Spinal electro-magnetic stimulation combined with transgene delivery of neurotrophin NT-3 and exercise: novel combination therapy for spinal contusion injury

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Petrosyan HA, Alessi V, Hunanyan AS, Sisto SA, Arvanian VL. Spinal electro-magnetic stimulation combined with transgene delivery of neurotrophin NT-3 and exercise: novel combination therapy for spinal contusion injury. J Neurophysiol 114: 2923–2940, 2015. First published September 30, 2015; doi:10.1152/jn.00480.2015.—Our recent terminal experiments revealed that administration of a single train of repetitive spinal electromagnetic stimulation (sEMS; 35 min) enhanced synaptic plasticity in spinal circuitry following lateral hemisection spinal cord injury. In the current study, we have examined effects of repetitive sEMS applied as a single train and chronically (5 wk, every other day) following thoracic T10 contusion. Chronic studies involved examination of systematic sEMS administration alone and combined with exercise training and transgene delivery of neurotrophin [adeno-associated virus 10-neurotrophin 3 (AAV10-NT3)]. Electrophysiological intracellular/extracellular recordings, immunohistochemistry, behavioral testing, and anatomical tracing were performed to assess effects of treatments. We found that administration of a single sEMS train induced transient facilitation of transmission through preserved lateral white matter to motoneurons and hindlimb muscles in chronically confined rats with effects lasting for at least 2 h. These physiological changes associated with increased immunoreactivity of GluR1 and GluR2/3 glutamate receptors in lumbar neurons. Systematic administration of sEMS alone for 5 wk, however, was unable to induce cumulative improvements of transmission in spinomuscular circuitry or improve impaired motor function following thoracic contusion. Encouragingly, chronic administration of sEMS, followed by exercise training (running in an exercise ball and swimming), induced the following: 1) sustained strengthening of transmission to lumbar motoneurons and hindlimb muscles, 2) better retrograde transport of anatomical tracer, and 3) improved locomotor function. Greatest improvements were seen in the group that received exercise combined with sEMS and AAV-NT3.

after spinal cord injury; magnetic stimulation; exercise; AAV

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2007), which have been attributed to greater plasticity through preserved fibers in young mammalian spinal cord (Dunlop 2008; Rossignol 2006).

Plasticity of neural circuits and transmission through these preserved fibers to lumbar motoneurons are diminished following lateral hemisection (Arvanian et al. 2009) and contusion (Arvanian et al. 2006; Hains et al. 2004; Hunanyan et al. 2013) SCI in rats. Our earlier electrophysiological studies revealed that synaptic connections from lateral white matter (LWM) to individual motoneurons could be strengthened by administration of neurotrophin 3 (NT3) using engineered fibroblast implantation (Arvanian et al. 2003). This action of NT3, however, required the reversal of the developmental loss of neuronal N-methyl-d-aspartate (NMDA) receptor activity at these synaptic inputs, which we achieved through herpes simplex virus 1 vector-mediated overexpression of the juvenile form of NMDA receptors (Arvanian et al. 2004; García-Alías et al. 2011; Schnell et al. 2011).

In search of less-invasive treatments, we recently found that noninvasive, repetitive spinal electromagnetic stimulation (sEMS; 0.2 Hz frequency train for 35 min) over the thoracic spinal cord could strengthen transmission and facilitate NMDA receptor function at motoneuron synaptic inputs from LWM and the dorsal corticospinal tract following lateral hemisection SCI (Hunanyan et al. 2012). In this study, we examined whether a similar train of repetitive sEMS would strengthen transmission at spinomuscular circuitry following thoracic contusion injury. The major goal was to examine whether chronic administration of repetitive sEMS (every other day for 5 wk) and exercise training applied alone, together, and in combination with NT3 would improve transmission and recovery of function following thoracic contusion SCI. Based on our recent results, demonstrating robust transduction of both neuronal and glial cell populations in injured spinal cord (Petrosyan et al. 2014a), we used recombinant human adeno-associated viral 10 (AAV-rh10) vector for transgene delivery of NT3, which is an improved strategy from our previous study (Schnell et al. 2011) to increase levels of neurotrophins in the spinal cord of animals with SCI (Blits et al. 2003; Boyce et al. 2012; Fortun et al. 2009; Hunanyan et al. 2013). Furthermore, we systematically compared the motor-evoked potentials (MEPs) and synaptic responses recorded from individual motoneurons following contusive SCI and above-mentioned interventions. Despite the diagnostic potential of this knowledge, systematic
comparisons of these electrophysiological measures in adult rats have never before been conducted.

Three sets of experiments were conducted that included a detailed examination of the following: 1) electrophysiological changes of transmission to lumbar motoneurons and hindlimb muscles through the spared fibers following thoracic contusion using simultaneous intracellular, extracellular, and intramuscular recordings; 2) the effects of a single train of repetitive sEMS on spinomuscular transmission and expression of glutamate GluR1 and GluR2/3 receptor subunits in spinal neurons, 6–8 wk following thoracic contusion; and 3) the effects of chronic administration of sEMS (every other day for 5 wk), alone and in combination with exercise training and transgene delivery of neurotrophin NT3 on synaptic transmission, anatomical plasticity, and recovery of function following thoracic contusion SCI (using multiple electrophysiological recordings, anatomical tracing, immunohistochemistry, and a battery of behavioral tests).

Some of these findings have been reported in abstract form [see Arvanian et al. (2011) and Petrosyan et al. (2013a, 2014b)].

MATERIALS AND METHODS

All experiments were carried out on adult female (~210 g) Sprague-Dawley rats. Animals were housed singly under a 12-h light/dark cycle with ad libitum access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook and Northport Veterans Affairs Medical Center.

Spinal Cord Injury

Animals were anesthetized initially with 3% isoflurane in 100% O₂, followed by 1.5% isoflurane in 100% O₂, delivered via nose cone to maintain anesthesia during surgeries. A subcutaneous injection of buprenorphine (0.01 mg/kg) was given to initiate postsurgical pain control. Animals were positioned on a water-circulating heating pad, and body temperature was monitored by a rectal thermometer. Contusion injuries were performed at the T10 level of the spinal cord using an IH-0400 Impactor device (Precision Systems and Instrumentation, Fairfax Station, VA), as described previously (Hunanyan et al. 2013; Petrosyan et al. 2014a). Briefly, a dorsal laminectomy was performed exposing the T10 spinal cord level. Vertebral bodies, rostral and caudal to the opening, were fixed with Adson forceps to stabilize the spine. The probe of the impactor was positioned in the center of the opening, 1 mm above the dorsal surface of the exposed spinal cord, and an impact force of 150 Kdyn was delivered. For experiments that examined the acute effects of repetitive sEMS, the wound was closed immediately after the SCI procedure was completed. For studies that examined effects of treatments (chronic sEMS, exercise training, neurotrophin NT3) immediately following injury, animals received intraspinal injections of AAV vectors expressing either green fluorescent protein (AAV-GFP) or NT3 together with GFP (AAV-NT3-GFP; described below). After injury and AAV vector administration, the muscles were sutured in layers, and the skin was closed with surgical clips, followed by subcutaneous injections of antibiotic (Baytril 5 mg/kg) and sterile lactated Ringer’s solution (5 ml). Baytril, Ringer’s solution, and buprenorphine were continued for 3 days post-op and then as needed.

Examination of Acute Effects of a Single Train of Repetitive sEMS in Chronically Contused Rats

Figure 1A illustrates the experimental design. One group of animals was used to determine the effects of T10 contusion injury and repetitive sEMS on electrophysiological responses recorded intracellularly and extracellularly from lumbar motoneurons and hindlimb muscles. Animals received contusion injury at T10, and 6–8 wk later, in vivo terminal electrophysiological recordings (intracellular, extracellular, intramuscular, see below) were conducted. Results were compared with noninjured animals. One subgroup of contusive animals received a single train of repetitive sEMS (2.8 T, 0.2 Hz frequency, for 35 min) during the recordings. The other subgroup received no repetitive sEMS, but similar electrophysiological record-
ings were conducted. After completion of electrophysiological recordings, spinal cords of animals from both subgroups were processed, and immunoreactivity of GluR1 and GluR2/3 glutamate receptor subunits in lumbar spinal cord (described below) was examined in chronic contused animals and contused animals after sEMS administration.

**Electrophysiological evaluation of acute effects of a single train of repetitive sEMS.** For electrophysiological recordings, animals were deeply anesthetized using a ketamine (80 mg/kg)/xylazine (10 mg/kg) mixture, injected intraperitoneally. Supplemental injections of one-fifth of the initial dose were administered intramuscularly if necessary. Animals were placed on a heating pad to maintain body temperature at 36°C. Heart rate and expired CO2 were monitored continuously during recording via an AM10 audio monitor (Grass Technologies, Natus Neurology, Warwick, RI) and a SurgiVet capnograph (Smiths Medical, St. Paul, MN). Stimulation and recordings protocols were described previously in detail (Arvanian et al. 2009; Hunanyan et al. 2012). Briefly, two dorsal laminectomies were performed to expose the spinal cord at T6 for electric stimulation and L1–L6 for recording.

For electric stimulation of LWM at T6, a tungsten stimulation electrode (300 kΩ; FHC, Bowdoin, ME) was inserted between the root entry zone and the lateral edge of the cord to a depth of ~1.7 mm via a micromanipulator, as described previously (Arvanian et al. 2009; Hunanyan et al. 2012). Electric stimulation of right LWM axons was performed using an A300 pulse master with an A360 stimulus isolator (World Precision Instruments, Sarasota, FL). The stimulus was delivered at 1 Hz using 50 μS stimulus duration.

To record MEPs, evoked either by electric stimulation or EMS, fur from the hindlimbs of the biceps femoris (BF) muscle region was shaved, two platinum subdermal electrodes (27-G) were inserted into the hindlimb BF muscle (Hunanyan et al. 2012), and a reference electrode was positioned under the skin close to the tail (Beaumont et al. 2006). MEP responses were acquired using a PS111 amplifier (Grass Technologies, Natus Neurology). The amplitude of the responses was measured between the first largest peak in either direction and the following peak of other polarity (Hunanyan et al. 2012). The average of peak-to-peak amplitude was computed from 30 consecutive responses.

Intracellular recordings were performed from L5 motoneurons using an Axoprobe amplifier (Molecular Devices, Sunnyvale, CA), as described previously in detail (Arvanian et al. 2009; Petrosyan et al. 2013b). Briefly, motoneurons were identified by their antidromic response to stimulation of the cut L5 ventral root, and glass microelectrodes (3M K-acetate; 50–70 MΩ resistance) were used to perform recordings. Motoneurons that had resting membrane potentials ranging from −45 to −65 mV were used for analysis.

Extracellular responses were recorded from right L5 ventral horn (VH) gray matter. A tungsten electrode (300 kΩ; FHC) was positioned at the right side of the spinal cord at the dorsal root entry zone and lowered to a depth of 1.3 mm. The electrode was placed with the tip, directed rostrally at an angle of 25° from vertical in the sagittal plane, as described previously (Arvanian et al. 2009). All signals were analyzed offline using pCLAMP 10 software (Molecular Devices). **Immunohistochemistry and image analysis.** After completion of electrophysiological recordings, rats were perfused, and spinal cords were processed as described below. Briefly, the spinal cord from every experimental animal was cut into five sets of 40 μm sections. Each individual set of serial sections spans the entire segment of interest, with respective sections of each separated by 200 μm. One complete set of transverse sections (40 μm thick) from the L5 spinal cord was used for immunofluorescence staining using rabbit anti-GluR1 (1:200; EMD Millipore, Billerica, MA) and mouse anti-Neu-N (1:600; EMD Millipore) or rabbit anti-GluR2/3 (1:200; EMD Millipore) antibodies. Briefly, sections were blocked in 0.1 m PBS containing 6% normal goat serum and 0.3% Triton X-100, incubated in one of the above antibodies overnight at 4°C, rinsed with 0.1 m PBS several times, and then incubated in goat anti-rabbit or goat anti-

mouse secondary antibody (1:800; Alexa Fluor 488, Alexa Fluor 594; Invitrogen, Thermo Fisher Scientific, Grand Island, NY) for 2 h at room temperature. Sections were then rinsed in PBS, dipped in dH2O, coveredps with Fluoromount-G (SouthernBiotech, Birmingham, AL), and then visualized with a Zeiss Axioskop 2 microscope, equipped with fluorescent capability. Images were captured with a Zeiss AxioCam MRm camera using identical settings within each antibody study. To quantify GluR1 and GluR2/3 immunoreactivity, somas within a fixed area (1.5 × 1.5 mm2) of the L5 VH were outlined manually, and mean signal intensities were measured using ImageJ software (National Institutes of Health, Bethesda, MD) for each animal. Each image was thresholded to determine background, and background values were collected as described previously (Petrosyan et al. 2013b). Normalized intensity was calculated as the mean intensity of each soma divided by the corresponding background value for each section/image.

**Chronic Experiments**

Figure 1B illustrates the design of the experiment with chronic treatments. In this study, we used spinally contused rats and examined the effects of chronic administration of sEMS in combination with other treatments that enhance plasticity: 1) exercise training (running in an exercise ball and swimming) applied during a period of enhanced synaptic transmission following sEMS application and 2) viral vector-mediated delivery of neurotrophin NT3. Briefly, all animals were uniformly pretrained for 2 wk to swim; walk in the exercise ball; and cross the irregular grid, narrowing beam, and CatWalk platform. Immediately after injury administration, animals received intraspinal injections of AAV-NT3-GFP or control AAV-GFP. We used the AAV-h10 serotype, because it has been shown to result in excellent transduction of both neurons and glial cells in the contused spinal cord of adult rats (Petrosyan et al. 2014a). It has also been shown to be an appropriate vehicle for neurotrophin NT3 delivery to the contusive spinal cord of adult rats (Hunanyan et al. 2013). AAV-mediated gene expression in the adult rat spinal cord is known to start ~1 wk following intraspinal injections and lasts for several months (Eaton et al. 2002). Treatments with sEMS and exercise were applied every other day for 5 wk, and behavioral testing was conducted once/wk. After 5 wk of treatment (sEMS and/or exercise), administration rats received a break for 4 wk, during which there was no treatment administration or behavioral testing. Following the 4-wk break period, all rats underwent behavioral testing to assess the retention of treatment effects and were then randomly assigned for either terminal electrophysiology or anatomical tracing experiments.

**Animal groups.** Following 2 wk of handling/pretraining, animals were randomly divided into five groups. Group 1 (Gr.1): one control group received contusion injury at the T10 level, intraspinal injections of AAV-GFP immediately after the injury, were kept under light isoflurane anesthesia for 35 min every other day (the same amount of time and level of anesthesia that animals would be exposed to during sEMS administration), and received no other treatments (SCI, no treatment). Group 2 (Gr.2): the second group received the same contusion injury, intraspinal injections of AAV-GFP, and chronic administration of sEMS for 35 min every other day for 5 wk under light isoflurane anesthesia (described below; SCI, chronic sEMS). Group 3 (Gr.3): the third group received the same contusion injury, intraspinal injections of AAV-GFP, and exercise training performed every other day, 1 h following exposure to light isoflurane anesthesia for 35 min (similar to Gr.1 and 2; SCI, Exercise only). Group 4 (Gr.4): the fourth group received contusion injury, intraspinal injections of AAV-GFP, and repetitive sEMS for 35 min every other day for 5 wk under light isoflurane anesthesia, followed by exercise training (SCI, sEMS + Exercise). Group 5 (Gr.5): the fifth group received contusion injury, intraspinal injection of AAV vector expressing NT3 (AAV-NT3-GFP), and repetitive sEMS for 35 min every other day for 5 wk under light isoflurane anesthesia, followed by exercise training (SCI,
sEMS + Exercise + NT3). The persons conducting behavioral, electrophysiological, and anatomical experimenters were blind with regard to treatment.

AAV viral vectors. AAV vectors used in this study were obtained from the Penn Vector Core at the University of Pennsylvania (Philadelphia, PA). AAV serotype rh10 encoding the neurotrophin NT3 gene and coexpressing GFP under the cytomegalovirus (CMV) promoter (AAVrh10.CMV.bGH.CMV.NT3.IRES.GFP.WPRE.bGH) using a 1.17 × 10^13 genomic copy (GC)/ml dosage or control AAV vector encoding the GFP gene under the CMV promoter (AAVRh10.CMV.PI.eGFP.WPRE.bGH) using a 3.99 × 10^13 GC/ml dosage was used for this study. AAV-mediated gene transfer has been used for safe and prolonged delivery of NT3 in injured spinal cord (Blits et al. 2003; Boyce et al. 2012; Fortun et al. 2009; Hunanyan et al. 2013). The procedure for intraspinal injections of AAV-NT3 has been described before (Hunanyan et al. 2013; Petrosyan et al. 2014a). Briefly, immediately after the injury, the contused rats received two intraspinal injections of AAV-rh10-GFP or AAV-rh10-NT3-GFP (1 µl each injection). Injections were made into the spinal cord at the midline, 1.2 mm rostral, and 1.2 mm caudal to the injury epicenter at the depth of 1 mm. Our recent results demonstrated an excellent transduction of both neurons and glial cells in the contused spinal cord of adult rats following similar injections (Petrosyan et al. 2014a). AAV-mediated expression of NT3 in this study was confirmed by observing the GFP signal (which is secondary to NT3 in the AAV-NT3-IRES-GFP system).

sEMS and exercise training. sEMS was applied for 35 min (see stimulation protocol below), every other day (Monday–Friday) for 5 wk, under light isoflurane anesthesia, starting 1 wk postinjury. Exercise training (swimming followed by running in a transparent ball) was performed after each sEMS session, beginning 1 h after recovery from isoflurane anesthesia. Animals from groups 1 and 3 were exposed to isoflurane anesthesia for the same amount of time as groups 2, 4, and 5 but received no sEMS, and animals from group 3 performed the same exercise training as groups 4 and 5. Behavioral experiments were conducted once/wk on days when no treatment was administered. After 5 wk of treatment with sEMS and exercise training, there was a 4-wk break period (no sEMS or exercise training), after which behavioral testing was conducted again. After completion of behavioral experiments, each group of animals was retested with exercise training protocol below, every other day (Monday–Friday) for 5–10 laps. All animals were assisted and prodded when necessary to ensure the completion of 10 laps per session, which typically lasted ~15 min. Following swim sessions, animals were dried, warmed, and allowed to rest for 15 min before initiating the next training protocol.

EXERCISE BALL. Animals were pretrained to become comfortable inside of the exercise ball (50 cm diameter; Run-About Ball; Super Pet, Elk Grove Village, IL) (Brown et al. 2011) and to walk and roll the ball freely before injury. After completion of swimming sessions, animals were placed inside of the exercise ball and permitted to exercise voluntarily by walking and rolling the ball in a large, open space for 15–20 min.

Behavioral Assessment

Irregular grid. Rats were pretrained to walk across a 1-m-long horizontal ladder before surgeries. The middle part (50 cm) of the ladder, where bars were spaced randomly ~1–4 cm apart, was chosen for scoring. For each animal, the number of foot slips from three trials was counted and normalized by the total number of steps, as described previously (Arvanian et al. 2009).

Narrowing beam. We used a progressively narrowing beam with a length of 1.5 m to evaluate locomotor function of hindlimbs. Briefly, the starting point of the beam is 50 mm wide and terminates at a narrow width of 10 mm. The entire length of the beam is divided into 30 consecutively numbered, equal units. The unit at which the first slip of either hindlimb was made was recorded and normalized for three runs.

CatWalk gait analysis. Gait print parameters were collected using the CatWalk (Noldus Information Technology, Wageningen, The Netherlands) apparatus (Hamers et al. 2001). Animals were pretrained to cross a CatWalk runway before the injury. For each animal, data from three uninterrupted runs were obtained, and measurements, such as Regularity Index, Base of Support, Stride Length, and Mean Paw Intensity, were analyzed and compared among groups, using CatWalk XT software, as described previously (Petrosyan et al. 2013b).

Retrograde Tracing

Tracer injections. Following behavioral testing, animals designated for retrograde axonal tracing were anesthetized with isoflurane, as described above, and a laminectomy was performed to expose the spinal L5 level. Unilateral injections (right side of the cord, corresponding to the position of the recording electrodes in electrophysiology experiments) of 10% Fluoro-Ruby in dH2O (FR; 10,000 MW; Fluorochrome, Denver, CO) were made into the gray matter in a series of three, 0.5-µl injections (1, 1.5, and 2 mm) from the dorsal surface of the spinal cord in each animal, as described previously (Petrosyan et al. 2013b). Injections were made over 5 min, using a Hamilton syringe attached to a micromanipulator to minimize leakage and

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maximize uptake of the tracer. Muscles were sutured, skin was closed with surgical clips, and analgesics and antibiotics were administered as described above. Survival time for FR tracer transport was 2 wk.

Tissue processing. Rats received an overdose bolus of urethane and were intracardially perfused with 200 ml 0.1 M PBS, followed by 4% paraformaldehyde in 0.1 M PBS. The spinal cords were dissected, removed, and postfixed overnight, followed by cryoprotection in 30% sucrose in 0.1 M PBS for several days. Transverse sections, 40 µm thick, were cut on a cryostat and collected serially onto ColorFrost Plus slides (Thermo Fisher Scientific) in five sets. One set of serial sections was stained with Cresyl Violet and used as a guide to determine spinal cord segment level and for injury reconstruction. An adjacent set of sections, used for reconstruction of FR injections at L5 and counting of FR-positive cells at L1–L2, was air dried overnight, rinsed in 0.1 M PBS to remove embedding medium, and then dried at 50°C for one-half hour. The slides were then cleared with xylene and coverslipped with DPX mounting media. Analyses were performed with a Zeiss Axioskop 2 microscope equipped with fluorescent capability, an AxioCam MRm camera (Carl Zeiss, Jena, Germany), and AxioVision Rel 4.8 software, as described previously (Petrosyan et al. 2013b).

Statistical Analysis

SigmaPlot 11.0 software (Systat Software, San Jose, CA) was used for statistical analyses. A one-way ANOVA or one-way ANOVA on ranks with Tukey’s or Dunn’s post hoc tests was used to compare the electrophysiological, behavioral, and immunohistochemical data among groups. Results are considered statistically significant for P < 0.05, and data are presented as means ± SE.

RESULTS

Effects of Contusion SCI on Different Types of Electrophysiological Responses

Contusion SCI in adult rats closely resembles many SCI in humans (Metz et al. 2000; Scheff et al. 2003). Although integrity of synaptic transmission through the surviving axons spanning the injury epicenter is crucial for coordinated locomotion (Jordan et al. 2008; Kremer and Lev-Tov 1997; Noga et al. 2003), in humans, electrophysiological evaluation of functionality of spinomotor pathways is limited to recordings of MEPs in response to noninvasive electric or magnetic stimulations (Ellaway et al. 2011; Petersen et al. 2012). These methods, however, cannot provide information about synaptic transmission or plasticity at specific motor tracts, including those in LWM, known to be involved in recovery following SCI (Bareyre et al. 2004; Hill et al. 2001; Hunanyan et al. 2013; Loy et al. 2002; Schucht et al. 2002). Examination of transmission through specific tracts to individual motoneurons can be achieved by in vivo intracellular recordings of synaptic responses from neurons evoked by electric stimulation of the descending pathways (Arvanian et al. 2009; Babalian et al. 1993; Jankowska et al. 1993). This type of electrophysiological recording, however, is impossible to accomplish in humans. To conduct detailed electrophysiological examinations and establish correlation between MEPS evoked by noninvasive EMS with responses recorded intracellularly and extracellularly from the spinal cord, we have conducted simultaneously several types of electrophysiological recordings following chronic mid-thoracic contusion injury.

Adult rats received mid-thoracic contusion (150 Kdyn) injury and after 6–8 wk, terminal electrophysiological recordings were conducted. We recorded from these SCI animals and noninjured animals the following responses: 1) MEPS recorded from the hindlimb BF muscle and evoked by a single electromagnetic pulse applied over the intact T2 spinal level, 2) MEPS recorded from hindlimb BF muscles and evoked by electric stimulation of ipsilateral LWM at the T6 level, 3) extracellular responses recorded from L5 VH and evoked by electric stimulation of LWM at the T6 level, and 4) intracellular excitatory postsynaptic potential (EPSP) responses recorded from L5 VH motoneurons and evoked by electric stimulation of LWM at the T6 level. In cases of chronically contused rats, all described responses were recorded before, immediately after 35 min of sEMS administration (over the T2 spinal level), and 2 h after stopping sEMS application.

MEPs evoked by EMS at the thoracic level were obtained reliably in all contused animals, although the amplitude of these MEPS evoked by sEMS was diminished significantly in contused animals (0.27 ± 0.02 mV, n = 10) compared with noninjured animals (0.78 ± 0.06 mV, n = 7, P < 0.05; Fig. 2A). MEPS evoked by electric stimulation at T6 LWM in contused animals were significantly diminished as well. Mean amplitude of MEPS in contused rats was 0.17 ± 0.04 mV and significantly smaller compared with noninjured rats (0.84 ± 0.11 mV, P < 0.05; Fig. 2B). Diminished MEP responses recorded from hindlimb BF muscles were associated with reduced amplitude of responses recorded from spinal cord, including extracellular responses recorded from L5 VH (Fig. 2C) and EPSP responses recorded from individual motoneurons in L5 VH (Fig. 2D). Electric stimulation of LWM at T6 evoked extracellular responses of 0.33 ± 0.04 mV in noninjured and 0.08 ± 0.02 mV in contused spinal cord (P < 0.05; Fig. 2C). Mean amplitude of intracellular responses recorded from noninjured animals was 5.22 ± 0.52 mV, and in contused animals, it was significantly smaller (0.86 ± 0.13 mV, P < 0.05; Fig. 2D).

These results suggest that assessment of MEPS in response to sEMS over spinal levels has potential for electrophysiological evaluation of transmission at spinomuscular circuitry after SCI. Application of sEMS is considered to be a safe and noninvasive approach for activating central and peripheral nervous systems in humans (Gerasimenko et al. 2010; Matsuboto et al. 2013). Moreover, our recent study emphasized the safety of spinal sEMS application and suggests that the presence of titanium spinal rods, typically used for spinal stabilization, 1) may not cause problems related to heating or movement of these implants during spinal EMS and 2) does not exert adverse effects on a number of physiological measurements, such as MEPS, heart rate, and respiratory rate during sEMS applied at the thoracic level (Petrosyan et al. 2015).

Effects of Single Trains of sEMS Application

Electrophysiological responses. In our previous study, we demonstrated that low-frequency (0.2 Hz), repetitive sEMS over the T2 level induces LTP-like facilitation of responses recorded from spinal cord and hindlimb muscles in the chronically HX spinal cord (Hunanyan et al. 2012). To examine effects of sEMS on electrophysiological responses in chronically contused spinal cord, we used the stimulation protocol (see MATERIALS AND METHODS) established previously to improve transmission following HX SCI (Hunanyan et al. 2012). Several types of electrophysiological responses evoked by either
sEMS or electric stimulation of the LWM were used to examine effects of repetitive sEMS. Electrically evoked 1) MEP responses recorded from hindlimb BF muscles, 2) intracellular responses recorded from L5 motoneurons, and 3) extracellular responses recorded from L5 VH were collected before and then after sEMS administration. MEPs evoked by sEMS were recorded continuously before, during, and after sEMS administration. The mean amplitude of these MEP responses increased significantly after 35 min of sEMS administration compared with responses measured before sEMS (0.62 ± 0.10 mV vs. 0.27 ± 0.02 mV, n = 6, P < 0.05; Fig. 2A). Facilitation of these responses sustained for at least 2 h after termination of sEMS application (0.43 ± 0.03 mV, 2 h after stop of repetitive sEMS, n = 6, P < 0.05; Fig. 2A). MEPs recorded from...
hindlimb BF muscles and evoked by electric stimulation of LWM at T6 were increased significantly as well (0.71 ± 0.07 mV vs. 0.17 ± 0.04 mV, n = 6, P < 0.05; Fig. 2B) after sEMS administration.

In parallel with the increase of amplitude of MEPs following sEMS, both extracellular (0.16 ± 0.03 mV vs. 0.08 ± 0.02 mV, n = 6, P < 0.05; Fig. 2C) and intracellular (1.68 ± 0.20 mV vs. 0.86 ± 0.13 mV, n = 6, P < 0.05; Fig. 2D) responses were significantly increased after sEMS administration. Two hours following the termination of sEMS, the mean amplitude of responses recorded extracellularly from the L5 motoneurons pool (0.11 ± 0.01 mV, n = 6, P < 0.05; Fig. 2C) and responses recorded intracellularly from individual L5 motoneurons (1.42 ± 0.20 mV, n = 6, P < 0.05; Fig. 2D) were still significantly higher compared with responses recorded before sEMS administration. These results provide the first evidence about effects of sEMS on synaptic transmission at spinomuscular circuitry in chronically contused adult rats.

Expression of glutamate receptor subunits. To investigate molecular mechanisms that possibly participate in the LTP-like facilitation of synaptic responses following sEMS administration in the damaged spinal cord, we examined and compared immunoreactivity of glutamate GluR1 and GluR2/3 subunits that are known to be involved in LTP-like action and synaptic transmission, respectively, in the brain (Nicol and Malekna 1995; Song and Huganir 2004; Triller and Choquet 2005). In the spinal cord, the excitatory synaptic input to L5 motoneurons from LWM is glutamatergic as well, and “fast” monosynaptic responses evoked by electric stimulation of LWM can be blocked by intraspinal injections of the glutamate receptor antagonist in the vicinity of the recording electrode (Arvanian et al. 2009; Hunanyan et al. 2011). GluR1 and GluR2/3 receptors are known to mediate fast transmission, and recent studies revealed that GluR1 and GluR2/3 subunits exhibit robust expression in the L5 lumbar segment of the rat spinal cord (Alvarez et al. 2000; Grossman et al. 1999; Nagy et al. 2004). In this study, we asked whether the reported facilitation of synaptic responses by sEMS (see Fig. 2) could occur, due to an alteration in density of GluR1 and GluR2/3 subunits on the neuronal soma in L5 VH. Thus after completion of terminal electrophysiological recordings (when the sEMS-evoked, LTP-like action was achieved), we examined immunoreactivity of GluR1 and GluR2/3 subunits in L5 gray matter and compared two groups of animals: one group received T10 contusion SCI (n = 4), and the other group received the same contusion injury and sEMS administration (n = 5; see Fig. 4, A and B). Note that all animals used in this study received the same contusive SCI; identical ketamine/xylazine anesthesia; and identical electrophysiology, perfusion, and tissue-processing protocols. In addition, care was taken to ensure that experiments and protocols were carried out with the most similar timeframes and durations as possible so that the only difference between the two groups was that one group received repetitive sEMS, and the other group did not.

Immunofluorescence experiments revealed good expression of GluR1 and GluR2/3 receptors throughout sections at all spinal levels, including dorsal, intermedial, and ventral. Double immunostaining with Neu-N, a neuronal marker, revealed the presence of these receptors at VH neurons, which are known to project to leg muscles (see example for GluR1 staining in Fig. 3). Results of examination of sections from chronically contused and chronically contused animals after sEMS administration revealed significantly higher normalized intensity of both GluR1 (3.12 ± 0.09, n = 5 vs. 1.72 ± 0.08, n = 4, P < 0.001; Fig. 4C) and GluR2/3 (1.45 ± 0.06, n = 5 vs. 1.18 ± 0.05, n = 4, P < 0.05; Fig. 4D) subunits in VH neurons of animals after a single sEMS application compared with control contused animals. These results demonstrate that LTP-like facilitation of responses recorded from L5 VH motoneurons and from hindlimb muscles following sEMS administration is associated with increased immunoreactivity of GluR1 and GluR2/3 receptors in lumbar VH neurons. These findings are consistent with the view that synaptic function at central synapses is activity dependent (Alvarez et al. 2010; EIBasiosy et al. 2010), and the loss of plasticity following denervation can be partially restored by activation of the afferent inputs in SCI subjects (Dimitrijevic et al. 1998; Harkema et al. 2011).

Chronic Experiments

AAV delivery confirmation. After completion of experiments (terminal electrophysiology and tracing), spinal cord tissue was processed to examine the severity of injury and transduction efficiency of viral vectors in the AAV-GFP and AAV-NT3-GFP-injected rats. Because the AAV viral vectors encoding the NT3 gene used in this study were also coexpressing GFP (see MATERIALS AND METHODS), the GFP signal was used to examine the distribution of transduced cells and transduction efficiency of AAV-rh10-NT3. Consistent with our previous results (Hunanyan et al. 2013; Petrosyan et al. 2014a), intraspinal injections of AAV-rh10 induced a robust transduction of spinal cord tissue in the vicinity of the injury. We observed a large number of transduced cells located in both the VH and dorsal horns of thoracic (T9–T11) gray matter, as well as many GFP-positive processes in white matter (Fig. 5A) of all AAV-injected animals. We also observed many GFP-positive cells and processes in gray matter and GFP-positive processes in white matter in lumbar levels (Fig. 5A). These results suggest delivery of gene product, not only to the vicinity of the injury but also in segments distant from the injections as well. Note that the GFP expression examined in this study was native and that no immunostaining was used to amplify the GFP signal.

Behavioral Assessment

Overview. During the first 2 wk after incomplete SCI (partial lesion, contusion, or compression SCI in rats), there is a spontaneous recovery of locomotor function in the open field (Basso, Beattie, and Bresnahan scoring) (Basso et al. 1995), which reaches a plateau at ~4 wk postinjury (Arvanian et al. 2009; Basso et al. 1996; Schucht et al. 2002). Challenging tests and gait analysis, however, demonstrated that hindlimb function is still severely impaired during the chronic stage of hemisection (Arvanian et al. 2009; Petrosyan et al. 2013b) and contusion (Fouad et al. 2000; Hunanyan et al. 2013) injuries. Therefore, in this study, the locomotor function of all animals was assessed using automated CatWalk gait analysis and challenging tests, such as Irregular Grid and Narrowing Beam. At the first week postinjury, the majority of injured animals showed similar poor performance in all tests, indicating consistency of injury in all groups. Four animals that showed abnormal physical impairment well outside typical variability for this location and severity of injury were removed from the
A post-SCI in all injured animals (Fig. 6A). The Irregular Grid test showed a major increase of hindlimb foot errors postinjury, function during the first 2 wk after injury and a plateau in (SCI, no treatment) showed partial, spontaneous recovery of 1 wk (Gr.1: 14 ± 0.73, n = 7; Gr.2: 14.7 ± 0.67, n = 8; Gr.3: 13.8 ± 0.47, n = 8, at 4 wk postinjury). There was no significant difference among these groups (P > 0.05; Fig. 6B). As in the case of the Irregular Grid test, animals from group 4 (SCI, sEMS + Exercise) showed significant improvement compared with groups 1, 2, and 3, starting from wk 4 (Gr.4: 16.6 ± 0.46, n = 7, at 4 wk postinjury, P < 0.05; Fig. 6B). Starting from wk 5 the Narrowing Beam were still significantly better compared with other injured groups (P < 0.05; Fig. 6B).

CatWalk gait analysis. CatWalk automated gait analysis offers a large number of dynamic and static gait parameters, such as Regularity Index, Base of Support Distance, Stride
Length, and Paw-Print Intensity. All animals showed a decreased number of coordinated steps after contusion SCI, as indicated by the Regularity Index score in CatWalk (Fig. 7A), which is a measurement of interlimb coordination, calculated as a degree of normal step sequences during one run (Koopmans et al. 2005; Petrosyan et al. 2013b). Other gait parameters that were markedly affected following SCI include the following: (1) increased Base of Support Distance between hindlimbs (Fig. 7B), which is the core-to-core distance between hind paws; (2) decreased paw pressure, indicated as Mean Paw Intensity, which is a measure of pressure exerted on the glass plate (Fig. 7C); and (3) reduced Stride Length of forelimbs (Fig. 7D), which is the distance between successive placements of the same paw. At wk 2, all injured groups exhibited significant impairments in all of these parameters compared with preinjury measurements (Fig. 7). During the recovery course, all groups showed improvements in locomotor function determined by these CatWalk measures. In addition, a plateau was observed for groups 1, 2, and 3 in all CatWalk measures at wk 4 and remained unchanged at that level for the rest of the experiment (Fig. 7). There was no significant difference among these three groups at any time point. Animals from groups 4 and 5 showed a significantly higher number of coordinated steps and other gait parameters vs. groups 1, 2, and 3, determined at wk 5 postinjury by the following measures (Fig. 7): increased Regularity Index (Gr.4: 91.6 ± 1.56%, n = 7, and Gr.5: 96% ± 1.46%, n = 8 vs. Gr.1: 85.6 ± 2.2%, n = 7, Gr.2: 87 ± 1.46%, n = 8, and Gr.3: 86 ± 1.42%, n = 8, P < 0.05); decreased Base of Support (Gr.4: 30.5 ± 1.0 mm and Gr.5: 28.6 ± 0.96 mm vs. Gr.1: 34 ± 1.3 mm, Gr.2: 33.6 ± 1.45 mm, and Gr.3: 35 ± 1.8 mm, P < 0.05); increased Stride Length (Gr.4: 94.8 ± 1.4 mm and Gr.5: 97.6 ± 1.46 mm vs. Gr.1: 91.4 ± 2.6 mm, Gr.2: 92.3 ± 2.1 mm, and Gr.3: 91.4 ± 1.69 mm, P < 0.05); and higher Paw-Print Intensity (Gr.4: 69.4 ± 2.6 and Gr.5: 70.4 ± 2.1 vs. Gr.1: 63.8 ± 2.95, Gr.2: 63.2 ± 2.69, and Gr.3: 64.6 ± 2.68, P < 0.05). These gait parameters still remained improved for groups 4 and 5 vs. groups 1, 2, and 3 after a 4-wk treatment (sEMS and exercise) break (P < 0.05; Fig. 7).

Electrophysiological Assessment

After completion of behavioral testing, one subgroup of animals from each group was dedicated for in vivo terminal electrophysiological evaluation of transmission to the lumbar motoneurons pool and hindlimb muscles from segments rostral to injury. MEPs were recorded from hindlimb BF muscles, and extracellular responses were recorded from the L5 VH motoneurons pool in response to electric stimulation of LWM at the T6 spinal level.

Mean amplitude of MEP responses recorded from hindlimb muscles was significantly decreased in all contused animals compared with noninjured animals (P < 0.05; Fig. 8A). There was no significant difference in the mean MEP amplitude among animals in groups 1–3 (Gr.1: 0.17 ± 0.03 mV, n = 4; Gr.2: 0.21 ± 0.03 mV, n = 5; and Gr.3: 0.19 ± 0.05 mV, n = 5, P > 0.05; Fig. 8A1). Consistent with results of behavioral experiments, animals from group 4 (Gr.4: 0.34 ± 0.04 mV, n = 4) and group 5 (Gr.5: 0.51 ± 0.05 mV, n = 5) showed significantly larger amplitudes compared with groups 1–3 (P < 0.05; Fig. 8A).

Consistent with the results of behavioral experiments and MEP recordings, animals from groups 4 and 5 showed markedly increased extracellular responses recorded from the L5 VH motoneurons pool compared with other injured groups.
Mean amplitudes of extracellular responses recorded from animals of group 4 (0.13 ± 0.008 mV, n = 4) and group 5 (0.16 ± 0.008 mV, n = 5) were significantly larger compared with responses recorded from animals of group 1 (0.092 ± 0.007 mV, n = 4), group 2 (0.095 ± 0.01 mV, n = 5), and group 3 (0.088 ± 0.01 mV, n = 5, P < 0.05; Fig. 8B). Both MEP and extracellular responses recorded from group 5 animals were significantly larger compared with group 4 animals.

These results suggest that improved locomotor performance for groups 4 and 5 animals, evident in challenging behavioral tests and CatWalk gait analysis, was associated with improved transmission to lumbar motoneurons and hindlimb muscles after chronic treatment administration.

**Correlation between Lesion Size and Electrophysiological Responses**

After completion of electrophysiological recordings, spinal cords were removed, and preserved tissue at the injury epicenter for all groups studied was examined. Injury reconstruction was used to examine the correlation between the amplitude of electrophysiological responses and the spared tissue at the injury epicenter for each animal (Fig. 8). Analysis revealed an absence of correlation between the percentage of spared tissue area at the injury epicenter and the amplitude of MEPs in group 4 ($r^2 = 0.39$, $P = 0.43$) and group 5 ($r^2 = 0.16$, $P = 0.75$) animals using Pearson's correlation test. There was also no correlation between spared tissue and extracellular responses recorded from the L5 VH (Gr.4: $r^2 = 0.32$, $P = 0.52$; Gr.5: $r^2 = 0.10$, $P = 0.84$) for these groups. Thus these results suggest that improved transmission in animals from groups 4 and 5 was apparently determined by the treatment that the rats received.

**Retrograde Tracing**

The other subgroup of animals from each group was injected unilaterally with FR retrograde tracer in L5 gray matter (corresponding to placement of recording electrodes in electrophysiology experiments). Two weeks after tracer injections, the number of retrogradely labeled cells was counted in lumbar L1–L2 segments, which is considered to be a central pattern generator (CPG) region (Cazalets et al. 1995). Consistent with previous work (Conta and Stelzner 2004; Petrosyan et al. 2004), improved locomotor performance for groups 4 and 5 animals, evident in challenging behavioral tests and CatWalk gait analysis, was associated with improved transmission to lumbar motoneurons and hindlimb muscles after chronic treatment administration.
locomotor circuitry, which is known to control hindlimb motor function (Courtine et al. 2009; Magnuson et al. 1999).

**DISCUSSION**

The results presented provide strong preclinical data demonstrating the efficacy of systematic administration of 1) repetitive sEMS (to open a window of synaptic plasticity in damaged spinal cord), 2) followed by exercise training (during a period of sEMS-induced plasticity) and 3) combined with AAV vector-based delivery of NT3 as a novel combination treatment for contusion SCI. These results are the first to suggest that chronic administration of this triple treatment can be an effective approach to induce cumulative improvements of transmission in spinomuscular circuitry and motor function following thoracic contusion injury. After injury and 5 wk of chronic sEMS/exercise or sEMS/exercise/AAV-NT3 treatment, enhanced performance in all behavioral tests sustained for at least 4 wk beyond the termination of treatment administration.

Additionally, these data provide several new insights regarding electrophysiological evaluation of transmission in a chronically contused spinal cord. Simultaneous in vivo recordings from the hindlimb muscles, spinal cord motoneurons pool, and individual motoneurons provide the first direct electrophysiological evidence that a single train of repetitive sEMS promotes synaptic plasticity in spinomuscular circuitry in a chronically contused spinal cord. These physiological changes associated with increased immunoreactivity of neuronal glutamate GluR1 and GluR2/3 receptor subunits.

**sEMS: a Reliable Approach for Electrophysiological Evaluation of Spinomuscular Activity following Contusion**

Thoracic contusion is a common type of injury in humans. Rat contusion models allow control over location and severity of the impact and are, therefore, used by many investigators as standardized models for preclinical examination of spinal trauma therapies (Beattie et al. 1997; Metz et al. 2000). The results reported here represent the first comprehensive comparison of MEPs evoked by noninvasive sEMS with several other types of electrophysiological responses recorded in the same animal, following thoracic chronic contusion injury and treatment administration. We found that all of these responses decline in parallel in the chronically contused spinal cord and increase in parallel following treatment with repetitive sEMS (Fig. 2). In contrast to MEPs evoked by sEMS, which were diminished but still could be reliably obtained in contused animals (see Fig. 2), transcranial magnetic stimulation (TMS) failed to induce reliable MEPs in leg muscles in contused rats. These results are consistent with other reports indicating that TMS failed to evoke responses in leg muscles in different SCI models (Hunanyan et al. 2012; Magnuson et al. 1999).

Our current electrophysiological experiments provide strong evidence that MEPs recorded from hindlimb BF muscles and evoked by noninvasive thoracic sEMS may be used reliably as an evaluation tool for documenting the physiological condition of spinomuscular pathways after chronic thoracic contusion SCI in adult rats. Consistent with this view, sEMS at thoracic levels evoked MEP responses recorded from leg muscles, and...
repetitive sEMS induced rhythmic leg movement in healthy humans (Gerasimenko et al. 2010).

**Transient Strengthening of Transmission in a Chronically Contused Spinal Cord by a Single sEMS Train**

Results of the current study demonstrate that administration of a single train of sEMS (over T2 for 35 min) promotes synaptic plasticity and induces an LTP-like action in the rat spinal cord during the chronic stage of contusion SCI. sEMS-evoked facilitation of synaptic responses lasted for at least 2 h after discontinuing stimulation (Fig. 2). At this time, it is difficult to specify spinal pathways that were activated by sEMS. Nevertheless, our results demonstrate that repetitive sEMS may strengthen synaptic projections through spared LWM to lumbar motoneurons, which contain important pathways related to coordination and locomotion. Propriospinal projections in LWM are proposed to be important in the recovery process following SCI (Bresnahan et al. 1991; Cao et al. 2005; Kim et al. 2012; Steencken et al. 2009). Anatomical experiments (Bareyre et al. 2004; Courtine et al. 2008; Ghosh et al. 2010; Hill et al. 2001) and recent electrophysiological studies (Hunanyan et al. 2013) suggest that propriospinal projections in LWM play a compensatory role in the recovery process.

Consistent with the current immunochemistry experiments (Fig. 4), our recent Western blot studies revealed decreased expression of GluR1 and GluR2/3 receptors in the chronically contused spinal cord, and these deficits have been partially reversed by repetitive sEMS treatment (Petrosyan et al. 2013b). Increased density of GluR1 and GluR2/3 glutamate receptors in cells of spinal cords that were exposed to repetitive sEMS (Fig. 4) suggest that this might be one possible mechanism underlying LTP-like action of repetitive sEMS. Consistent with these views, repetitive trans-sEMS was found to improve...
locomotor function in SCI mice, and this recovery correlated with an elevated release of radioactive glutamate analog in spinal tissue (Leydeker et al. 2013). We hypothesize that these changes in the density of GluR subunits in spinal neurons could be determined by trafficking of GluR receptors to and from synaptic sites depending on synaptic activity, which has been postulated to be a major mechanism leading to changes in synaptic strength (Hayashi et al. 2000; Lüscher et al. 1999). The level of synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors can be regulated rapidly by activity-dependent mechanisms (Harms et al. 2005; Liao et al. 2001; Turrigiano 2000), and synaptic activity can regulate AMPA receptors at the postsynaptic membrane over time scales from minutes to days (Ehlers et al. 2007). Regulated trafficking of AMPA receptors is also involved in multiple forms of synaptic plasticity, including LTP, long-term depression, and depotentiation (Kerchner and Nicoll 2008; Shepherd and Huganir 2007). The reported results, demonstrating rapid distribution of AMPA receptors following activation of the synaptic NMDA receptors (Shi et al. 1999), are consistent with our previous observation that activation of NMDA receptors is an important component for sEMS-evoked, LTP-like facilitation in the damaged spinal cord (Hunanyan et al. 2012).

Sustained Improvements Induced by Chronic sEMS Combined with Exercise Training

Effects of repetitive sEMS on synaptic transmission were, however, transient and lasted ~2 h after stopping the train of repetitive magnetic stimulation (Fig. 2); the amplitude of responses declined to prestimuli levels even when sEMS was applied chronically every other day (Fig. 8). Consistent with our current reports of the transient effects of sEMS on synaptic...
transmission, chronic administration of sEMS alone did not induce significant improvements of locomotion (Figs. 6 and 7). When combined with exercise, however, chronic repetitive sEMS induced even better improvements of locomotion (Figs. 6 and 7). The ability of physical rehabilitation/exercise, particularly rhythmic patterned activity, to induce limited recovery of function has been reported in humans with SCI (Forrest et al. 2008; Harkema et al. 2012; Roy et al. 2012) and in animal models (Beaumont et al. 2004; De Leon et al. 1999; Sandrow-Feinberg et al. 2009).

In the current study, we used swimming exercise as a therapy that provides hindlimb rhythmic exercise with little load. Partial water immersion provides unique body weight support that cannot be achieved by harness systems (Stevens et al. 2015). In addition to the body weight support provided by the harness system, water provides buoyancy to the legs, which can be essential for movement initiation, particularly after partial spinal cord injuries. A recent study demonstrates improved physical function and walking ability following underwater treadmill training in adults with incomplete spinal cord injuries (Stevens et al. 2015). As a second component of our exercise training, we used voluntary walking in the exercise ball as a rhythmic exercise with full load, which was shown to induce better locomotor function in rats with contusion spinal cord injuries (Brown et al. 2011). Effects of swimming and other forms of rehabilitation exercise applied separately and combined with sEMS and AAV-NT3 are to be examined further.

The combination of exercise and electric stimulation is a developing treatment in the SCI field. Beneficial effects of electric fields applied epidurally or directly to lesioned spinal cord on functional outcomes are well documented (Ahmed 2014; Brus-Ramer et al. 2007; Carmel et al. 2010; Courtine et al. 2009; Fehlings et al. 1988; Ichiyama et al. 2005). A disadvantage of this stimulation technique, however, is that it requires surgery and electrode implantation, resulting in a high risk of adverse effects (Dam-Hieu et al. 2010; Franzini et al. 2005).

In the current study, we demonstrate that chronic administration of repetitive sEMS, combined with exercise applied during a period of sEMS-induced LTP-like facilitation, induced sustained strengthening of synaptic transmission (Fig. 8). These cumulative improvements of synaptic transmission associated with improved anatomical plasticity in lumbar spinal cord revealed by an increased number of retrogradely labeled cells in L1–L2 segments (Fig. 9) and recovery of motor function in multiple behavioral tests (Figs. 6 and 7).

The L1–L2 segment of the spinal cord is considered a CPG region (Cazalets et al. 1995), plays a key role in the interlimb coordination and stepping paradigm (Kiehn 2006), and can produce locomotion of hindlimbs, without or with just small input from the brain (Rossignol et al. 2006). Activity of commissural interneurons, located at L1–L2 segments and projecting to L4/L5 motoneurons in rats, mediates CPG function (Kiehn 2006). Current results of retrograde tracing, demonstrating more labeled cells in the intermediate level of lumbar L1–L2 segments following FR injection in L5 of groups 4 and 5, correlate well with recent reports demonstrating the significance of anatomical and synaptic plasticity of local lumbar circuits for better coordination and locomotor function in multiple behavioral tests (Figs. 6 and 7).

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function after SCI (Blits et al. 2003; Courtine et al. 2008; Petrosyan et al. 2013b). In particular, the AAV vector induced transgene delivery of NT3, and the brain-derived neurotrophic factor induced improved hindlimb function in transected animals and was associated with an increased number of retrogradely labeled interneurons in L1–L2 segments (Blits et al. 2003).

Administered singly, however, sEMS and exercise had no or little effects. It has been reported before that exercise applied in the form of either swimming (Smith et al. 2009) or training in an exercise ball (Brown et al. 2011) induced minor or no effects on recovery of function following contusion injury in rats. Consistent with our current report of the transient effects and the lack of cumulative action observed following repetitive sEMS applied alone in rats, lumbar repetitive magnetic stimulation was found to reduce the increase in the spastic tone of legs in patients with spinal lesions for no more than 4–24 h after stimulation (Krause et al. 2004). Results of behavioral evaluation in rodents are consistent with the current behavioral results and demonstrate that combined treatment of repetitive sEMS and exercise, but not these treatments applied separately, improved functional recovery after SCI (Ahmed et al. 2011; Ahmed and Wieraszko 2008; Hou et al. 2014). Thus although application of activity-based treatments is not a novel idea in the SCI field, our study does provide novel and important knowledge about appropriate parameters, sequence/timing, and site of application of these treatments.

AAV-NT3 Treatment Combined with sEMS and Exercise Induced Further Improvement

Another important finding was that AAV vector-mediated delivery of neurotrophin NT3 further improved the beneficial effects of combinatorial sEMS and exercise treatments on synaptic transmission, FR retrograde transport, and recovery of function following contusion SCI (Figs. 6–9). Neurotrophin NT3 is an essential factor required for growth of new connections and has been implicated to enhance regenerative sprouting and reorganization of damaged fibers in the injured spinal cord (Alto et al. 2009; Schnell et al. 1994; Tuszynski and Gage 1995). Electrophysiological experiments, however, revealed that NT3 may enhance plasticity at synaptic inputs to lumbar motoneurons in neonates (Arvanian et al. 2003) but not in damaged spinal cord of adult rats when applied alone (García-Alías et al. 2011; Schnell et al. 2011). Consistent with these results, chronic administration of NT3 alone via AAV vectors had only limited effects on locomotion in transected animals, although the combination of NT3 with cutaneous stimulation was able to induce plantar stepping in paraplegic rats (Boyce et al. 2012). Other experiments demonstrated that NT3 is an important component of combination treatment that strengthens synaptic connections following hemisection (García-Alías et al. 2011; Schnell et al. 2011) and contusion (Hunanyan et al. 2013) SCI in adult rats. As modulation of synaptic plasticity induced by neurotrophins requires activation of NMDA receptors at postsynaptic terminals (Arvanian et al. 2006), the combination of transgene delivery of NT3 with sEMS, which was shown to activate NMDA receptors at neuronal synapses (Hunanyan et al. 2012), can be one possible explanation of better functional outcomes in animals that received delivery of NT3 in addition to sEMS and exercise.

In summary, the most important feature of the current study is that it provides insight into the recovery of electrophysiological control at neuromuscular circuitry, underlying the beneficial effects of combined administration of repetitive sEMS and exercise. Results clearly demonstrate that repetitive sEMS may open a window of plasticity, and exercise performed while this window is open induced improvements of transmission and function following SCI. The expectation is that the proposed strategy of applying exercise training during an LTP period induced by repetitive sEMS or combining exercise with repetitive sEMS and AAV-NT3 will find clinical applications.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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