier, et al., 2008). Proprioceptive fibers also may come into play by reconnecting the fore and hind limb since the ipsilateral hind limb of the animals that received Ex made fewer mis-steps on the grid walk and demonstrated the ability to make coordinated steps in an open field compared to the control. A study by Bareyre et al. (2004) investigated neuronal reorganization within the spinal cord after a thoracic SCI and found that the spontaneous recovery seen was due to new connections created by sprouting propriospinal interneurons. A more recent study by Courtine et al. (2008) investigated return of function after a severe SCI and the contributions of reorganizing propriospinal neurons in spontaneous recovery.

HSPs act as molecular chaperones with the ability to detect and correct mis-folded proteins. Given their important role in normal cells, their functional significance is magnified in cells undergoing stress. HSP27 is one member of this class

of proteins found to upregulate after peripheral nerve damage (Plumier et al., 1997; Costigan et al., 1998; Benn et al., 2002) and HSP70 expression is increased rapidly (<6 h) after a weight-drop injury in adult rats (Gower et al., 1989). At 5 days after SCI, we found that HSP27 levels were increased by Ex, but there was no change in expression of HSP70, which suggests different roles for the HSP isoforms. It is possible that Ex-induced HSP27 serves as a neuroprotective molecule against secondary tissue damage and apoptosis that occurs in the days to weeks after SCI (Alford et al., 2007). HSP70 is comprised of constitutive and inducible forms of protein, with specific targets within cells, and likely has a more immediate effect since there is a rapid increase in expression in response to injury. In our experimental paradigm, the level of expression HSP70 in the injured and injured with Ex groups remained unchanged suggesting that the post-injury interval (10 days) was too long to observe a change due to SCI.

Inflammation is a prominent contributing factor to secondary degeneration after SCI (Schwab and Bartholdi, 1996). To expand our cellular results, we examined the effects of Ex on the cytokine response in and around the lesion site (Sandrow et al., *Abst. Soc. Neurosci.* 2006;762.4) and found a trend towards a reduction of interleukin-1 β , interleukin-2, interleukin-6, and interferon- γ , all pro-inflammatory messengers. Increased levels of interleukin-4, an anti-inflammatory cytokine were measured in and around the lesion site. These findings indicate a decreased inflammatory response after 5 days of forced Ex, but we did not investigate the cytokine response after 8 weeks of injury since levels return to baseline within several days of injury. There does not appear to be a long-term consequence of Ex on the presence of phagocytic cells in or around the lesion; however, the expression of GFAP as a marker of astrocytic reactivity was reduced at 8 weeks after injury in the Ex group.

In summary, these findings show a high degree of spontaneous recovery occurring after a C4 unilateral contusion with accelerated recovery during early stages of the post-injury response with a forced Ex treatment strategy. The increase in local levels of BDNF and HSP27 indicate possible mechanisms for this transient recovery but it is apparent that additional interventions are needed to expand the recovery phase and promote long-term effects of a combined treatment strategy.

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