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Functional recovery of stepping in rats after a complete neonatal spinal cord transection is not due to regrowth across the lesion site

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Abstract

Rats receiving a complete spinal cord transection (ST) at a neonatal stage spontaneously can recover significant stepping ability, whereas minimal recovery is attained in rats transected as adults. In addition, neonatally spinal cord transected rats trained to step more readily improve their locomotor ability. We hypothesized that recovery of stepping in rats receiving a complete spinal cord transection at postnatal day 5 (P5) is attributable to changes in the lumbosacral neural circuitry and not to regeneration of axons across the lesion. As expected, stepping performance measured by several kinematics parameters was significantly better in ST (at P5) trained (treadmill stepping for 8 weeks) than age-matched non-trained spinal rats. Anterograde tracing with biotinylated dextran amine showed an absence of labeling of corticospinal or rubrospinal tract axons below the transection. Retrograde tracing with Fast Blue from the spinal cord below the transection showed no labeled neurons in the somatosensory motor cortex of the hindlimb area, red nucleus, spinal vestibular nucleus, and medullary reticular nucleus. Retrograde labeling transsynaptically via injection of pseudorabies virus (Bartha) into the soleus and tibialis anterior muscles showed no labeling in the same brain nuclei. Furthermore, re-transection of the spinal cord at or rostral to the original transection did not affect stepping ability. Combined, these results clearly indicate that there was no regeneration across the lesion after a complete spinal cord transection in neonatal rats and suggest

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that this is an important model to understand the higher level of locomotor recovery in rats attributable to lumbosacral mechanisms after receiving a complete ST at a neonatal compared to an adult stage.

Keywords

locomotion; retrograde labeling; spinal cord injury; training; rubrospinal; corticospinal

Rats receiving a complete spinal cord transection (ST) neonatally spontaneously recover a significant level of stepping ability, whereas minimal recovery is attained in rats transected as adults (Murray et al., 2004). Rats transected prior to, but not after, P15 showed good recovery of motor function (Weber and Stelzner 1977). Rats transected at P7 showed the best, whereas rats transected after P14 showed the worst, recovery of stepping (Commissiong and Toffano 1989). These data indicate that there is a critical period between P7 and P15 during which the rat spinal cord loses its intrinsic ability to generate locomotor activity. The mechanisms involved in this process are unclear.

The amount of functional recovery related to regrowth of spinal tracts across the lesion leading to the restoration of functional contacts or to intrinsic adaptations in the spinal circuitries below the lesion that control hindlimb locomotor activity is controversial. Some anatomical evidence that regeneration does not occur after a neonatal ST exists. No signs of regeneration were observed through the site of a ST at P1–P2 or P21–P25 when evaluated 3–6 months post-transection (Bernstein et al., 1981). No ascending fibers crossed the lesion three months after ST (mid-thoracic) at P0–P1 (Bryz-Gornia and Stelzner; 1986), corticospinal axons after ST (mid-thoracic) at P0–P4 (Cummings et al., 1981), or descending axons from several supraspinal nuclei seven weeks after ST (low-thoracic) at P2 (Hase et al., 2002). Re-transection studies also have provided functional evidence for a lack of regeneration after neonatal ST. Rats were transected (mid-thoracic) at P2 and trained to walk bipedally for 5–10 weeks (Miya et al., 1997). After re-transection just rostral to the initial lesion, rats showed an initial period of depression of stepping but recovered to pre-re-transection levels of performance. Similarly, several hindlimb responses (including stepping) after re-transection (low-thoracic level) in weanling rats that had been transected neonatally (mid-thoracic) were similar to that observed pre-re-transection (Stelzner et al., 1975).

Some results, however, suggest that there is some spontaneous regeneration after a neonatal ST. For example, Wakabayashi et al. (2001) reported that some rats undergoing a ST at P14 regained some hindlimb function by 5 weeks post-ST. Muscle-evoked potentials could be elicited in selected hindlimb muscles of some spinal rats and, based on retrograde labeling it appeared regeneration had occurred in some descending tracts.

In the present study, we hypothesize that recovery of stepping after a mid-thoracic ST in neonatal (P5) rats can be attributed to changes in the lumbosacral circuitry and not to regeneration across the lesion. We use extensive anatomical evidence through anterograde, retrograde, and transsynaptic labeling, and re-transection experiments to substantiate this hypothesis. We also include a group of step-trained ST rats to enhance the regeneration potential. Our data clearly indicate that there is no regeneration across the lesion after a complete ST in neonatal (P5) rats and suggest that this is an appropriate model to use to understand the higher level of locomotor recovery in rats receiving a complete ST at a neonatal compared to an adult stage.

Materials and Methods

To test that recovery of stepping after a complete mid-thoracic ST in neonatal rats can be attributed to changes in the lumbosacral circuitry and not to regeneration across the lesion, we performed a complete ST in rat pups at P5 and assessed their stepping ability using a battery of behavioral and kinematics analyses from the time of weaning (26 days) to 12 weeks of age. During this period, some of the spinal rats were step trained on a treadmill to improve their stepping ability and to enhance any potential for regeneration across the lesion. Six days prior to the end of the study, the spinal cords of some of the spinal rats in the non-trained groups were re-transected above, at, or below the initial transection site (See Figure 1 and Table 1). At the same time, Fast Blue soaked gelfoam was inserted at one of the transection sites in each rat to retrogradely label spinal pathways reaching specific regions of the brain. The non-trained spinal rats then were retested for stepping ability during 5 days of recovery. A group of spinal rats step trained for 8 weeks were tested for stepping ability, re-transected at a level rostral to the initial transection site, and retested for stepping ability. In another group of step-trained and non-trained spinal rats, biotinylated dextran amine (BDA) was injected into the somatosensory motor cortex of the hindlimb area and red nucleus to anterogradely label spinal pathways that may have traversed the lesion. Finally, in another group of trained and non-trained spinal rats pseudorabies virus (PRV, Bartha) was injected into the soleus and tibialis anterior muscles to transsynaptically label functionally connected neurons. Age matched intact rats were used for Fast Blue, BDA and PRV injections. The number of rats undergoing each experimental condition is summarized in Table 1.

Animals

Data were collected from 128 young adult Sprague-Dawley female rats (~3 months of age) (Table 1). All procedures, including the handling of the PRV, were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles and followed the American Physiological Society Animal Care Guidelines.

Surgical and animal care procedures

All surgical procedures were performed under aseptic conditions. The spinal cords of 5-day old (P5) rat pups were transected completely at a mid-thoracic level as previously described (Kubasak et al., 2005). The pups were anesthetized with isoflurane gas, 1.0 to 2.5% via facemask as needed. Surgery was performed with the pups on a water-circulating heating pad maintained at 37° C to prevent hypothermia. A dorsal midline skin incision was made from ~T6 to T10 and the paravertebral muscles and fascia from ~T7 to T9 were reflected laterally to expose the vertebrae. Small spring microscissors and small blunt forceps were used to remove the dorsal portion of the T8 and T7 vertebrae to expose the spinal cord. The dura was picked up using fine forceps and microscissors were used to completely transect the spinal cord (including the entire extent of the dura). Small cotton balls were used to separate the cut ends of the spinal cord and to clean the transection site. Two surgeons independently verified a complete transection by gently passing a fine glass probe through the transection site and then lifting the cut ends of the spinal cord. Gelfoam was inserted in the transection site to minimize bleeding and to separate (~2 to 3 mm) the two cut ends of the spinal cord. The muscle/fascia and skin overlying the spinal cord were closed in layers using 6.0 chromic gut and 6.0 silk suture, respectively. After surgery the rat pups were placed in an incubator maintained at 37° C until fully recovered. All rat pups within a litter were taken away and returned to their mothers as a group. Female littermates not undergoing the ST surgery were used as non-injured intact controls. Rat pups were weaned at ~26 days after birth and then housed individually in polycarbonate cages in a room maintained at 26 ± 1° C with 40% humidity and a 12:12 h light:dark cycle. The cage floors were covered with CareFresh bedding. Dry kibble and water were provided *ad libitum* and pieces of fruit were given once daily.

Step training procedures

Some of the ST rats were step trained bipedally and tested on a treadmill using a robotic device (Table 1). A detailed description of the robotic device used for treadmill locomotion has been published previously (de Leon et al., 2002a,b;Timoszyk et al. 2002). Briefly, the upper body of the rats was supported in a harness that allowed placement of the feet on a treadmill belt. Robotic arms were attached to the ankles of the rat in a passive mode and a counterbalanced body weight support system was used to provide the amount of weight support by the rat required to optimize hindlimb stepping. Prior to training, the rats were acclimated to the robot training system. Beginning 26 days after ST (31 days of age), the rats were trained 9 min/day, 5 days/wk for 8 wks. Treadmill speed (6, 11, or 20 cm/s) and the percentage (between 30% and 90%) of the animal's body weight supported by the robotic system were adjusted to enable each rat to execute plantar surface stepping. Stepping ability was assessed after 4 and 8 wks of training and consisted of a series of 1-min tests typically at speeds of 6, 11, and 20 cm/s and at 50% and 75% body weight support. For consistency, all results reported are for 11 cm/s and 75% body weight support. All tests were performed blindly by assigning a code letter to each rat, and deciphering this code only after all of the data analyses were completed.

Determination of maximum number of consecutive steps using video analysis

Video data of stepping were recorded as described by de Leon et al. (1994). During all tests, the movements of the left and right hindlimbs were recorded with video cameras (Panasonic System Camera, WV D5100 Panasonic, Cypress, CA) placed orthogonal to the treadmill. Two individuals reviewed the videotaped stepping sequences and visually counted the number of consecutive successful steps at each given speed and body weight support (see Fig. 2). A successful step was defined as any weight-bearing step with obvious swing (forward movement of the limb with some lift) and stance (backward movement of the limb) phases.

Kinematics and principal component analyses (PCA) based on robotic data

The details for identifying individual steps from the robotic data have been described in detail (Timoszyk et al. 2002). Briefly, the beginning of the stance and swing phases were identified based on changes in the horizontal velocity in each hindlimb, i.e., the change from positive to negative horizontal velocity was defined as the beginning of stance, whereas a change from negative to positive horizontal velocity was defined as the beginning of swing. Once these events were identified, the duration of the swing and stance phases, step length, and step height were calculated.

PCA was performed on behavioral data using a technique adapted from Cai et al. (2005). In short, PCA is a multivariate analysis method that picks out patterns in a dataset, reducing dimensionality in the data without a significant loss of information. PCA analysis extracts the fundamental trajectory from a given series of steps and defines it as the first principal component. The analysis returns values in percentage of total variance that is captured by the first principal component. Therefore, higher values correspond with more consistent trajectories, and thus more consistent stepping in both space and time. This analysis was performed on X vs. Y plots of stepping of each leg to determine the consistency of stepping. The first principal component and PCA variances were calculated using a Matlab script adapted from Cai et al. (2005). Analyses were performed on the entire stepping session for each rat.

Anterograde labeling procedures and tissue processing

Some intact and ST rats were anesthetized with isoflurane as described above and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The dorsal surface of the skull was exposed and a 1 mm × 1 mm square burr hole was made on both sides. Injections were made bilaterally into the somatosensory motor cortex of the hindlimb area and red nucleus

(Fig. 1A). Five injections were made on each side: each injection consisted of 2.0 μl of 10% BDA (10,000 MW, Molecular Probes, Eugene, OR). The coordinates used for the injections relative to bregma for the somatosensory cortex were: anterior-posterior 1.0 mm, medial-lateral 2.5 mm, and dorsal-ventral 1.0 mm. BDA was injected into the red nucleus at anterior-posterior 4.9 mm, medial-lateral 1.4 mm, and dorsal-ventral 7.8 mm at a 5° angle (Paxinos and Watson 1998). Each BDA injection was given over a 5-min period via a glass micropipette attached to a Hamilton 10 μl syringe. Following each injection, the micropipette was left in place for two min to allow for diffusion of the BDA. Gelfoam was placed over the burr hole and the skin was closed with 4-0 Ethilon suture. Post-operatively, the rats were placed in an incubator (37° C) until fully awake and given Buprenex (0.5 to 1.0 mg/kg, subcutaneously, bid) for two days.

Fourteen days after the BDA injections, the rats were given Eutha-6 (100 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde. The entire spinal cord was removed and impregnated with 30% sucrose for 2 days. Blocks from the thoracic (rostral to the transection site) and lumbar (caudal to the transection site) regions of the spinal cord were embedded in Polyfreeze (Polysciences, Warrington, PA) and Transverse (for red nucleus injected) and horizontal (hindlimb cortex injected) spinal cord sections (40 μm thick) were cut using a cryostat.

To detect BDA-labeling, free-floating spinal cord sections were first incubated in 0.3% H_2O_2 in phosphate buffered saline solution (PBS) (15 min), rinsed in PBS, and incubated in Vectastain Elite avidin-biotin-peroxidase (ABC) complex (1:200 in PBS for 1 h; Vector Labs, Burlingame, CA). Following ABC incubation, the sections were rinsed in PBS, incubated in diaminobenzidine (DAB) (SigmaFast DAB kit for 20 min; Sigma-Aldrich, St. Louis, MO), and stored in PBS. The sections were mounted (ColorFrost Plus slides, Fisher Scientific, Pittsburgh, PA), air-dried, counterstained with methyl green, and coverslipped with Permount (Fisher Scientific). The sections were examined using a Zeiss Axiophot microscope (Carl Zeiss) and Apogee KX-85 camera (Apogee Instruments). Detailed images were acquired using Image Pro Plus software (Media Cybernetics) to visually assess the presence or absence of BDA labeling in the spinal cord sections.

Retrograde labeling procedures and tissue processing

One day after the final testing of stepping ability, some ST non-trained rats were divided randomly into three groups and completely re-transected either one segment caudal to, one segment rostral to, or at the initial lesion site (Fig. 1B; Table 1). In addition, some rats were transected for the first time in the mid-thoracic region of the spinal cord (Table 1). The new lesion sites were packed with gelfoam soaked with a retrogradely transported tracer, i.e., 2% Fast Blue (Sigma-Aldridge), for 90 min. The gelfoam then was removed and replaced with dry gelfoam, and the incision closed as described above. Stepping ability was assessed 5 days after the re-transection of neonatal ST rats or initial transection of intact rats at this adult stage.

After stepping ability was assessed, the rats were given a lethal dose of Eutha-6 and decapitated. The spinal cord and brain were removed immediately, frozen on dry ice, and stored at −80° C. After freezing, the spinal cord (one segment above, at, and one segment below the transection site) and brain were embedded in Polyfreeze. Serial sections of the brain (40 μm thick) were cut and immediately mounted on slides. Neurons were quantified in the somatosensory motor cortex of the hindlimb area, red nucleus, spinal vestibular nucleus, and medullary reticular nucleus. The labeled neurons were counted using a modified optical fractionator technique (Tobias et al., 2003; Williams et al., 1988; West, 1993; West et al., 1991, 1996). Neurons were counted in every second section for the medullary reticular and spinal vestibular nuclei, every third section for the red nucleus, and every fourth section for the hindlimb region of the sensorimotor cortex, respectively. The first section to be counted was chosen randomly so that it was equally likely that any given set of sections would be counted, e.g., odd or even sections.

The vestibulospinal nucleus and reticulospinal nucleus were subdivided into four quadrants, and one quadrant in each section was selected randomly by using a random number generator. A labeled neuron was counted if it displayed clear neuronal morphology with a nucleus.

PRV injection procedures and tissue processing

After the final session to assess stepping ability, the soleus or tibialis anterior muscle of some intact and ST rats were injected with PRV (Bartha) at 4 sites (0.5 μl/site, total virus of 2.5×10^8 pfu/ml) (Table 1; Fig. 1C). After three days, the rats were perfused and the tissues processed as described above. Brain sections (40 μm thick) containing the somatosensory motor cortex of the hindlimb area, red nucleus, spinal vestibular nucleus, and medullary reticular nucleus were processed with immunohistochemistry to detect cells positive for PRV (PRV+). Brain sections were incubated overnight at room temperature with a primary antibody (Rb 133 antibody made in rabbit) that detects PRV at a 1:2000 dilution in 1×PBS. PRV (Bartha) virus and Rb 133 antibody was a gift from Dr. Lynn Enquist at Princeton University, NJ. After extensive washing the sections were incubated for one hour in the secondary antibody labeled with fluorescein isothyiocyanate (FITC) (1:200). The processed sections were mounted on slides and cover slipped using VectaShield mounting media. The slides were evaluated for PRV+ neurons under an FITC filter and digital images were acquired with an Apogee CCD camera using Image Pro Plus software.

Statistics

One-way analysis of variance (ANOVA) followed by Tukey post hoc tests were used to compare the number of labeled neurons across the groups (GraphPad Prism, Version 4, GraphPad Software Inc., San Diego CA). Pre-post re-transection comparisons of the kinematic analyses were made using dependent two-tailed Student t-tests. A repeated-measures ANOVA was used to determine differences in the kinematics analyses among the three testing periods (weeks 0, 4, and 8). Bonferroni post-hoc tests were used to determine significant differences between testing periods and experimental groups. Significance was determined at $P < 0.05$.

Results

Hindlimb stepping after a neonatal ST improves with step training

Quantitative analyses of the video and robotic data generated during locomotor tests confirmed that step training improved the stepping ability of neonatal ST rats. For example, before training (week 0) the maximum consecutive steps/min was similar in trained and non-trained ST rats (Fig. 2). After 4 weeks of training, however, the performance was greater in trained than non-trained ST rats. The trained rats significantly improved from 0 to 4 weeks post-lesion, but there was no further improvement in stepping with an additional 4 weeks of training, suggesting that the training effect had reached a plateau by 4 weeks.

Re-transection at a level rostral to the original transection site has no effect on the stepping ability of trained ST rats

Figure 3 illustrates robotic data from a representative ST trained rat before and after a complete re-transection at a level rostral to the original transection site after 8 weeks of training. Plots of horizontal and vertical movements recorded at the ankle in one hindlimb (left traces), of ankle vertical movement displayed over time (middle traces), and of ankle vertical movements in the left and right hindlimbs (interlimb coordination, right traces) were similar before and after a re-transection at a rostral level. In addition, there were no differences in the consistency of stepping in both space and time (1^{st} PC analyses for step height and length) and in average step height, length, and duration before and after the re-transection rostral to the original transection site (Fig. 4).

The anterograde tracer BDA injected into the brain did not cross the transection site into the lumbar spinal cord

Longitudinal sections of the spinal cord were used to examine the corticospinal tract, and transverse sections were used to examine the rubrospinal tract (red nucleus) for BDA labeling (Fig. 5). In intact rats, positive staining was observed in both tracts at spinal cord levels that would be rostral (Fig. 5A, E, I, and M) or caudal (Fig. 5B, F, J, and N) to the transection site. Positive staining for BDA, however, was observed only rostral (Fig. 5C, G, K, and O) to the lesion site in all ST trained or non-trained rats examined. The absence of positive staining caudal (Fig. 5D, H, L, and P) to the lesion site was indicative of a lack of re-connectivity of these tracts across the transection site.

Fast Blue did not cross the transection site

Fluorescent imaging of the brain for Fast Blue (inserted via gel foam at each transection site) revealed robust staining of neurons in the somatosensory motor cortex of the hindlimb area, red nucleus, spinal vestibular nucleus, and medullary reticular nucleus of non-trained ST rats that had been re-transected rostral (Fig. 6B, F, J, and N) to the initial transection site and in intact rats that received a complete spinal cord transection at the same time that the ST rats were re-transected (Fig. 6A, E, I, and M). In contrast, the same brain regions in the ST non-trained rats that were re-transected at the initial transection site (Fig. 6 C, G, K and O) or one segment caudally (Fig. 6D, H, L, and P) showed no Fast Blue staining. Mean cell counts verify these differences and indicate that no re-connectivity occurred across the initial transection site (Fig. 7). The stepping abilities of these ST non-trained rats were not affected by the re-transection (data not shown).

The transsynaptic retrograde tracer PRV is not observed in the brains of ST rats

Consistent PRV staining was observed in the somatosensory motor cortex of the hindlimb area (Fig. 8A), red nucleus (Fig. 8B), spinal vestibular nucleus (Fig. 8C) and medullary reticular nucleus (Fig. 8D) of intact rats. In contrast, there was no PRV staining in these same brain regions in ST trained and non-trained rats (Fig. 8F–I, data for a representative ST trained rat), indicating that a reconnection of descending tracts was not established across the lesion site. Spinal cord sections below the lesion of both intact and ST rats contained PRV (+) neurons showing that the absence of PRV (+) cells in the brains of ST rats was not due to a failure of infection (Fig. 8E, J). It also is clear from these sections that the overall pattern of infectivity of the PRV in the spinal cord is not different in intact and ST rats.

Discussion

Importance of the problem

The importance of the present results is several-fold. Firstly, there is a clear difference in the overt locomotor behavior in a rat that has received a complete ST at a neonatal compared to adult stage. Secondly, to explore the hypothesis that the difference in motor behavior can be attributed solely to the lumbosacral circuitry, it is important to know whether this difference in locomotor behavior can be attributed to any remaining axons that may not have been interrupted in the original surgery. The potential benefits of testing this hypothesis are critical from the standpoint of understanding the basic mechanism of neural control of locomotion and providing new insight as to how to enhance locomotor capacity after a complete ST. Knowledge of the differences in the lumbosacral locomotor circuitry after a chronic injury induced at a neonatal vs. an adult stage of development is likely to provide some critical information. To address this issue, however, one has to be assured that there is no supraspinal influence. The present results clearly demonstrate that the improvements in locomotor behavior in rats receiving an ST at P5 cannot be attributed to supraspinal input.

Verification of a complete ST

A critical element for determining the possibility for regeneration in the present study was the verification of a complete ST. The surgical procedures included cutting the spinal cord including the dura, lifting the cut ends of the spinal cord for visual verification, and inserting gelfoam in the gap between the two cut ends. A subsequent complete spinal re-transection at a level above the original transection site did not affect stepping ability in either non-trained or step-trained rats, i.e., poor steppers continued to step poorly and successful steppers maintained their stepping ability. The lack of anterograde labeling in the spinal cord after BDA injection into the brain, transsynaptic labeling in specific brain regions from PRV injected into muscles, and of retrograde labeling in specific brain regions after administration of Fast Blue into the spinal cord at or below the original transection site are consistent with the absence of any anatomical re-connectivity between the brain and spinal cord post-transection. The combined results provide considerable assurance that our procedures resulted in a complete ST and that the completeness of the transection was maintained throughout the experimental period.

Extent of spontaneous recovery of locomotion in neonatal ST rats

Previous studies have reported spontaneous recovery of locomotor ability after a neonatal ST in rats. In a thorough qualitative behavioral study, Stelzner et al. (1975) compared the effects of a complete ST at a mid- to low-thoracic level in neonates (mostly P4–P5) or as weanlings (P21–26) on locomotor ability and on a number of motor responses. Neonatal ST rats showed motor responses, including locomotion and weight support capabilities, that were closer to those observed in age-matched non-injured littermates than rats transected at as weanlings (Stelzner et al. 1975). Spinal cords in several neonatal ST rats were re-transected below the original transection at a weanling stage and “the final behavior of most of the hindlimb responses approximated the preoperative state”, suggesting that spontaneous recovery of motor function was not due to reestablishment of brain-spinal cord connectivity. Subsequent studies (Weber and Stelzner 1977; Commissiong and Toffano 1989) assessed a wide range of behavioral tasks in rats spinally transected between P0–P28 and reported a critical period near P15 after which minimal spontaneous recovery of motor function was observed. Rats spinally transected at a mid-thoracic level at P2 showed a wide range of recovery between 5–8 weeks post-transection: based on BBB scores 65% were non-weight-bearing and 35% weight supporting and based on a treadmill test 88% and 12% were poor and moderate steppers, respectively (Kao et al., 2006).

Although the spontaneous recovery of locomotion after a neonatal ST does not approximate that observed in non-injured rats, the stepping ability of neonatal ST rats can improve significantly with step training. De Leon et al. (2002a) studied rats that received a complete ST at P5 and at P25 were assigned to a non-trained or trained (hindlimbs stepped on a treadmill 10 min/day, 5 days/week for 12 weeks while supporting 25%–50% of their body weight) group. All spinal rats showed some level of spontaneous locomotion prior to the training period. The maximum number of consecutive steps performed in 1 min was greater in trained than non-trained rats after 6 and 12 weeks of training and the total number of steps performed was higher in trained than non-trained rats after 8 weeks of training. The amount of training also appears to be closely related to improvement in stepping ability. Rats receiving a complete ST at P5 were trained to step on a treadmill 5 days/week using a robotic device beginning at P28 (Cha et al. 2007). After 4 weeks of training, the quality of stepping at various speeds and weight support levels was significantly better in spinal rats receiving 1000 than 100 steps per training session. Similarly, ~30% of the rats receiving a ST at P0–P2 and trained to step on a treadmill and to walk across a narrow runway 3–5 days/week for 5–10 weeks regained unassisted weight-bearing stepping ability with some spinal rats performing similarly to intact rats during bipedal treadmill locomotion (Giszter et al. 1998; Miya et al., 1997).

Spinal cord and cortical adaptations associated with recovery of locomotion after a complete neonatal ST

The mechanisms associated with recovery of limited stepping ability in non-trained and the enhanced stepping ability of step-trained neonatal ST rats are not well understood. Since these functionally recovered rats showed no evidence of axonal regeneration across the lesion site, recovery must be mediated by spinal cord plasticity below the lesion. Functional recovery could be mediated by injury- and training-induced plasticity of local spinal neurons and their targets through mechanisms such as modulation of synaptic architecture, dendritic morphology, synaptic connections, and/or neurotransmitters and their receptors. These changes may occur in several spinal cord regions and/or in a variety of cell types, e.g., motoneurons, premotor pattern-generating neurons, and/or non-neuronal cells.

Improved recovery with training also may be related to cortical plasticity affecting the forelimbs and the axial musculature (Giszter et al. 2007, 2008; Kao et al. 2009). For example, although there is little hindlimb representation in the somatosensory cortex after a neonatal ST, forelimb and axial musculature representation increases. Enhanced forelimb use and axial musculature recruitment may improve body balance that, in turn, may improve hindlimb stepping ability.

Neurochemical changes—There is significant reorganization of glycine and GABA inhibitory systems in the spinal cord in response to neonatal ST (Edgerton et al. 2001; Khristy et al 2009). For example, the levels of gamma 2 (γ-2) subunit of $GABA_A$ receptor are higher in tibialis anterior and lower in soleus motoneurons in spinal than intact rats. Step training for 8 weeks restores γ2 levels towards control values in both motoneuronal pools. Since the γ2 subunit is involved with $GABA_A$ receptor trafficking and synaptic clustering, γ2 could be an important component of activity-dependent responses after a spinal cord injury. The α1 subunit of the glycine receptor and gephyrin, a protein associated with the glycine receptor, are significantly higher in the spinal cord in neonatal ST than intact rats and return to control values after 12 weeks of step training (Edgerton et al., 2001). Changes in other neurotransmitter and receptor systems also have been observed in neonatal ST rats. For example, Kim et al. (1999) reported that 5-HT_{2C} receptor binding was upregulated 8 weeks after ST in the ventral horn of the lumbar spinal cord caudal to the transection in rats receiving a ST at P2. Subsequently, Kim et al. (2001) showed that injection of m-chlorophenylpiperazine, a 5-HT_{2C} agonist, 8 weeks after ST increased the number of hindlimb weight-supporting steps performed on a treadmill.

Changes in synaptic architecture and connections—The lumbar locomotor spinal cord circuitries in P5 rats are still developing and not all supraspinal connections are formed. Dendritic bundles on motoneurons have not matured at this stage of development, e.g., dendritic bundles in the soleus motoneuronal pool develop around P15 (Gramsbergen 2001; Westerga and Gramsbergen 1992). These and other incompletely matured pathways may undergo compensatory changes after ST. For example, there are more ventral lateral funiculus projections to hindlimb motoneurons in neonatal than adult spinal rats (Petruska et al., 2007). The effectiveness of step training and amplitude of both the action potential afterhyperpolarization and synaptic inputs to motoneurons from peripheral nerves and ventral lateral funiculus are strongly correlated. These changes are absent if step training is unsuccessful, but other spinal projections, apparently inhibitory to step training, become evident.

Conclusions

The significance of the present data is that the impressive level of recovery of stepping in adult rats spinalized as neonates is not necessarily attributable to regeneration. For example, no evidence of anatomical or functional connectivity was observed in descending or ascending tracts and locomotor function was similar before and after a spinal re-transection. Therefore, the spontaneous recovery of locomotion and the training-induced improvement in recovery appears to be attributable to changes in the spinal circuitry and not to regeneration across the lesion.

List of abbreviations

α	alpha
ABC	avidin-biotinylated enzyme complex
BBB	Basso, Beattie, and Bresnahan open-field locomotor rating scale
BDA	biotinylated dextran amine
DAB	diaminobenzidine
γ	gamma
5-HT	5 –hydroxytryptamine
P5	postnatal day 5
PCA	Principal component analysis
PBS	phosphate buffered saline
PRV	pseudorabies virus
ST	complete spinal cord transection
T	Thoracic segment

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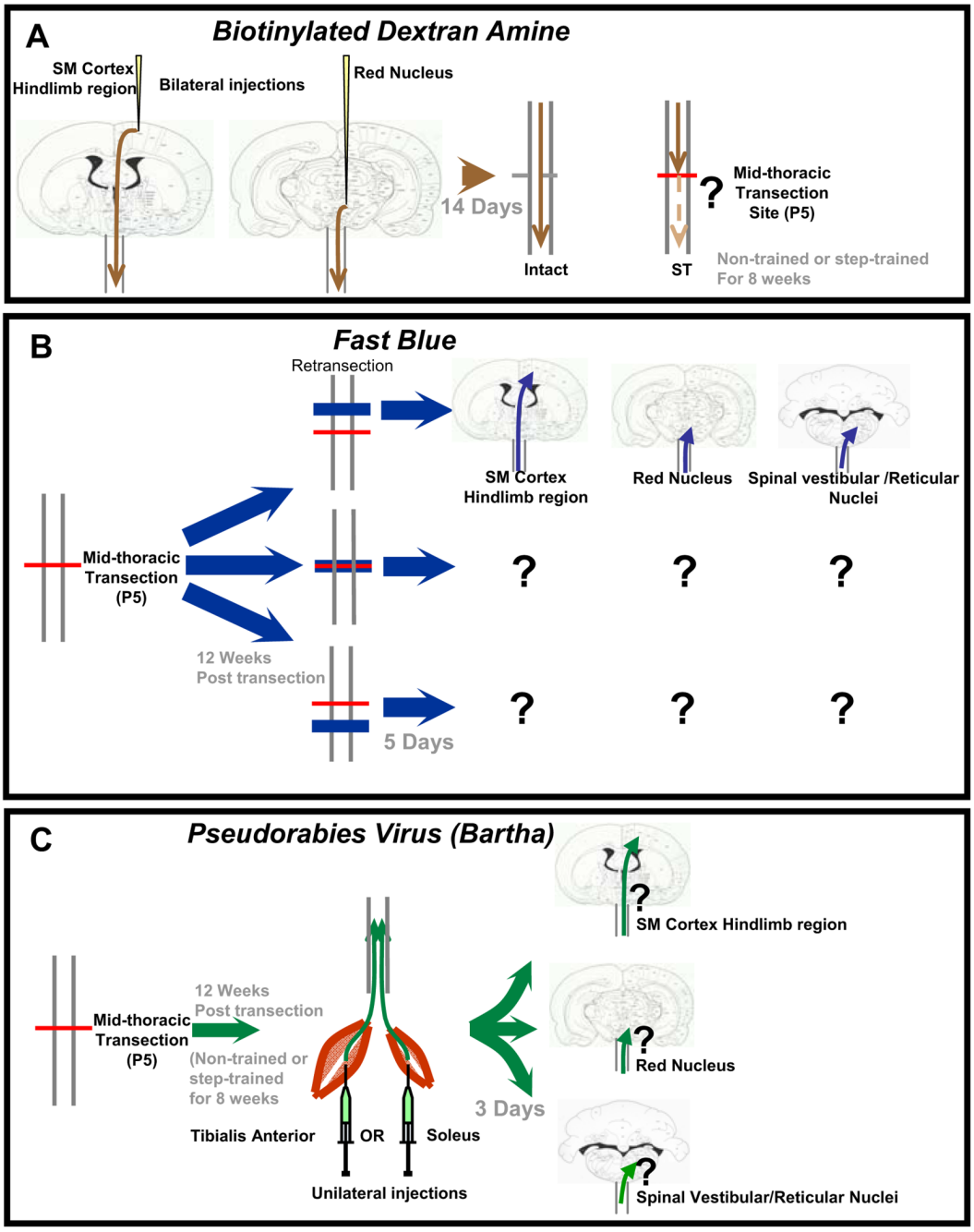

Figure 1.

Schematic of anatomical labeling techniques. *A*, biotinylated dextran amine (BDA) was bilaterally injected into the somatosensory motor cortex of the hindlimb area (SM HL) and red nucleus: the presence or absence of the anterograde tracer rostral and caudal to the transection site was examined In both intact and ST rats. *B*, ST animals were given a second spinal cord transection (re-transection) either rostral to, caudal to, or at the original transection site at 12 weeks after the initial transection. Gelfoam soaked in Fast Blue then was inserted into the transection site, and after 5 days the presence or absence of Fast Blue was examined in specific brain nuclei. *C*, intact and ST rats were given an injection of pseudorabies virus (PRV; Bartha

strain) unilaterally into the tibialis anterior or soleus muscles: the presence or absence of the retrograde tracer in the brain was examined.

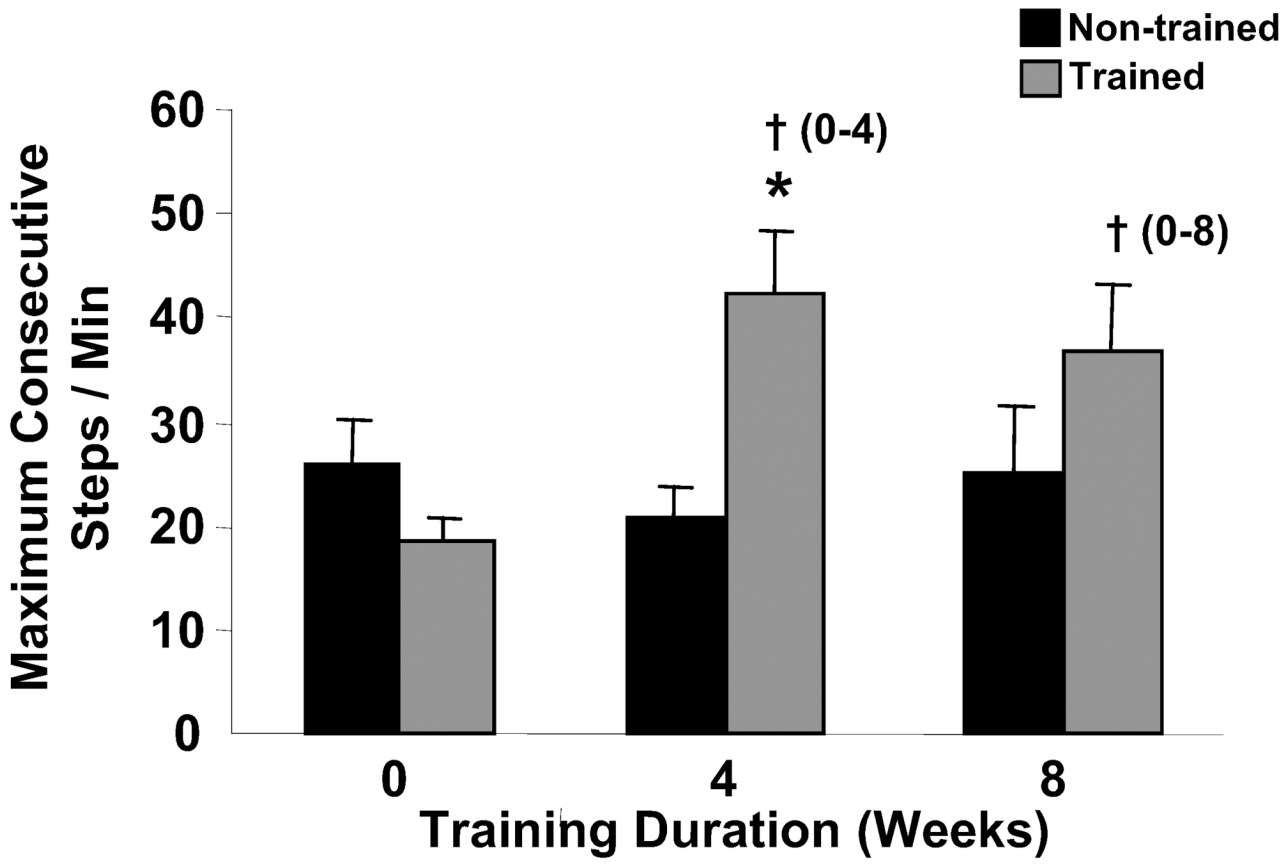

Figure 2.

Step training improves hindlimb stepping in ST rats. The maximum number of steps performed by non-trained (black bars) and trained (gray bars) ST rats at 0 (30 days post-injury), 4, and 8 weeks after the initiation of step training is shown. The data are mean (±SEM) values from one min tests at a treadmill speed of 11 cm/s with 75% body weight support determined using video analysis. * and †, significant difference between non-trained (N = 14) and trained (N = 14) groups and between testing dates (in parentheses), respectively, at $P < 0.05$.

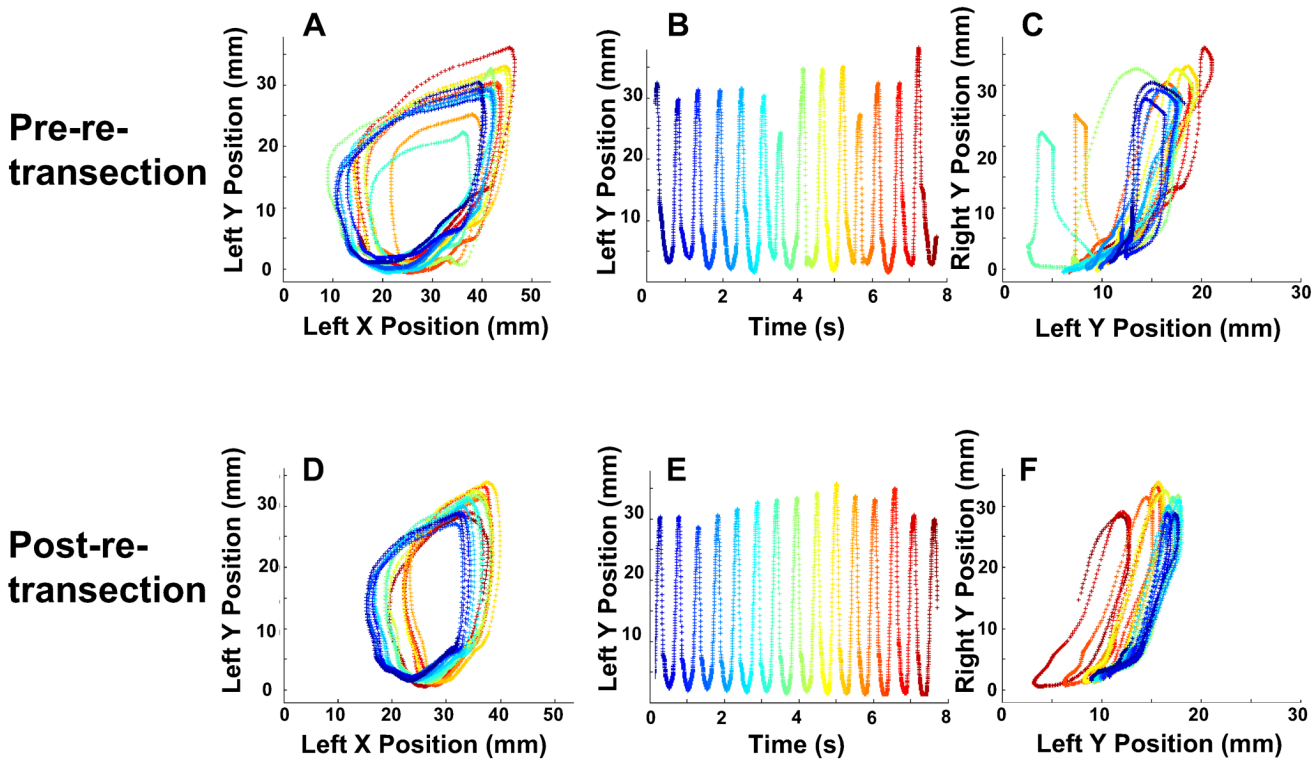

Figure 3.

Re-transection rostral to the original transection site causes no apparent loss in stepping performance in ST trained rats. Stepping ability was determined by plotting the ankle position as recorded by the robotic arm attached to the rat's ankle as it stepped on a treadmill. The stepping characteristics (X, step length; Y, step height) of a representative ST trained rat before and after a re-transection rostral to the original transection site are shown. *Column A*, graphs of X vs. Y of the left leg (step shape); *column B*, graphs of Y vs. time of left leg (step rhythm); and *column C*, graphs of right Y vs. left Y (interlimb coordination). Note that the rat exhibits nearly identical stepping performance under the two conditions. Chronological progression of stepping is indicated by the gradual change in color from blue (beginning) to red (end). All three graphs were plotted using the same set of ~15 steps as determined by left ankle position.

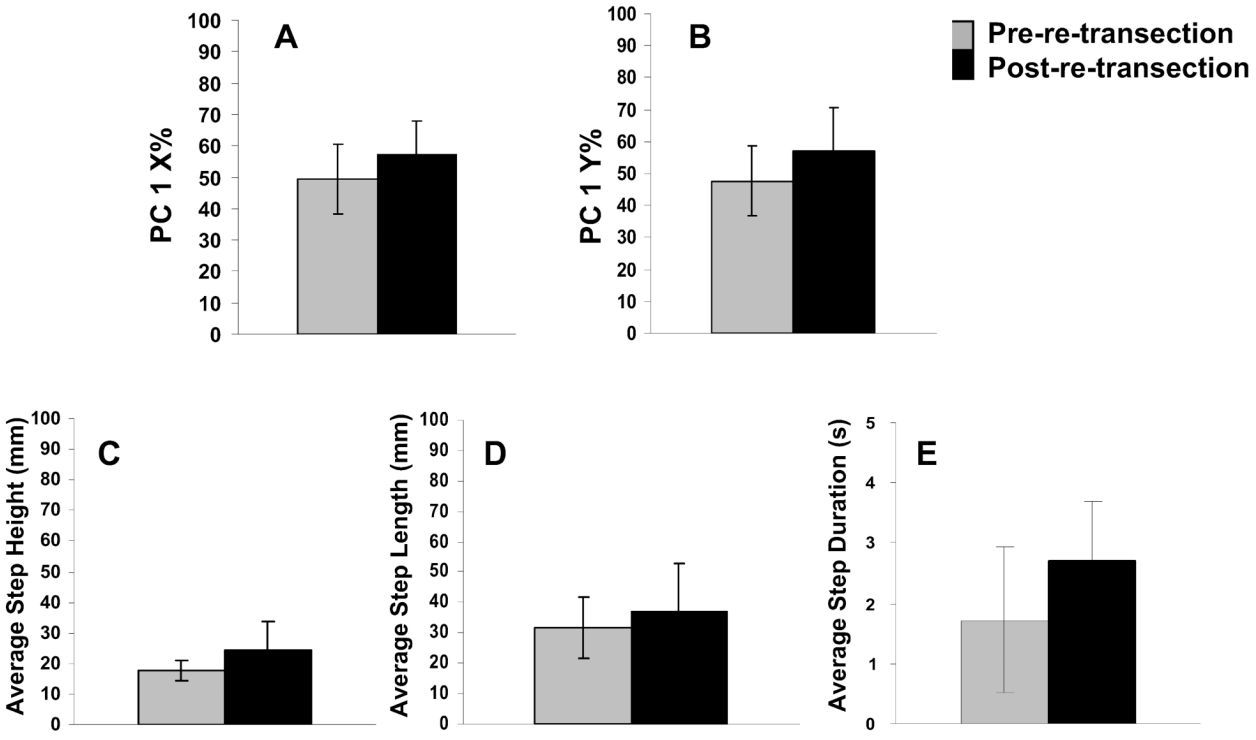

Figure 4.

The kinematics of stepping in ST-trained rats are unaffected by a re-transection rostral to the original transection site. There was no significant difference in the PC1 X% (*A*) or Y% (*B*), and average step height (*C*), length (*D*), or duration (*E*) before and after the re-transection. Values are mean ± SEM for 5 rats.

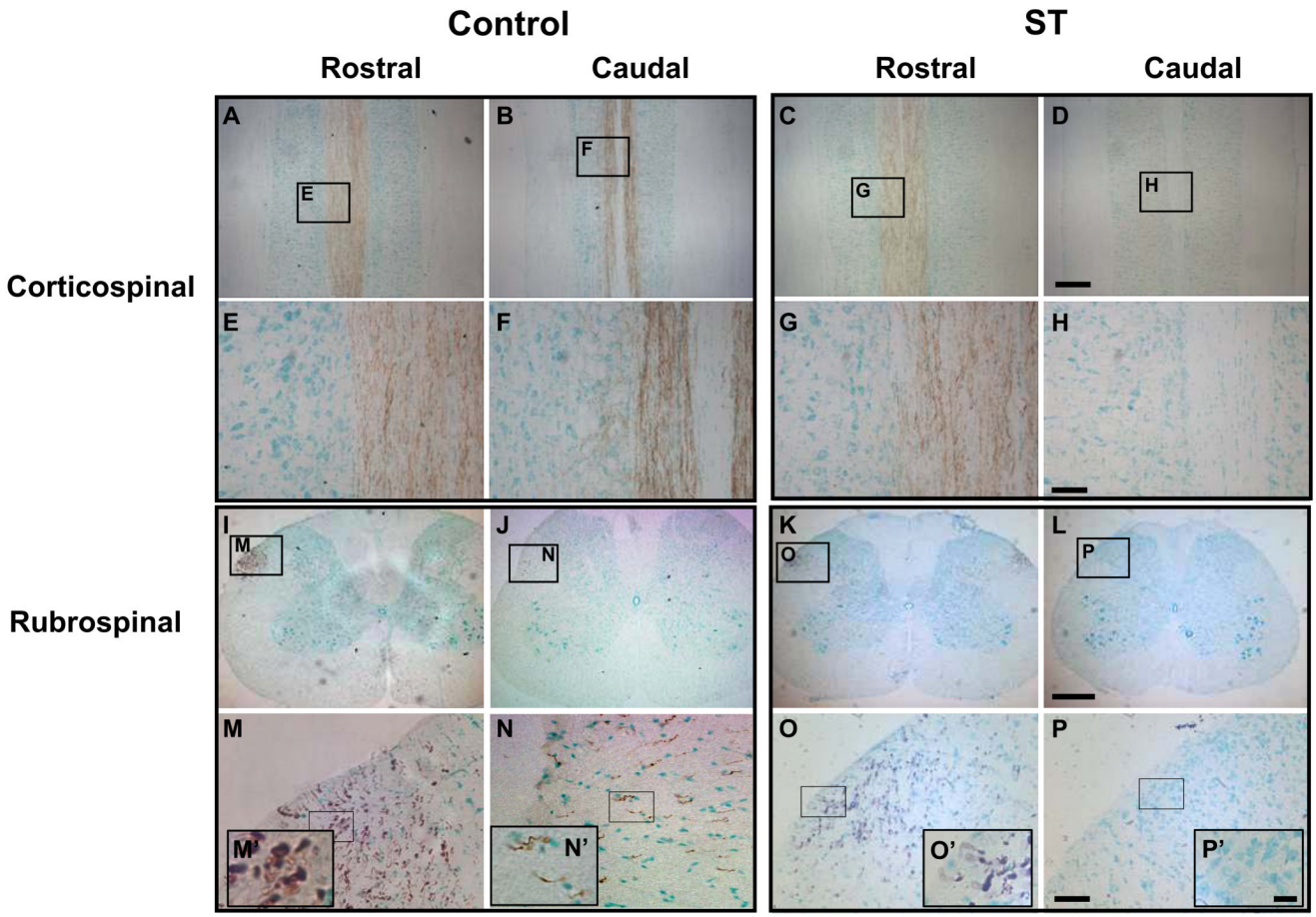

Figure 5.

BDA is not present in the spinal cord caudal to the transection site in both non-trained and trained ST rats. *A–H*, horizontal sections of the spinal cord from a representative intact (*A, B, E, and F*) and a representative ST trained (*C, D, G, and H*) rat injected with BDA in the somatosensory motor cortex of the hindlimb area. BDA staining is visible in sections rostral, but not caudal, to the transection site in the ST trained rat. *I–P*, transverse sections of the spinal cord from a representative intact (*I, J, M, and N*) and ST trained (*K, L, O, and P*) rat injected with BDA in the red nucleus. Again, staining is visible in sections rostral, but not caudal, to the transection site in the ST trained rat. *E–H, M–P and M'–P'* are higher magnification images of the inset boxes in *A–D, I–L and M–P*, respectively. Scale bars in *D*=500 μm, *H*=100 μm, *L*=300 μm, *P*=30 μm and *P'*=5 μm and apply to *A–D, E–H, I–L*, M–P and *M'–P'*, respectively.

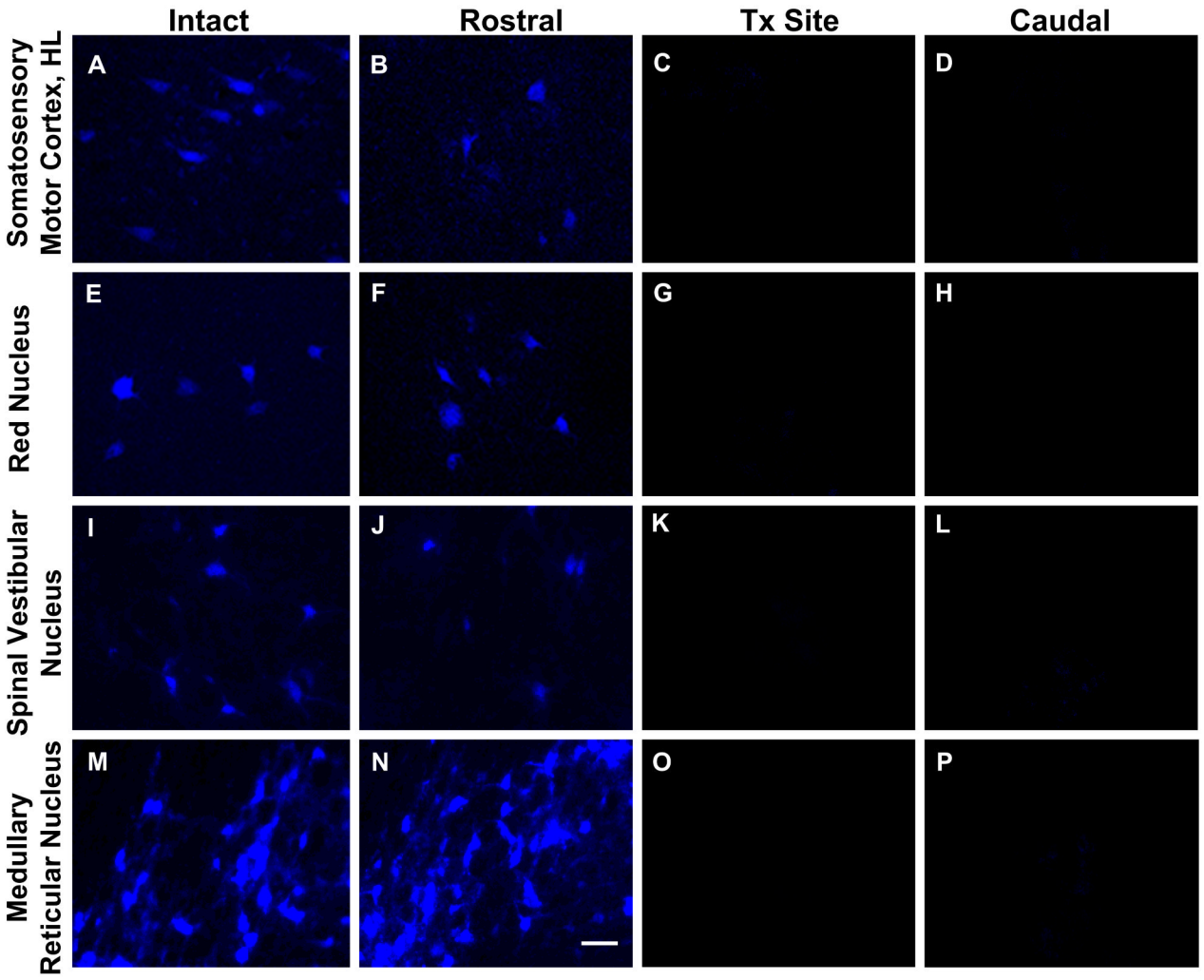

Figure 6.

Fast Blue is present in brain nuclei of intact and ST non-trained rats re-transected rostral, but not caudal, to the original transection site. Coronal sections of the brain from a representative intact (*A, E, I, and M)*, ST non-trained plus re-transection at a rostral level (*B, F, J, and N*), ST non-trained plus re-transected at the original transection site (*C, G, K, and O*), and ST non-trained plus re-transected at a caudal level (*D, H, L, and P*) rat are depicted. Staining for the somatosensory motor cortex of the hindlimb area (*A–D*), red nucleus (*E–H*), spinal vestibular nucleus (*I–L*), and medullary reticular nucleus (*M–P*) is shown. Labeled cells are observed in all regions in the intact and ST non-trained plus rostral re-transection, but not the ST non-trained plus re-transection at the original or caudal levels. Scale bar in *N* is 25 μm and applies to all panels.

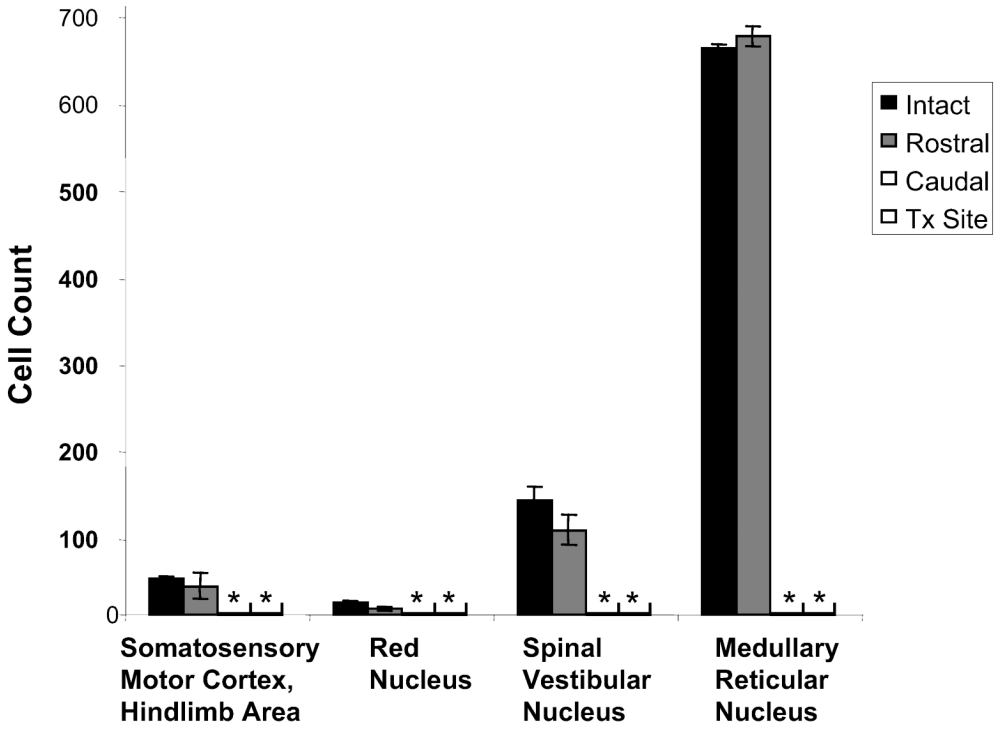

Figure 7.

No Fast Blue labeled neurons are observed in the brain of ST non-trained rats receiving a re-transection at the original transection site or at a level caudal to the transection site. Asterisks indicate a cell count of zero. Spinal rats that were re-transected either at or caudal to the original transection site (Tx Site) showed no Fast Blue stained neurons in any of the four nuclei examined. In contrast, intact and ST non-trained rats that were re-transected rostral to the original transection site showed a similar number of Fast Blue stained neurons in each of the four regions of the brain. Values are mean ± SEM for 7 intact and 15 ST non-trained rats.

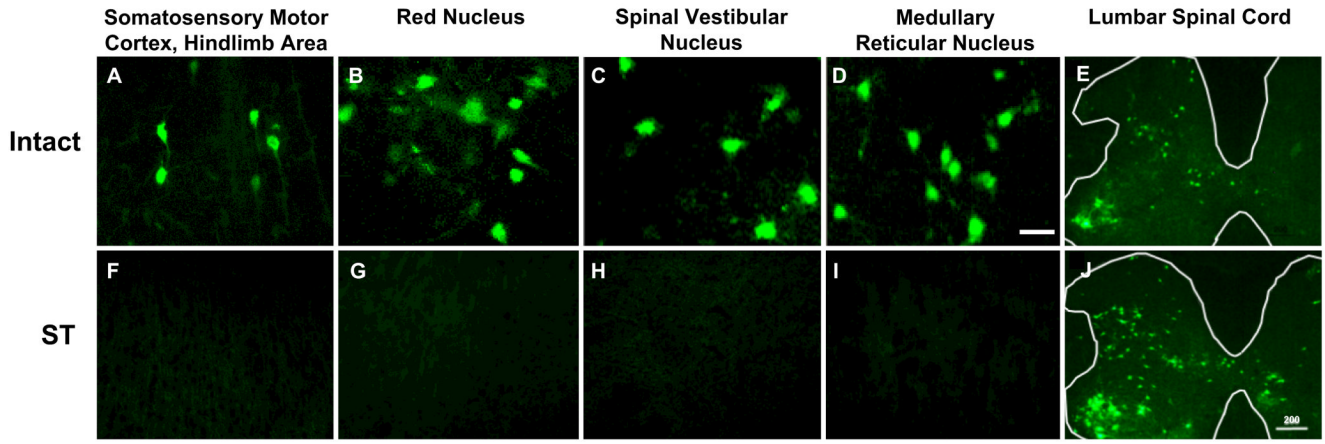

Figure 8.

PRV is present in four motor nuclei in the brain of intact, but not ST trained or non-trained rats. Coronal sections of the brain from a representative intact (*A–D)* and a representative ST trained (*F–I*) rat are depicted. Staining for the somatosensory motor cortex of the hindlimb area (*A* and *F*), red nucleus (*B* and *G*), spinal vestibular nucleus (*C* and *H*) and medullary reticular nucleus (*D* and *I*) is shown. Labeled cells are observed in all regions in the intact, but not the ST trained, rat. Transverse sections of the lumbar spinal cord from a representative intact (*E*) and ST trained (*J*) rat are shown. PRV-labeled cells are observed in both the intact and ST trained rats in the spinal cord below the lesion site. Scale bar in D is 50 μm and applies to *A–I*; scale bar in *J* is 200 μm and applies to *E* and *J*.

Table 1

Summary of experimental groups, training status, and anatomical labeling Procedures

Site of dye insertion/injection	Number of rats	Anatomical Labeling
Intact		
Mid-thoracic	7	Fast Blue
SM Cortex, hindlimb area	8	BDA
Red Nucleus	5	BDA
Soleus muscle	11	PRV
Tibialis anterior muscle	9	PRV
Transected, non-trained		
Rostral to original ST-site	5	Fast Blue
Original ST-site	5	Fast Blue
Caudal to original ST-site	5	Fast Blue
Red Nucleus	5	BDA
SM Cortex, hindlimb area	5	BDA
Soleus muscle	13	PRV
Tibialis anterior muscle	7[a]	PRV
No dye injection	7[a]	N/A
Transected, step-trained		
Red nucleus	5	BDA
SM Cortex hindlimb area	5	BDA
Soleus muscle	7	PRV
Tibialis anterior muscle	7 [a]	PRV
No dye injection	7 [a]	N/A
Retransected rostral to original ST-site	5 [b]	N/A

Total number of rats: 128; BDA, Biotinylated dextran amine; SM, somatosensory motor; PRV, pseudorabies virus (Bartha); ST, spinal cord transected;

[a] behavior data for Fig 2;

[b] behavior data for fig 3 and 4.