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Tail Nerve Electrical Stimulation-Induced Walking Training Promotes Restoration of Locomotion and Electrophysiology in Rats with Chronic Spinal Cord Injury

—TANES Promotes Functional and Conductive Recovery in Chronic SCI Rat

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Abstract

Functional recovery is the final goal in the treatment of spinal cord injury. However, to date, few treatment strategies have demonstrated significant locomotor improvement in animal experiments. By using tail nerve electrical stimulation (TANES) as an open-field locomotor training method combined with glial scar ablation and cell transplantation, we have successfully promoted locomotor recovery in rats with chronic spinal cord contusion injury. The purpose of the present study is to further investigate the mechanism of TANES and its effect on electrophysiology. Spinal cord segment T10 of female, adult Long-Evans rats was contused using the NYU impactor device with 25 mm height setting. After injury, rats were randomly divided into three groups. Group I was used as a control without any treatment, group II and group III were subjected to basic treatment including glial scar ablation and transplantation of olfactory lamina propria 6 weeks after injury, and group III received TANES-induced open-field locomotor training weekly after basic treatment. All animals were allowed to survive 22 weeks, except some rats which were transected. Basso, Beattie, and Bresnahan (BBB) open-field locomotor rating scale, horizontal ladder rung walking test, and electrophysiological tests were used to assess the restoration of functional behavior and conduction. Results showed that TANES significantly improves locomotor recovery and spinal cord conduction, reflex, as well as significantly reduces the occurrence of autophagia. Additionally, after transection, trained rats still main-

tained higher BBB score than that of control rats. This may be related to the activity-dependent plasticity promoted by TANES-induced locomotor training.

Keywords

Spinal Cord Injury, Tail Nerve Electrical Stimulation (TANES), Horizontal Ladder Rung Walking Test, Electrophysiology, Autophagia, Rat

1. Introduction

Restoration of locomotor function from paralyzed people is one of the final goals of the research on spinal cord injury, which scientists have worked for decades with a variety of animal models. However, to date, few treatment strategies including cellular transplantation to the chronically injured, especially contused, spinal cord have yielded significant functional improvement in animal experiments [1] [2]. A large number of questions need to be addressed for the treatment of chronic spinal cord, such as tissue repair, glial scar, inhibitory molecules, axonal regeneration, neuropathic pain, locomotor recovery, etc.

In our previous studies, we have reported that an existing glial scar in the chronically contused rat spinal cord can be removed by using rose Bengal-dependent phototoxic ablation. We also observed that photochemical scar ablation did not prominently damage the spared tissue, motor and sensory functions, or spinal electrophysiological conduction, and may allow migration of more Schwann cells into the spared tissue [3] [4]. Immediately after scar ablation, we transplanted the olfactory lamina propria (OLP) alone or together with cultured olfactory ensheathing cells (OECs) into the lesion cavity. Later we found that the transplanted OLP or/and OECs remarkably promoted the tissue repair and axonal regeneration. However, tissue repair and axonal regeneration did not directly result in locomotor recovery [5], which could be due to the lack of motor training and activity-dependent plasticity.

Recently, we developed a noninvasive electrical stimulating technique, tail nerve electrical stimulation (TANES). It can directly trigger the activation of the central pattern generator (CPG) below the lesion level through the tail nerves, resulting in active movements of two hind limbs in rats with chronically contused spinal cord. During the TANES, these rats with motor defects acquire a temporary ability to step or walk with some characteristic features of normally walking, such as body weight-support, alternation of left-right hind limbs, and even coordination of front-hind limbs. It has been demonstrated that the temporary ability to walk is associated with the activation of CPG by TANES [6]. We hypothesized that this temporary ability to walk could be transformed to permanent ability; therefore, we used TANES as a locomotor training paradigm for locomotor recovery from rats with chronic spinal cord injury.

In our previous studies, we combined TANES-induced open-field locomotor

training with basic treatments including glial scar ablation and transplantation of LP plus OECs, significantly improving locomotor recovery in rats with chronic spinal cord contusion injury [7]. In addition, we found that application alone of TANES-induced open-field locomotor training also can promote the locomotor recovery from rats with chronic [7] or sub-acute [8] spinal cord injury. Motor function improvement either in chronic or sub-acute injury models, is most likely attributed to activity-dependent plasticity promoted by TANES through the activation of the CPG below the level of injury [9] [10] [11] [12] [13].

Electrophysiology represents one way to reliably quantify the functionality of motor and somatosensory pathways [14]. For the further understanding of the mechanisms in the present study, in addition to use our traditional methods, such as glial scar ablation, LP transplantation, and TANES-induced open-field locomotor training, we will add electrophysiological tests including motor evoked potential (MEP), somatosensory evoked potential (SSEP), and Hoffmann reflex (H-reflex) to evaluate the effect of TANES on electrophysiological change and the relationship between functional recovery and electrophysiology.

In our previous observations, autophagia was found in rats with chronic spinal cord injury and SCI rats with basic treatments including glial scar ablation plus OEC/OLP transplantation, but almost none was found in rats receiving TANES-induced open-field locomotor training. In this study, we will continue to observe the incidence of autophagia in rats with different treatment, and try to determine if TANES or TANES-induced open-field locomotor training has an effect on preventing/reducing the occurrence of autophagia.

2. Materials and Methods

2.1. Animal

Adult, female, Long-Evans rats (200 - 220 g) from Charles River Laboratory were used for this study. They were singly housed in plastic cages on 12 hours light/dark cycles and were allowed to freely access the drinking water and standard food. All rats were subjected to spinal cord injury and were randomly divided into 3 groups. Group I (SCI Control, n = 13) received contusion injury only. Group II (SCI + BT, n = 12) and Group III underwent basic treatment (BT) including scar ablation and transplantation of olfactory lamina propria 6 weeks after spinal cord injury. Additionally, Group III (SCI + BT + TANES, n = 13) started to receive TANES-induced locomotor training one week after basic treatment. All rats were allowed to survive 22 weeks after injury, except 3 - 6 rats from each group, which received spinal cord transection.

All surgical and therapy procedures, including interventions, pre- and post-surgery care, electrical stimulation, evaluation of locomotor function, and electrophysiological tests, were approved by the Institutional Animal Use and Care Committee of CARE Research, Inc. and are consistent with the Guide for the Care and Use of Laboratory Animals (1996).

2.2. Spinal Cord Contusion and Post-OP Care

The contusion injury to the rat spinal cord was produced by the New York University impactor device as previously described [5]. In brief, under deep anesthesia with sodium pentobarbital (50 mg/kg, ip), the back skin was cut and the muscles were separated from the spinous processes; then the spinous processes of vertebra thoracic (T) level T7 and T10 were clamped to stabilize the vertebral column. After spinal cord was exposed through a dorsal laminectomy on T8-9, the 10 g impact rod was dropped on the cord segment T10 from a height of 25 mm, and it resulted in hind limb locomotor deficits as described previously [15]. Afterwards, the muscles and skin were closed with sutures. Additionally, 22 weeks after injury 3 rats from Group I, 3 rats from Group II, and 6 rats from Group III underwent complete spinal cord transection at T8 and survived 2 more weeks.

After injury/surgery analgesia, buprenorphine (0.02 mg/kg, sc) was injected twice a day for the first 2 days to relieve pain. The antibiotic cephazolin (20 mg/kg, sc) was used twice a day for one week to prevent potential infection at the surgical wound and urinary bladder. Urinary bladders were carefully emptied by hand twice a day until adequate spontaneous voiding returned. Additionally, 10 ml of saline solution (0.9% NaCl) was administered daily for one week to prevent dehydration.

2.3. Preparation of Lamina Propria for Transplantation

The method to prepare the olfactory lamina propria for transplantation was described previously [16]. In brief, under surgical microscope, the olfactory mucosa, devoid of the respiratory mucosa, was isolated from the nasal septum of a donor rat and was placed in Dispase II (Roche) for 35 - 40 min at 37˚C. The olfactory epithelium was then gently removed using forceps thereby exposing the lamina propria. For transplantation, the lamina propria was cut into 0.5 × 1.0 mm and kept in L-15 on ice until transplant. Two pieces of lamina propria were used for one rat.

2.4. Scar Ablation and OLP Transplantation

Six weeks after spinal cord injury, glial scar ablation was performed at the injury epicenter as previously described [3]. Briefly, under deep anesthesia with sodium pentobarbital, the injury site was reopened to expose the injured spinal cord. One (1.0) microliter of 2% rose Bengal in saline was slowly injected stereotaxically into the lesion cavity through a narrow bore (~100 μm) of glass pipette connected to a Digital Microdispenser (Drummond Scientific Co., Broomall, PA, USA). Then, the rose Bengal was allowed to diffuse for 5 min in order to permeate through the cytoplasm of the superficial astrocytes that composed the glial scar; the injury site was illuminated for 5 min with the focused full spectrum light of a halogen bulb (150 W, 7 cm distance). To prevent damage to the cord tissue by the heating source from the halogen light, the exposed spinal cord was

bathed with room temperature saline solution with fresh change each 30 - 40 seconds during the illumination.

Immediately after scar ablation, two pieces of olfactory lamina propria were carefully delivered into the lesion cavity through a small hole made with an 11 blade. After that, the dura was closed by suturing with a 10-0 Novafil nylon suture (Ethicon, Somerville, NJ, USA), which was put on the dura and ready to tighten up immediately after the delivery of olfactory lamina propria. Then the muscles and skin were closed with proper sutures, respectively [5].

2.5. TANES-Induced Open-Field Locomotor Training

One week after the basic treatment, TANES-induced open-field locomotor training started with a physical therapy instrument (Type J18A1, Quan-Ri-Kang Company, China). The instrument has been widely used and demonstrated neither risk nor side effect in the application in clinic and home (http://www.quanrikang.net/Aboutus.asp?Title=%C6%F3%D2%B5%BC%F2%BD%E9). It has a middle frequency carrier wave of 2.5 - 8 kHz and low adjustable frequency 0 - 150 Hz with a maximal output current 100 mA. The stimulating strength can be adjusted according to animals' response to the stimulation, usually between 10 - 40 mA at a frequency of 4 kHz. The locomotor training was performed as described previously [6]. Briefly, the rat was placed in an open field (plastic basin with a diameter of 4 feet). The rat's tail was attached to two electrodes connected to the instrument. Once the stimulation started, the rat's two hind limbs started to move extensively and alternatively, thus enabling rat to stand up, step, and even freely walk around the open field. To prevent possible fatigue due to over contract of the leg muscles, the moving frequency of the hind limbs was limited to 40 - 60 steps per minute by modulating the stimulating strength. The TANES-induced locomotor training lasted 20 min per session, 5 sessions a week, for a total of 16 weeks.

2.6. Locomotor Recovery Assessment with BBB Scale

The behavior outcomes were evaluated by two independent researchers using the well-known Basso, Beattie, and Bresnahan open-field locomotor rating scale (BBB scale) [17] [18]. BBB scores were collected day 1, day 3, day 7 after injury and once a week afterward until the end of experiment (22 weeks). In addition, a video record (1 - 2 minute long each time) was shot weekly for each test rat. The moving patterns of rat's hind limbs were reviewed repeatedly and carefully with the help of slow motion video records. The final BBB scores for each time point were determined by combining the BBB paper record with the results of the video record review as our previous study [7].

2.7. Horizontal Ladder Rung Walking Test

The apparatus used for the horizontal ladder rung walking test consists of two side walls made of Plexiglass (9 mm in thickness) and 47 metal rungs (3 mm in

diameter), which are inserted to the side walls to create a floor with irregular spaces (8 - 30 mm) between two rungs. The side walls are 100 cm long and 21 cm high measured from the height of the rungs. The space between two side walls is 55 mm. During the test, rats were allowed to walk across the ladder from one end to another; a video camera was positioned at the level of rungs, so positions of all four limbs could be recorded simultaneously and recognized clearly. The video recordings were analyzed using frame-by-frame analysis [19].

Before test rats were not allowed to practice walking on the rungs in order to prevent them from learning the pattern [19]. A footfall error is defined as the misplacement of the rat's foot such that the hind paw to ankle falls completely below the level of the rungs, rather than being placed onto the rungs [20] [21]. The number of footfall errors and the number of steps were counted separately for each hind limb and presented as a ratio of errors. Each animal was allowed to walk on the rungs three times in different direction, and the ratio of errors per step was averaged as the error rate for the animal tested [19] [22].

In order to confirm the objective reality of methods we used to assess the locomotor recovery, a potential correlation between BBB scores and the result of horizontal ladder rung walking test was analyzed using linear regression. The average percentage of footfall errors per step made in horizontal ladder rung walking test each rat 22 weeks after spinal cord injury were plotted against the BBB scores.

2.8. Electrophysiological Tests

Electrophysiological tests including motor evoked potentials (MEPs), somatosensory evoked potentials (SSEPs), and Hoffmann reflex (H-reflex) were performed to examine the spinal motor conductions, somatosensory conductions, and supraspinal control. A Magstim 200^2 monophasic magnetic stimulator (Magstim, UK) with a stimulating frequency 0.25 Hz and maximum voltage of 2.80 kV was used to stimulate the nerves and neurons by inducing a magnetic field which can pass through tissue and bone to reach otherwise inaccessible areas. The adapted Magstim stimulating coil (Magstim, UK) is a single coil with a diameter of 50 mm. Transcranial/cutaneous magnetic stimulation (TMS) is a non-invasive and painless method of stimulating human or animal tissue using strong, time varying magnetic fields to induce small currents in nerve tissue. A Sierra Wave System (Cadwell Laboratories, Inc., Kennewick, WA) was utilized to record and analyze the waveform. In addition, 12 mm long monopolar needle electrodes with attached lead wire (Cadwell Laboratories, Inc., Kennewick, WA) were used for recording, reference, and ground electrodes. The interelectrode impedances were kept less than 2.5 kOhms. Electrophysiological tests were carried out on each rat without anesthesia [23] before injury and weekly after injury.

Motor evoked potentials. The recording electrode was placed in the belly of gastrocnemius muscle in the hind limbs, the reference electrode was placed near

the distal tendon of the muscle, and the ground electrode was placed subcutaneously in the tail root [24]. MEPs were recorded following stimulation of motor cortex in order to measure the integrity of the motor neuron output from the brain to the spinal cord level below the injury site (T10). In this type of monitoring, the motor cortex is stimulated with 100% strength of TMS through the scalp and skull over the motor cortex.

Somatosensory evoked potentials. A modified method was used to record the SSEPs [23]. Briefly, the needle electrodes were carefully inserted into the scalp and placed on the surface of the skull, parallel with the long axis of the rat body. The recording electrode was located on the midline of the skull and crossed bregma, covering the positions equal to CPz, Cz and Fz according to the 10-20 International System of EEG electrode placement [25]. The reference electrode was placed on the skull surface over the olfactory bulb, while the ground electrode was placed in the shoulder. SSEPs were recorded through a recording electrode on the skull surface over the somatosensory cortex following stimulation of tibial nerve with 60% maximal strength of TMS.

Hoffmann reflex. To record the H-reflex in rats, the recording electrode was inserted into the belly of gastrocnemius muscle in the hind limb, the reference electrode was placed into the muscle tendon, and the ground electrode was placed subcutaneously near the tail root [26]. The magnetic stimulating coil was placed over the L4-5 roots of spinal nerve and stimulated the nerves with 35% of maximal strength of TMS. In general, for the analysis of H-reflex data, the H-wave latency, H-wave amplitude, M-wave latency, M-wave amplitude were recorded and measured, and then the H/M amplitude ratio was calculated separately.

2.9. Histology and Immunohistochemistry

At the end of the experiment, all rats were transcardially perfused with phosphate-buffered 4% paraformaldehyde under deep anesthesia with sodium pentobarbital (60 mg/kg, ip). Spinal cords (10 mm) centered on the injury epicenter were processed for histological evaluation, *i.e.*, the paraffin sections (10 μm thick) were stained routinely (hematoxylin and eosin, H.E.) and immunohistochemically.

Method used for P0-immunostaining has been described previously [3]. Briefly, deparaffinized and rehydrated sections were boiled in the Antigen Retrieval Citra solution (BioGenex, San Ramon, CA) for 15 min. Sections were incubated with 3% H_2O_2 in 50 mM Tris-buffered saline containing 0.1% Triton X-100 (TTBS) pH 7.5, for 30 min at room temperature (RT) to quench the endogenous peroxidase. The normal goat serum (2% in TTBS) was added (60 min at RT) to the tissue to block non-specific antigens. After being incubated with primary antibody, monoclonal antibody against peripheral myelin protein (P0, 1:1000; courtesy of Dr. Archelos, Austria) of Schwann cells, diluted in TTBS with 1% normal goat serum in a humidified chamber overnight at RT, sections were

rinsed and incubated in 1:200 biotinylated goat anti-mouse IgG (Jackson) for 60 min and then in 1:500 streptavidin peroxidase (Jackson) for 60 min in the same buffer. The immunoreactive product was visualized by incubating in 3, 3'-diaminobenzidine solution (DAB, 0.5 mg in 1 ml TTBS) with 0.01% H_2O_2 for 1 - 3 min. Sections were rinsed, dehydrated, cleared, and coverslipped after counter-staining with hematoxylin. Negative controls were treated similarly, except the lack of the primary antibody.

2.10. Statistical Analyses

All quantitative data were presented as Mean ± SEM. Open field locomotor scores (BBB scores) for the hind limbs from the same group were averaged to yield one score for each time point. Repeated-measures ANOVAs (analysis of variance) were used to analyze BBB scores, horizontal ladder rung walking tests, and electrophysiological measures for all groups. The BBB scores were compared using a two-way ANOVA between groups. Other statistical analyses of differences between groups were carried out by one-way ANOVAs. In most cases, the analysis of quantitative data was performed for overall significance followed by a post hoc LSD Student's t test; differences were considered significant if $p < 0.05$. In addition, the linear regression was used to analyze the potential correlation between BBB scores and the result of horizontal ladder rung walking test. More, Chi-square (χ^2) test was used to analyze the incidence rate of autophagia occurred in rats with various treatments.

3. Results

3.1. TANES Improved Locomotor Recovery

Six weeks after spinal cord injury, rats in three groups had consistent and nearly equivalent BBB scores. Rats in the control group showed a little change in BBB score from 6 weeks after injury to the end (22 weeks) of this experiment (SCI Control: 8.65 ± 0.14→9.12 ± 0.56). Similarly, rats with basic treatment including scar ablation and OLP transplantation did not have their BBB score increased remarkably in the last 16 week duration of the experiment (SCI + BT: 8.92 ± 0.19→9.21 ± 0.43). However, one week after receiving TANES-induced open-field locomotor training, treated rats showed BBB scores overtaking and exceeding that of the rats in the other two groups. By the end of the experiment, TANES-treated rats' BBB scores increased remarkably (SCI + BT + TANES: 8.96 ± 0.24→11.12 ± 0.54), and significantly higher than that of SCI Control group ($p = 0.016$) and SCI + BT group ($p = 0.011$). In addition, after transection at T8 the BBB scores dropped prominently in all three groups; however, 2 weeks later the BBB score of rats with locomotor training was still much higher (SCI + BT + TANES: 6.92 ± 0.47) than that of rats in injury control group (SCI Control: 2.83 ± 1.59, $p = 0.014$) and that of rats in basic treatment group (SCI + BT: 5.00 ± 1.00, $p = 0.084$) (**Figure 1**).

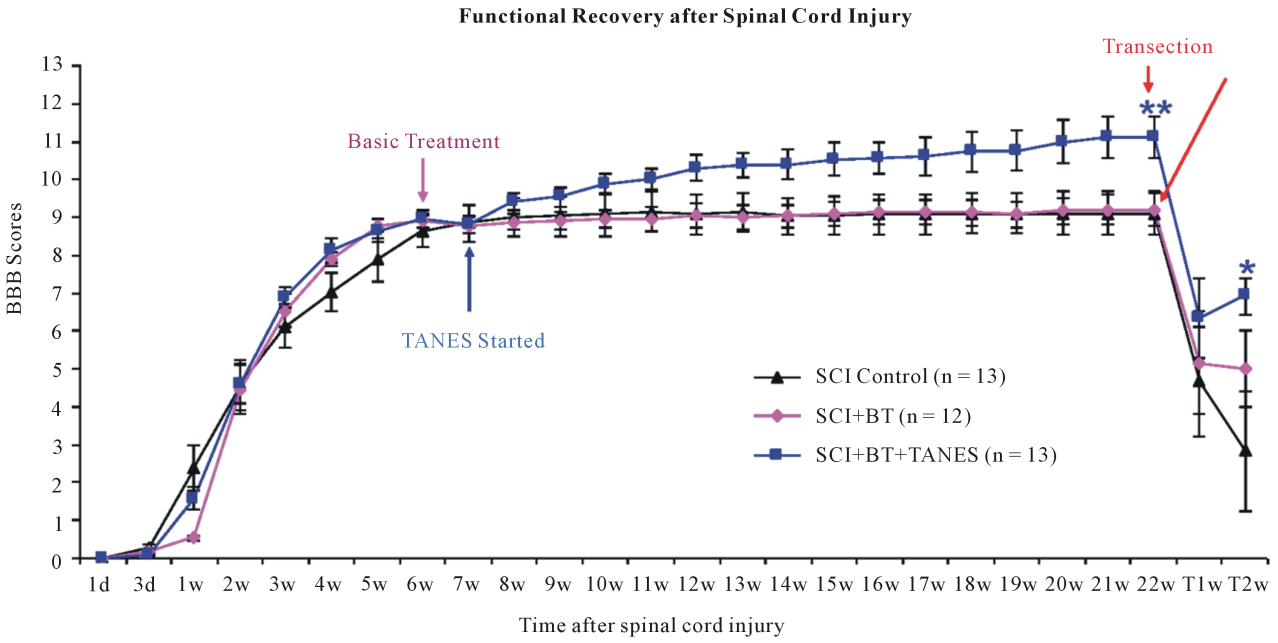

Figure 1. Locomotor recovery after SCI evaluated with BBB scale. BBB scores of both control rats (SCI Control) and rats receiving basic treatment (SCI + BT) show little change 6 wks after SCI. However, rats receiving both basic treatment and TANES-induced open-field locomotor training (SCI + BT + TANES) showed a significant increase in their BBB scores (**p = 0.016 vs SCI Control, p = 0.012 vs SCI + BT). In addition, 2 wks after transection at T8, there was a significant decrease in BBB scores, but the BBB score in the full treatment group (6.92 ± 0.47) is still higher than that of SCI Control group (2.83 ± 1.59, *p = 0.014) and basic treatment group (5.00 ± 1.00, p = 0.084).

3.2. TANES Reduced Footfall Errors

Results of horizontal ladder rung walking tests showed that before spinal cord injury rats were able to cross the horizontal ladder quickly with a very low ratio of footfall errors (4.94% ± 0.37%, n = 38). However, after spinal cord injury rats took a longer time and made more errors while crossing the horizontal ladder. By the end of the experiment, the ratio of footfall errors of rats in injury control group (SCI Control: 71.20% ± 3.28%) was very close to that of rats in basic treatment group (SCI + BT: 70.93% ± 2.76%). However, the ratio of footfall errors of rats in locomotor training group (SCI + BT + TANES: 50.98% ± 2.42%) was significantly lower than that of rats in two other groups ($p < 0.01$) (**Figure 2**).

The result of linear regression analysis showed a significantly negative correlation between BBB scores and horizontal ladder rung walking footfall errors ($r = -0.53$, $p < 0.01$; **Figure 3**).

3.3. MEPs Were Sensitive to the Spinal Cord Contusion

The latency and amplitude of MEPs (**Figure 4(A)**) were analyzed in this study. Before spinal cord injury, the average latency of MEPs was 6.40 ± 0.08 ms (millisecond) and the average amplitude was 5.61 ± 0.72 mV (millivolt). Immediately after spinal cord injury, the MEP amplitude dropped sharply (0.1 - 0.3 mV); the MEP latency also delayed significantly (11.74 - 13.19 ms), but was reduced slowly afterward. At the end of the experiment (22 weeks after injury), the MEP

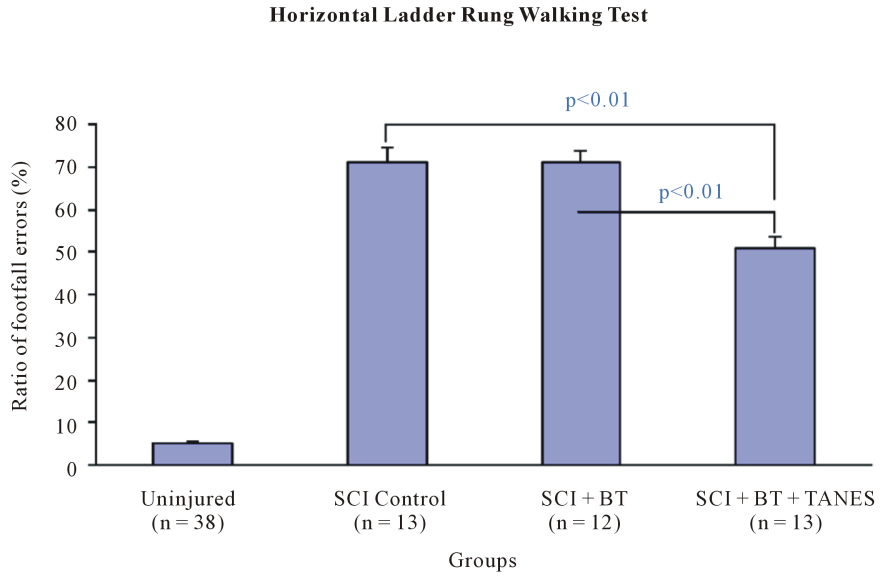

Figure 2. Horizontal ladder rung walking test before and 22 weeks after injury. The histogram shows that the normal rats (Uninjured, n = 38) have a very low ratio of footfall errors. On the other hand, 22 weeks after spinal cord injury, the ratio of footfall errors of rats with TANES-induced open field locomotor training (SCI + BT + TANES) was significantly lower than that of rats in injury control group (SCI Control) and in basic treatment group (SCI + BT).

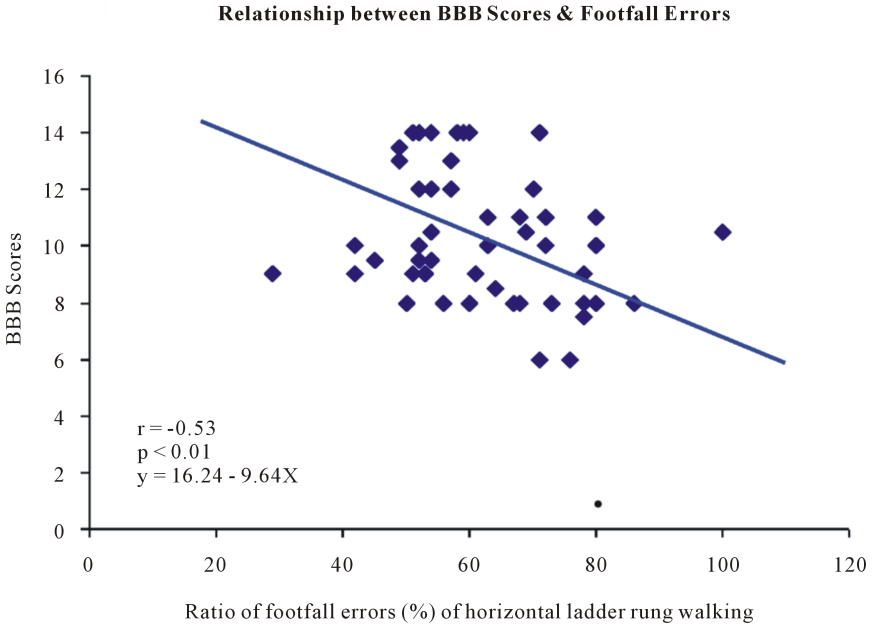

Figure 3. Linear regression analysis of correlation between BBB test and horizontal ladder rung walking test 22 weeks after spinal cord injury. The average percentage of footfall errors was plotted against the BBB Scores. The result shows a significantly negative correlation between them.

latency of rats in SCI Control group showed little restoration (11.53 ± 0.45 ms), while significant restoration of the MEP latency was found both in basic treatment

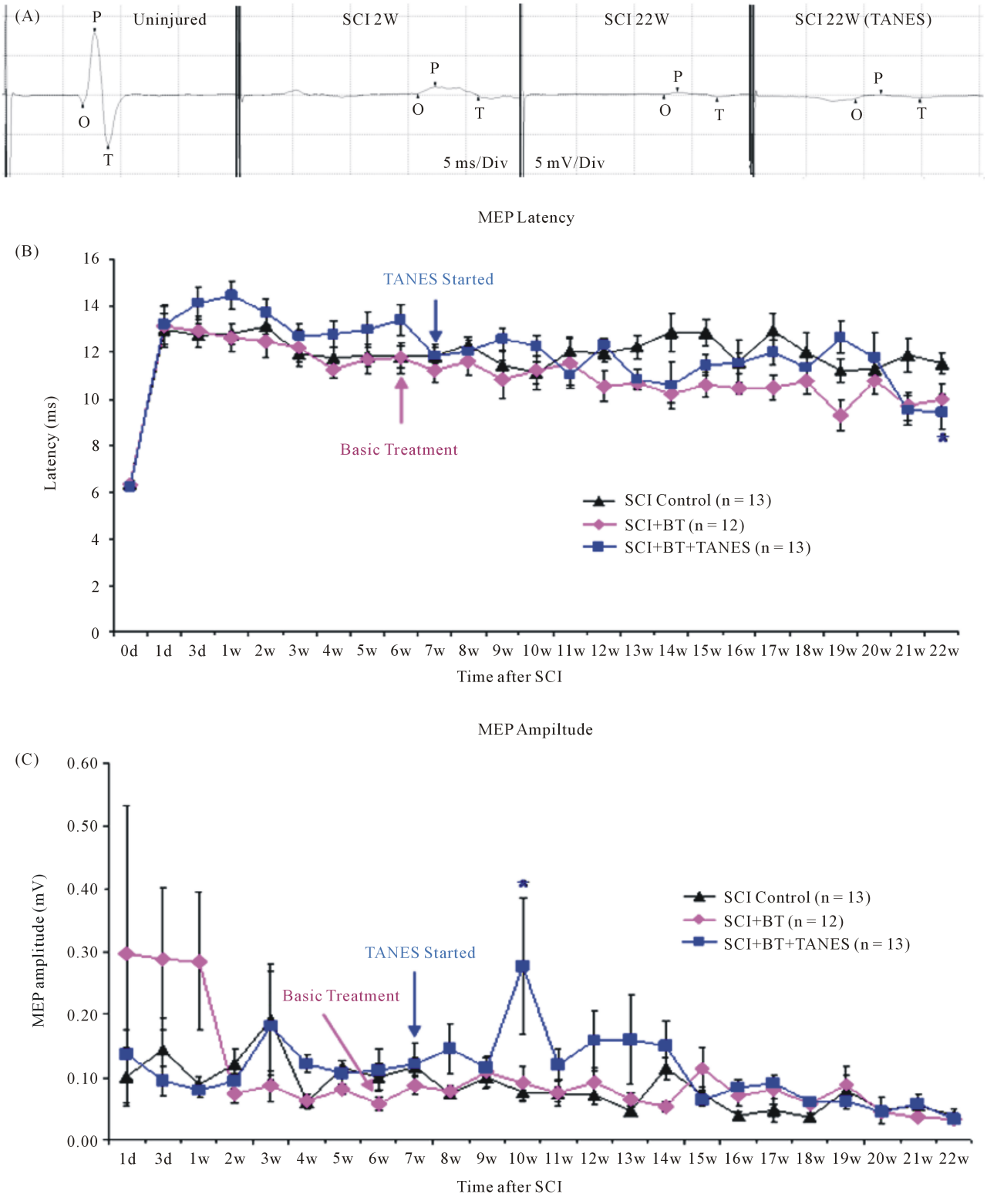

Figure 4. Changing course of MEP latency and amplitude after SCI. Representative wave forms of MEPs are shown in (A). Immediately after SCI the MEP latency was delayed significantly, but was reduced slowly afterward (B); the MEP amplitude dropped significantly, but did not show remarkably recovery even at the end of observation in all groups (C). The MEP latency in rats from groups both of SCI + BT and SCI + BT + TANES is shorter than that from rats in SCI Control group (*$p < 0.05$ in (B)). It was noted that TANES may induce temporary raise of MEP amplitude (*$p < 0.05$ in (C)).

group (SCI + BT: 9.98 ± 0.68 ms; p = 0.032) and locomotor training group (SCI + BT + TANES: 9.45 ± 0.75 ms; p = 0.016) as compared with the control animals (**Figure 4(B)**). Interestingly, all rats in three groups after spinal cord injury showed a tendency of continuous reduction of MEP amplitude without any restoration (**Figure 4(C)**), although rats with TANES-induced open-field locomotor training significantly improved their locomotor performance as assessed by BBB scale and horizontal ladder rung walking test. However, we have found that TANES also may make a temporary recovery of MEP amplitude (**Figure 4(C)**).

3.4. TANES Promoted SSEP Restoration

Under the normal condition, rat's SSEPs consist of a series of waves that reflect sequential activation of neural structures along the somatosensory pathways. A typical SSEP trace contains a positive P1 peak and a negative N1 peak (**Figure 5(A)**). Our analysis was focused on P1 latency and P1-N1 amplitude. The results showed that in uninjured rats the P1 latency was 8.40 ± 0.12 ms and P1-N1 amplitude was 120.98 ± 7.05 μV (microvolt). Immediately after spinal cord injury, the P1 latency delayed significantly (11 - 13 ms; **Figure 5(B)**) and the P1-N1 amplitude decreased drastically (16 - 37 μV, day 1 post-injury; **Figure 5(C)**), and then recovered slowly. By the end of the experiment, the SSEP latency in SCI Control group showed some recovery (10.41 ± 0.44 ms), while that in both basic treatment group (SCI + BT: 9.12 ± 0.39 ms, $p < 0.05$) and locomotor training group (SCI + BT + TANES: 9.25 ± 0.38 ms, $p < 0.05$) showed a significant recovery. On the other hand, the SSEP amplitude in SCI Control group showed a certain degree of recovery (46.82 ± 15.31 μV), while that in basic treatment group (SCI + BT) showed a moderate recovery (61.38 ± 10.95 μV). However, the SSEP amplitude in locomotor training group (SCI + BT + TANES) showed a significant recovery (97.16 ± 15.70 μV, p = 0.015) when compared with the control rats (**Figure 5(C)**).

3.5. TANES Promoted Further Recovery of H-Reflex

The latency and amplitude of both H-wave and M-wave (**Figure 6(A)**) were analyzed, and the H/M amplitude ratio was considered as an index to evaluate the recovery of H-reflex after spinal cord injury. In uninjured rats the H/M amplitude ratio was 25.25% ± 1.03%. After spinal cord injury, due to the increase in H-amplitude and the decrease in M-amplitude, the H/M amplitude ratio significantly increased (average 39.17% ± 3.09%) 2 weeks post-injury. Along with the endogenous recovery of the locomotor function, the H-reflex also spontaneously recovered to some degree. By the end of the experiment, the H/M amplitude ratio in SCI Control group was 33.54% ± 2.88%, while that in basic treatment group (SCI + BT) was 28.74% ± 1.45%. However, the H/M amplitude ratio in rats receiving TANES-induced open-field locomotor training was significantly lower (SCI + BT + TANES: 25.92% ± 2.51%, p = 0.0288) than that in rats of the control group, quite close to the normal level (**Figure 6(B)**).

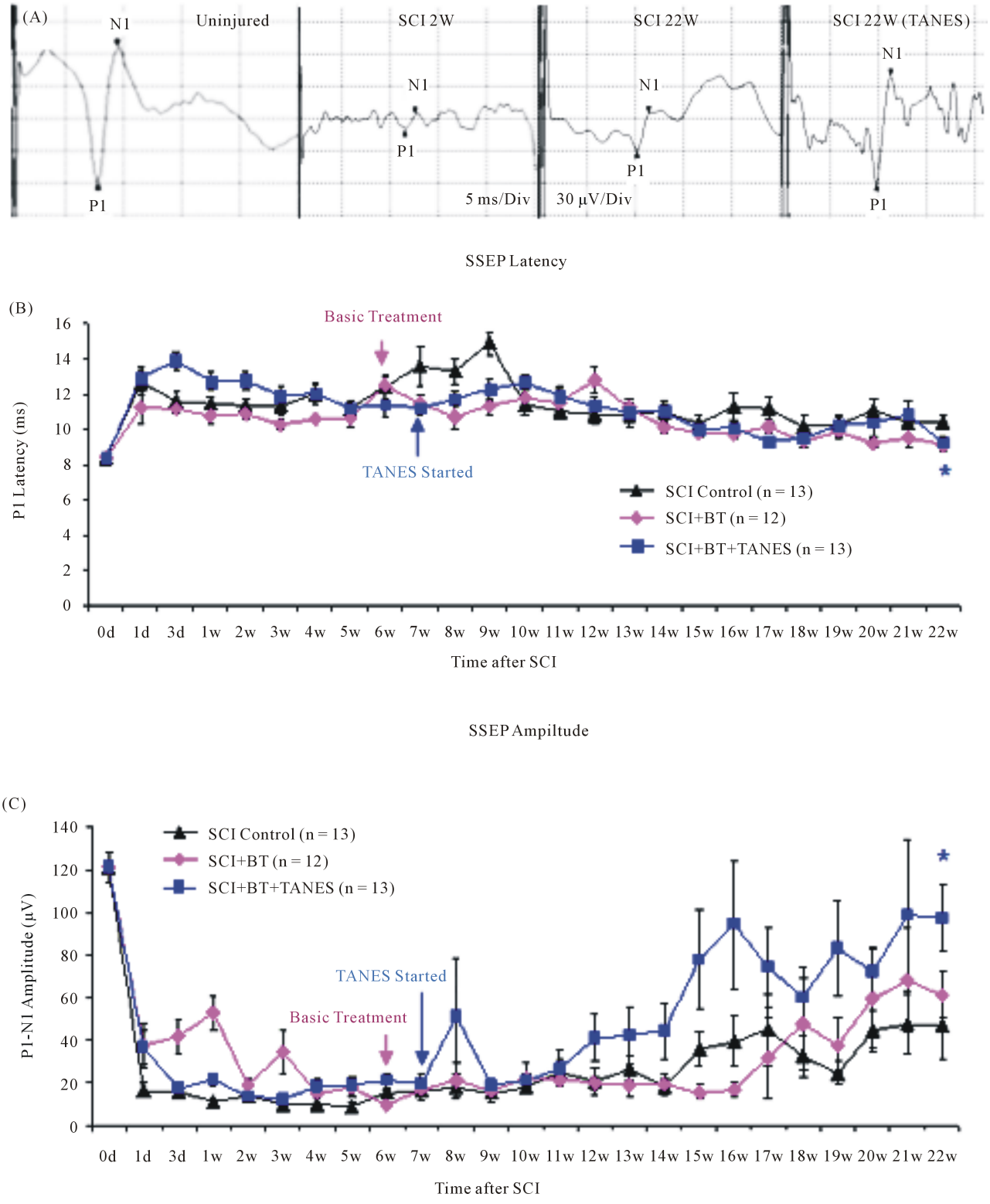

Figure 5. Changing course of P1 latency and P1-N1 amplitude of SSEP after SCI. Representative wave forms of SSEPs are shown in (A). Immediately after SCI the P1 latency was delayed significantly, but reduced slowly afterward (B); the P1 latency in rats from groups both of SCI + BT and SCI + BT + TANES is shorter than that from rats in SCI Control group (*$p < 0.05$). The P1-N1 amplitude also dropped significantly immediately after SCI, but recovered slowly afterward (C). The P1-N1 amplitude in rats from the group of SCI + BT + TANES is much higher than that from rats in two other groups (*$p < 0.05$).

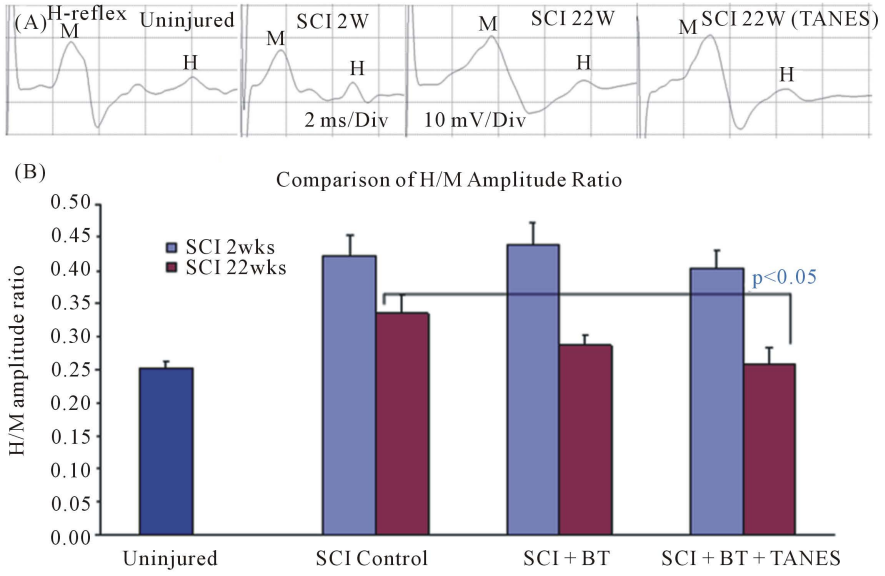

Figure 6. H-reflex test with comparison of H/M amplitude ratio. Representative wave forms of H-reflex are shown in (A). Following spinal cord injury, H/M amplitude ratio increased (SCI 2 wks) compared with that of uninjured rats. Rats in SCI Control group show an endogenous restoration at the end of the experiment (SCI 22 wks). Significant recovery of the H/M amplitude ratio was found in rats with basic treatment and TANES-induced open field training (SCI + BT + TANES) ($p < 0.05$, in (B)), compared with that in rats with injury alone (SCI Control) at 22 weeks.

3.6. Tissue Repair and P0-Positive Myelination

Similar to our previous observation [5], in SCI Control group, the lesion cavity, small sized initial repaired tissue, and glial scar with sharp edge can be identified clearly 22 weeks after injury (**Figure 7(A)**, **Figure 7(B)**). In both basic treatment group (SCI + BT) and locomotor training group (SCI + BT + TANES), the expanded repaired tissue almost completely occupied the damaged area, resulting in prominent reduction and nearly disappearance of the lesion cavity, as well as the integration of repaired tissue with spared tissue; simultaneously, almost no more glial scar with sharp edge could be identified (**Figures 7(C)-(F)**).

Most P0-positive myelin sheaths formed by the host Schwann cells are found in repaired tissue while some in spared tissue (**Figures 7(G)**). The myelinated fibers may include regenerating axons (in the repaired tissue) or demyelinated axons (in the spared tissue). The counts (Mean ± SEM) of P0-positive myelin sheaths in each group are shown as below: SCI Control group: 1999 ± 198 (n = 5); basic treatment group (SCI + BT): 3419 ± 424 (n = 5); locomotor training group (SCI + BT + TANES): 4511 ± 406 (n = 5). Differences between SCI Control group and SCI + BT group, between SCI Control group and SCI + BT + TANES group, and between SCI + BT group and SCI + BT + TANES group are statistically significant ($p < 0.05$) (**Figure 7(H)**).

3.7. Occurrence of Autophagia

In our animal experiments, autophagia with an area of 1 - 4 cm^2 could be found

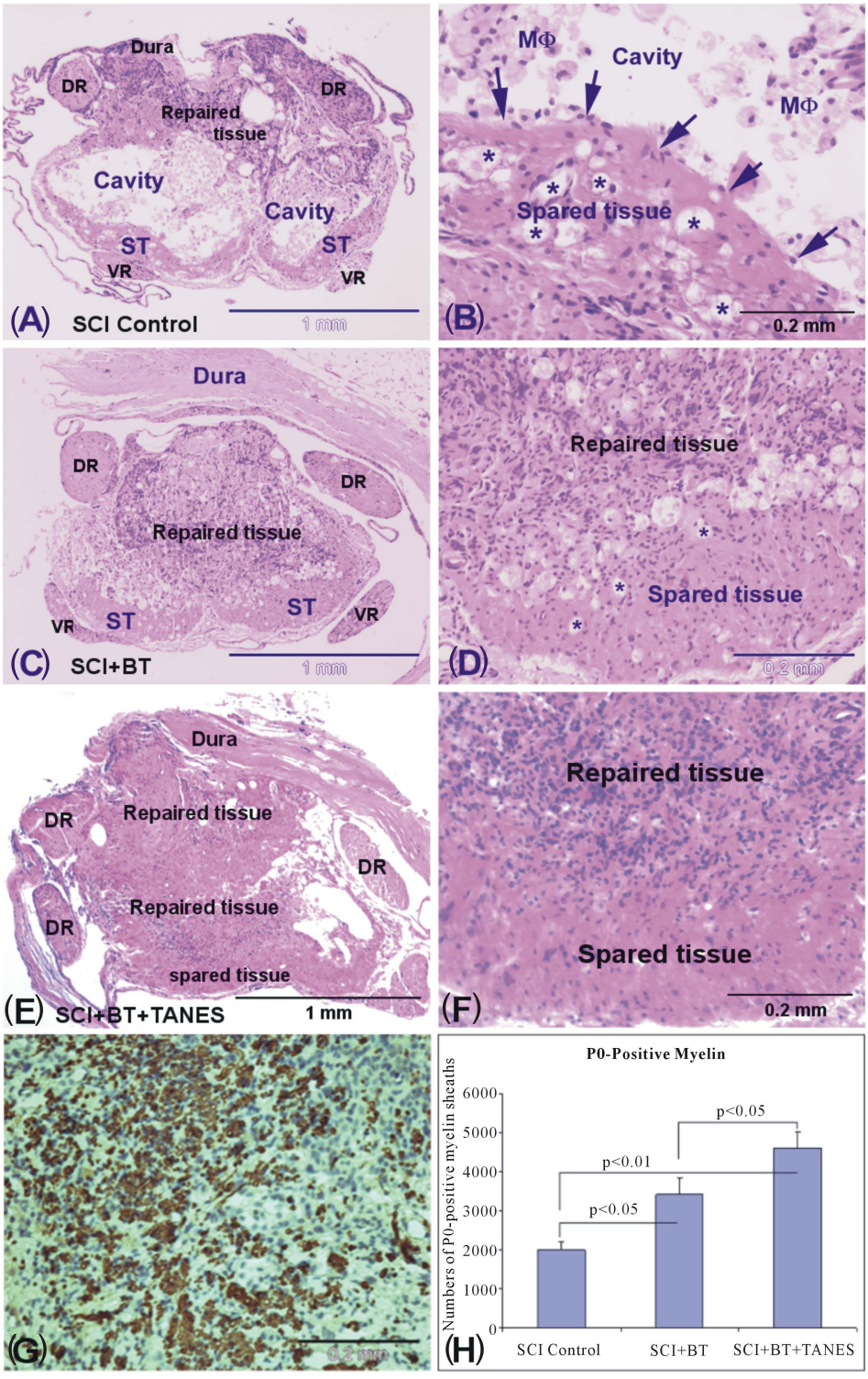

Figure 7. Tissue repair and P0-positive myelination. Representative images (A), (C), and (E) (H.E. staining) are cross sections at the injury epicenter from different groups and (B), (D), and (F) are their local magnifications, respectively. Clearly labeled are spared tissue, lesion cavity, repaired tissue, glial scar with sharp edge (arrows), and demyelinated or degenerated axons (*). Development of repaired tissue and its integration with spared tissue, and reduction and disappearance of the lesion cavities indicate the repair in anatomy. (G) (P0-immunostaining) reveals P0-positive myelin sheaths (brown circles or dots) in the repaired tissue. Histogram (H) shows the average numbers of P0-positive myelin sheaths in individual groups. ST: spared tissue; DR: dorsal root; VR: ventral root; Mϕ: macrophage.

time to time in rats with chronic spinal cord injury. In the last three similar experiments, 9 rats with autophagia were found among 37 rats in SCI control groups (incident rate 24.3%); 7 rats with autophagia were found among 35 rats receiving basic treatments (incident rate 20.0%). However, we have found only one case of autophagia occurred in 56 rats receiving basic treatments plus TANES-induced open-field locomotor training (incident rate 1.8%). Data indicate the difference in incidence rate between trained rats and untrained rats is statistically significant ($p < 0.01$) according to the Chi-square test ($\chi^2 = 14.30$, n' = 2). However, there is no significant difference ($p > 0.50$) between two untrained groups, although the incidence rate in rats with scar ablation and neural transplantation is lower than that in rats with injury only (Table 1).

4. Discussion

Our results from this study further demonstrate that TANES-induced open-field locomotor training significantly promotes locomotor improvement in the chronic model of rat with spinal cord contusion injury, promotes the electrophysiological restoration of spinal cord conduction and spinal cord reflex, and also may prevent/reduce the occurrence of autophagia. In addition, this study suggest again that the temporary ability to step or walk of rat with chronic spinal cord injury can transform into permanent ability, may be due to the activity-dependent plasticity promoted by the activation of CPG triggered by TANES [27] [28] [29].

Consistent with our previous observation [16] [30], rats in SCI Control group and SCI + BT group showed their BBB scores without remarkable change within the later 16 weeks of this experiment, indicating that only scar ablation and neural transplantation, which may induce tissue repair and axonal regeneration, do not directly result in locomotor recovery in rat with chronic spinal cord injury. However, rats receiving both basic treatment and TANES-induced open-field locomotor training showed significant improvement of their locomotor recovery. Data from horizontal ladder rung walking test in the present study support this observation. Horizontal ladder rung walking test is sensitive to chronic movement deficits after spinal cord injury of rat [19] and has been used as a stringent test of sensorimotor function to assess skilled walking and measure both of forelimb and hind limb placing, stepping, and inter-limb coordination [22]. Results of horizontal ladder rung walking test demonstrate that

Table 1. Incidence Rate of Autophagia.

Treatments	SCI	SCI + BT	SCI + BT + TANES
Number of rats	37	35	56
Number of autophagia	9	7	1
Incidence rate	24.3%	20.0%	1.8%**

**$p < 0.01$ compared to rats with two other different treatments, respectively. Note: This table contains collecting data from multiple animal experiments performed in our lab.

TANES can significantly reduce the deficits in hind limb sensorimotor function following spinal cord injury that may not be assessed with ratings of open-field locomotion [31]. In addition, linear regression analysis of correlation between BBB scores and horizontal ladder rung walking test helps confirm that these two methods are reliable and consistent to evaluate the locomotor performance after spinal cord injury. All these observations indicate that TANES-induced open-field locomotor training is a useful paradigm to promote locomotor recovery from rat model of chronic spinal cord injury.

It has been reported that after complete transection of thoracic spinal cord in rat at the beginning of the experiment or several weeks after contusion, slight movement of one or two hind limb joints could be found [18]. Interestingly, after complete transection at T8 in our contusion model, the BBB scores dropped dramatically in all rats, however, the BBB score of rats receiving TANES-induced open-field locomotor training was still much higher than that of control rats. Activity-dependent plasticity occurs in the spinal cord throughout life and plays an important role in the acquisition and maintenance of motor skills and in the effects of spinal cord injury and other CNS disorders [32]. The phenomenon observed in this study may be associated with the activity-dependent plasticity promoted by TANES or TANES-induced locomotor training [13]. This finding further demonstrates that the temporary ability to step or walk induced by CPG activation triggered through the TANES could be transformed into permanent ability which was not completely controlled by brain and brainstem.

It is well known that electrophysiology represents one way to reliably quantify the functionality of motor and somatosensory pathways [14]. In this study, MEPs, SSEPs, and H-reflex were drastically impaired by the spinal cord contusion, although most of them showed limited endogenous recovery later. Instead of recovery, however, MEP amplitude showed a tendency of continuous reduction after injury in all rats, including those which had their BBB score increased significantly after receiving TANES-induced open-field locomotor training. It has been reported that there was a correlation between BBB score and MEP amplitude after spinal cord injury [33]; rats with higher values of BBB score also showed higher MEP amplitude, however, it was also noted that most of rats with a BBB score of 10 - 15 or less had no recordable MEPs, suggesting that electrophysiological tests are more sensitive to the effects of injury than function behavior. Recovery of MEPs may be mainly correlated to the preservation of gray matter, while locomotor recovery requires preservation also of descending tracts in the white matter [33]. In our contusion model, almost no gray matter and dorsal corticospinal tract were preserved at the injury epicenter [16] and the BBB score ranged from 8 to 14, with an average of 11.12 ± 0.54 in rats with TANES-induced open-field locomotor training. The MEP amplitude did not increase along with the increase in BBB score, thus there is no difference between SCI + BT + TANES group and control groups. This observation is consistent with other studies [14] [34]. Based on this finding, MEPs may be sensitive to

spinal cord injury but may not give a faithful representation of motor functional recovery.

The categorized SSEP responses by latency and amplitude can be used as an indicator for the extent of ultrastructural damage of the spinal cord after chronic compressive injuries and correlate well with the disability and recovery in patients with cervical spondylotic myelopathy [35]. In the present study, rats in SCI Control group showed slow and minimal restoration of SSEP latency and limited recovery of P1-N1 amplitude, while rats in SCI + BT group showed better performance at these aspects. This may be associated with the spared tissue, endogenous recovery, and recovery of both anatomy and motor function promoted by scar ablation and neural transplantation. However, significant recovery both of SSEP latency and P1-N1 amplitude were observed in rats with TANES-induced open-field locomotor training, suggesting that TANES may stimulate the restoration of somatosensory conduction velocity and may also stimulate the restoration of P1-N1 amplitude. On the other hand, it may also indicate that the restoration of P1-N1 amplitude may be associated with the tissue repair and axonal regeneration [14] [34] [35].

Previous studies show that initial H-wave amplitude changes are likely due to spinal shock and greater responses eventually lower, approaching normal [36]. Our data showed that after spinal cord injury the H/M amplitude ratio increased significantly due to the increase in H amplitude. By the end of this experiment H/M amplitude ratio recovered remarkably, but did not approach to normal level. The discrepancy between our study and other study may be attributed to the instrument and animal model. Our results showed that TANES-induced open-field locomotor training significantly improved the restoration of H/M amplitude ratio, indicating that TANES or TANES-induced open-field locomotor training may benefit the recovery of supraspinal control or H-reflex. These results agree with other studies that passive exercise helps recover the frequency-dependent depression of the H-reflex in animals and humans after spinal cord injury [37] [38] [39] [40] [41].

Autophagia is a self-injurious behavior which can lead to significant tissue damage, jeopardizing animals' health even causing animal loss, as well as interfering with functional recovery from spinal cord injury. The autophagia is thought to be due to paresthesia (an abnormal sensation, as prickling, itching, etc), or numbness, of the paws or skin [42]. In other word, autophagia is considered to result from neuropathic pain in animals and humans with central nervous system lesions [43] [44]. According to the analysis of clinical cases and the human literature experience evidence was provided that compulsive targeted self-injurious behavior in humans with neuropathic pain and painful dysesthesiae is consistent with the concept that animal autophagia/autotomy may result from chronic neuropathic pain after experimental peripheral or CNS lesions [43]. In our own studies, autophagia has been observed in rats with chronic spinal cord injury and rats receiving scar ablation and OEC/LP transplantation.

However, autophagia has been very seldom found in rats receiving TANES. Our observation suggests that TANES may directly or via locomotor training prevent (preclude)/reduce the occurrence of autophagia in rats with spinal cord injury through attenuating the neuropathic pain. It may have a potential value in clinical trial. However, we do not have direct evidence to prove it so far; the mechanism is unknown yet and needs further investigation.

In summary, TANES as a locomotor training method significantly improves the restoration of locomotor function in rats with chronic spinal contusion injury, evaluated with BBB scale and horizontal ladder rung walking test. After complete transection, trained rats still maintain higher BBB score than that of control rats. This may be related to activity-dependent plasticity promoted by TANES-induced open-field locomotor training. TANES also promoted the recoveries of spinal cord conduction and reflex. However, MEP amplitude was sensitive to the spinal cord injury but not to the restoration of locomotor function. In addition, TANES may prevent/reduces the occurrence of autophagia which may result from neuropathic pain, a symptom of chronic spinal cord injury. Results from this study may shed light into the investigation of chronic spinal cord injury, however the effect and mechanism of TANES need to be further studied.

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No competing financial interests exist.

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