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Original Article

Electro-acupuncture promotes neuroplasticity associated to systematic expressions of NTFs in spinal cord transected rats

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Abstract: This study evaluated the effects of electro-acupuncture (EA) on neuroplasticity associated with expression changes of neurotrophic factors (NTFs) and their receptors in rats subjected to spinal cord transection (SCT) at T_{10}-T_{11} vertebral levels. Behavior evaluation showed that EA gave rise to a significant recovery in hindlimb locomotor (indicated by the scores of Basso, Beattie, Bresnahan) and sensory functions (assessed by the recording of cortical sensory evoked potential stimulation). The results of RT-PCR showed that mRNA expressions of CNTF, FGF-2 and TrkB were significantly upregulated, while these of NGF, PDGF, TGF-β1, IGF-1, TrkA, TrkC were concomitantly down-regulated in the spinal segments caudal (CSS) to the site of transection following EA. Immunohistochemistry (IHC) staining demonstrated an increased number of CGRP fibers, GAP-43 fibers and Synaptophysin fibers in the CSS in EA rats. At the same time, few corticospinal fibers indicated by axonal tracing after injection of biotinylated dextran amine into the precentral gyrus, were found in the CSS in EA group. Together, our findings demonstrated that EA plays an important role in neuroplasticity in rats subjected to SCT. This could be attributed to the establishment of local circuits that may depend on the systematic regulation of NTFs and their receptors after longer EA treatment.

Keywords: EA, spinal cord transection, NTFs, sensory and locomotor functions, rats

Introduction

Functional recovery of neurons in the spinal cord after physical injury is essentially abortive in clinical cases. Spinal cord injury (SCI) often results in cell death, inflammation, tissue degradation and the formation of a number of inhibitory molecules and extracellular matrix. The failure of axonal regeneration following SCI has been attributed to the non-permissive environment and lack of neurotrophic support. Numerous therapeutic strategies attempt to overcome these negative factors and promote axon regeneration, including administration of growth factors [1, 2], tissue bridges [3, 4], various cells types [5-8], artificial scaffolds [9, 10] and other various combinational treatments [11-15]. However, there is still a lack of effective treatments for spinal cord injuries.

Acupuncture has been practiced in China for thousands of years, which is availabe to treat patients suffering from SCI [16, 17], apoplexy [18, 19], ischemia [20] and inflammatory diseases [21, 22]. Electro-acupuncture (EA) as an effective treatment method, has been shown to bring about functional recovery in patients with nervous system injury [23-25]. It was reported that EA could increase the number of synaptic terminals in lamina II of the spinal cord and nucleus dorsalis of the spinal cord in experimental animals [26, 27], but the underlying mechanisms are largely unknown.

Many factors are known to influence neuronal plasticity following nerve injury. These include neurotrophic factors (NTFs) [28-35], pro- or anti-apoptotic factors [36], AMPA receptor activation [37], NMDA receptor activation [38], Nogo

and Nogo receptor activation [39] and GAP-43 expression [40]. The changes induced by EA to the central nervous system (CNS) is based on the changes in one or more of the above factors. A number of evidences were proposed that EA could alter the expression of endogenous NTFs [41], proteins involved in the apoptotic pathway [42] and inflammation factors [43], which might enhance neuroplasticity.

Although previous studies have shown that EA could induce the expression of NTFs and enhance spinal neuroplasticity after SCI [44, 45], they often focused on the effects of one or two NTFs. No systematic analyses of NTFs after spinal cord transection (SCT) have been studied, especially using EA. The present study was therefore designed to investigate whether EA could induce systematic regulation of multiple NTFs and their partial receptors in the spinal segments caudal (CSS) to the site of transection in rats, the possible link to the recovery of sensory, motor functions and neuroplasticity was also investigated.

Materials and methods

Animals

Sprague-Dawley rats (approximately 200 g each) were obtained from the Laboratory Animal Center of Sichuan University. All experimental procedures complied with the Guidelines for the care and use of animals stipulated by the National Institute of Health (NIH), USA. The animals were divided into three groups. Group I rats served as sham-operated controls. Group II rats as SCT group were subjected to surgical spinal cord transection between the T_{10}-T_{11} vertebral levels. Group III rats as EA group were received SCT as above and EA treatment was administrated at the acupoints of *zusanli*, *xuanzhong*, *futu* and *sanyinjiao*, all located in the lower limb.

Rats in Group I was subjected to *in situ* hybridization (ISH) and immunohistochemistry (n = 8) to get the data of location of NTFs and their receptor. Another rats were divied into four subgroups consisting of 8 rats each in respective time point (1, 3, 7, 14 days), respectively, and then performed reverse transcription polymerase chain reaction (RT-PCR) to determine the mRNA expression level of gene.

Other animals consisting of sham, SCT, EA rats (n = 8 for each group) were used to evaluate the restoration of motor functions by scores of Basso, Beattie, Bresnahan (BBB), sensory functions by cortical somato-sensory evoked potentials (CSEP) at 14, 21, 28 days post operation (dpo). Then all animals were perfused with 4% paraformaldehyde and morphological changes were investigated by BDA and IHC to evaluate the axonal regeneration and synaptic formation.

Surgical procedures

Rats were deeply anaesthetized with 3.6% chloral hydrate (1 ml/100 g). A midline incision was made on the back and the muscles retracted. T_{10} vertebral lamina was removed, and the spinal dura mater incised. The spinal cord between T_{10}-T_{11} vertebral levels was then transected with a pair of micro scissors, and the incision was sutured. Procedures for sham-operation were similar to that of spinal cord transection except that the spinal cord was untouched. All operated animals were allowed to recover without the administration of any drugs and thereafter housed in individual cages. During the postoperative time period, the bladders of the animals were manually voided three times daily.

Electro-acupuncture

The acupoints of *Zusanli-Xuanzhong* and *Futu-Sanyinjiao* were selected, known to lie in L6 dermatome. *Zusanli* is located 5 mm caudal to the anterior end of the fibular head; *Xuanzhong*, 10 mm rostral to the anterior end of the lateral malleolus; *Futu*, 1.5 cm rostral to the lower end of the patella; and *Sanyinjiao*, 10 mm rostral to the posterior end of the medial malleolus. The acupoints were stimulated in pairs (*Zusanli* and *Xuanzhong*, or *Futu* and *Sanyingjiao*). And each pair was stimulated on alternate days at a frequency of 98 HZ for 30 minutes every day. The stimulating electrodes were changed reversedly after 15 min.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was used to determine the expression of NTFs and their receptors in each group. According to the manufacturer's instruction, total RNA was extracted from each sample (weighing 100 mg) with Trizol reagent (Molecular Research Center, Inc, Cincinnati, USA). The con-

Table 1. Primers for RT-PCR

Gene	Primers	Production (bp)	Annealing temparature (°C)
β-actin	Sense: 5'GTAAAGACCTCTATGCCAACA3' Antisense: 5'GGACTCATCGTACTCCTGCT3'	227	52.5
NGF	Sense: 5'AAGCCCACTGGACTAAACT3' Antisense: 5'ACCTCCTTGCCCTTGATG3'	370	51
BDNF	Sense: 5'TCCCTGGCTGACACTTTT3' Antisense: 5'ATTGGGTAGTTCGGCATT3'	466	50
NT-3	Sense: 5'CGTCCCTGGAAATAGTCATACGG3' Antisense: 5'GACAGATGCCAATTCATGTTCTT3'	857	54
PDGF	Sense: 5'CTGCTGCTACCTGCGTCTGG3' Antisense: 5'GCACTGCACATTGCGGTTATT3'	391	55
TGF-β1	Sense: 5'GTGAGCACTGAAGCGAAAGC3' Antisense: 5'TAATGGTGGACCGCAACAAC3'	332	54
CNTF	Sense: 5'CTTTCGCAGAGCAAACACCT3' Antisense: 5'CATCCCATCAGCCTCATTTT3'	422	52
IGF-1	Sense: 5'GGCACTCTGCTTGCTCACCTT3' Antisense: 5'GCCTGTGGGCTTGTTGAAGTAAAA3'	130	57
FGF-2	Sense: 5'TCCCAAGCGGCTCTACT3' Antisense: 5'ACTCCAGGCGTTCAAAGA3'	301	51
TrkA	Sense: 5'GCTGGGAGCAGGAGGATTT3' Antisense: 5'GATGCTGTTCCACGGCTTT3'	417	54
TrkB	Sense: 5'GGTTCTACAACGGAGCCATAC3' Antisense: 5'GTCTTCATAGAGGACTTCAGGGT3'	248	56
TrkC	Sense: 5'AAGCCCACCCACTACAACAAT3' Antisense: 5'AAAGAGGACCACCAGAAGGAC3'	252	54

Table 2. Probes of NTFs and receptor for *In situ* hybridization

NTFs	Probes	Length (bp)
NGF	5'GCTGTGATCAGAGTGTAGAACAACATGGAC3'	30
BDNF	5'CCCAATGAAGAAAACAATAAGGACGCA3'	27
NT-3	5'ATTACCAGAGCACCCTGCCCAAAGC3'	25
PDGF	5'CCACACCAGGAAGTTGGCATTG3'	22
TGF-β1	5'TAGATTGCGTTGTTGCGGTCCACCATTAGC3'	30
CNTF	5'TCTCTTGGAGTCGCTCTGCCTCAGTCATCT3'	30
IGF-1	5'AACGATCAGAGTAGTGGTATTTCACC3'	26
FGF-2	5'CGGGAAGGCGCCGCTGCCGCC3'	30
TrkA	5'CCTCCCACACGGTAATAGAT3'	20
TrkB	5'CTTGGCTATTAGTGAGTCCCCATTGTTCA3'	29
TrkC	5'CCTTGAGATGTCCGTGATGTTGATACTGGCGT3'	32

centration was measured by using a Nanodrop spectrophotometer (ND-1000, VanoDrop, Wilmington, USA). Reverse transcription was performed using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania, EU). This was followed by further PCR amplification of each cDNA sample using rat specific primers as shown in **Table 1**. The PCR reaction products were run on 1% agarose gels, and the size of the reaction products determined by ethidium bromide staining. β-actin mRNA PCR product was used as an internal control.

In situ hybridization

To determine the location of mRNA expression of NTFs, the spinal cords in the sham operated rats was harvested, and 20 μm thicknesses frozen sections were got in cryostat microtome (Leica CM-1900, Germany). In situ hybridization was performed at room temperature unless otherwise indicated. The sections were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.2, then further treated with 0.3% TritonX-100 solution for 10 min and proteinase K (5 μg/ml) at 37°C for 25 min. They were then re-fixed with 4% paraformaldehyde for 5 min, then immersed in 0.1 M PBS, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) to prevent non-specific binding of the probes. This was followed by washing in 2× SSC (pH 7.0), then prehybridized in a hybridization solution (50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 0.2 mg/ml Herring sperm DNA, and 10 mM dithiothreitol) without probes at 37°C for 2 hr before hybridization. They were then hybridized in 100 μl hybridization solution containing 1 μl probes of NTFs at 37°C for 12-16 hr in a moist chamber, followed by washing in decreasing concentrations of SSC, from 4× SSC (pH 7.0) at 37°C for 20 min, 2× SSC (pH 7.0) at 42°C for 20 min, 1× SSC (pH 7.0) at 48°C for 20 min and ending with 0.5× SSC (pH 7.0) at 50°C for 20

Table 3. Polyclonal antibodies of NTFs for IHC

Primary antibody	Dilution	Source	Company
NGF	1:1,00	Rabbit	Chemicom
BDNF	1:500	Rabbit	Santa
NT-3	1:1,000	Rabbit	Chemicom
PDGF	1:1,000	Rabbit	Chemicom
TGF-β1	1:20,000	Rabbit	Chemicom
CNTF	1:2000	Rabbit	Santa
IGF-1	1:200	Rabbit	Chemicom
FGF-2	1:100	Rabbit	Santa
TrkA	1:1000	Rabbit	Santa
TrkB	1:500	Rabbit	Santa
TrkC	1:800	Rabbit	Chemicom
GAP-43	1:10,000	Rabbit	Santa
Synaptophysin	1:500	Rabbit	Chemicom
5-HT	1:1000	Rabbit	RD
CGRP	1:500	Rabbit	Chemicom

min. Sections were incubated at 37°C in 1% blocking buffer (Roche) for 1 hr, and reacted in 1:1,000 sheep anti-digoxygenin-alkaline phosphatase (AP) antibody in 1% blocking buffer at 4°C overnight. The AP activity was detected using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Roche). The reaction productions were visualized with blue and purple sedimentation and observed with a light microscope (**Table 2**).

Immunohistochemistry

Sections in CSS were processed for immunohistochemistry as described previously [45]. They were subsequently incubated at 4°C overnight with 2% goat serum containing rabbit polyclonal antibodies, ascribed as **Table 3**. This was followed by incubation in Reagents I and II from the PV-9000 Reagent Kit (Chemicon, Anti-Rabbit/Mouse Poly-HRP IHC Detection Kit, USA). Finally, sections were detected by DAB staining. Negative control was performed by replacing the primary antibody with 2% goat serum to ascertain the specificity of antibody staining. Immunoreactive (IR) products were observed and photographed with a light microscope (Leica, DMIRB, Germany) coupled with a computer assisted video camera.

BDA tracing

Animals were anesthetized at 4 weeks postoperation, and biotinylated dextran amine (BDA: Molecular Probes: 10% wt/vol solution in sterile saline) was stereotaxically injected through craniotomy holes at depths of 1.5 mm below the cortical surface at 4 points distributed over the bilateral sensorimotor cortex (0.5 μl per injection). The animals were sacrificed after 2 weeks and prepared for histology staining. The spinal cord was removed and post-fixed for 1 day in 4% paraformaldehyde in 0.1 M PBS (pH 7.2). Transverse sections (30 μm) were made on the injured site and spinal segments rostral and caudal to the lesion. Some of the sections were processed by strep-avidin-cy3 secondary antibody to detect the possible presence of BDA-labeled regenerating corticospinal tract. The remaining sections were used for immunohistochemistry.

Functional evaluation

Hindlimb locomotor functions: Hindlimb locomotor function was evaluated using the Basso, Beattie, Bresnahan (BBB) rat rating scale [46]. Motor performance of each animal was evaluated during free movement in an open-field arena placed 90 cm above the ground to aid close observations of the rats. Assessments were performed at 1, 2, 3, and 4 weeks after injury. All the behavior evaluations were performed at 8-9 am. Bladder expression was done, before commencing the testing. Scoring was done by three investigators who were trained professionally and had no knowledge of the operative procedure and survival time. At last the scores were averaged.

Measurement of cortical somatosensory evoked potentials (CSEP)

The right common peroneal nerve was located based on surface landmarks and a stimulating electrode place over the nerve. A recording electrode was placed over the dura overlying the somatosensory cortex through a small craniotomy (2 mm left of the midline and 2 mm anterior to the posterior fontanele). The reference electrode was inserted on the nose, and the ground electrode inserted on the tail. CSEP was recorded using a keypoint instrument (Medtronic, Minnesota, USA). The stimulus intensity was set high enough to produce a marked muscle twitch in the hind limb (amplitude ~ 1.1 mA, duration of stimulation ~ 0.2 ms, and frequency ~ 3 Hz). The CSEP tracing represented the average of 200 responses.

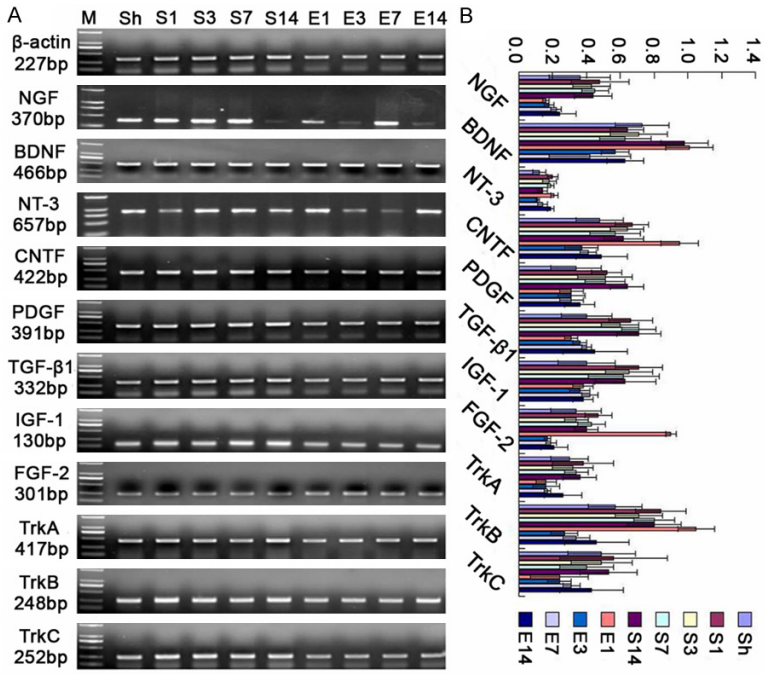

Figure 1. Gene expression changes in the CSS. A. mRNA level of NTFs and tyrosine kinase receptors in sham operated rats, SCT rats and EA rats, β-actin as the internal control. B. Quantitative analyses for above genes. M, marker; Sh, sham operated rats; S1, S3, S7 and S14: 1dpo, 3dpo, 7dpo and 14dpo of SCT rats. E1, E3, E7 and E14: 1dpo, 3dpo, 7dpo and 14dpo of EA rats.

Statistical analysis

Statistical analyses were performed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL., USA). The variables were investigated using visual (histograms, line graph) and analytical methods (Student's t-test for BBB score, One-way ANOVA and LSD-q test for other data). Continuous variables were expressed as mean ± standard deviation. The statistical significance was defined as $P<0.05$.

Results

Gene expression changes in the CSS

mRNA expression for NGF, BDNF, NT-3, PDGF, CNTF, TGF-β1, IGF-1, FGF, TrkA, TrkB and TrkC could be detected in the spinal cord of rats in three groups. Following SCT, it showed a significant increase in level of BDNF mRNA at 14dpo ($P<0.05$), FGF-2 mRNA at 1dpo ($P<0.05$), CNTF mRNA at 1 and 3dpo (P<0.05), PDGF and TGF-β1 mRNA at 1, 3, 7 and 14dpo ($P<0.05$), and TrkB mRNA at 1, 7 and 14dpo ($P<0.05$) in the CSS. There was no statistic significance in the level of the NGF, NT-3, IGF-1, TrkA and TrkC mRNA expression after lesion. Comparatively, in EA rats (receiving SCT and EA treatment), the results showed the most changes. Significant increases in level of CNTF, FGF-2 and TrkB mRNA at 1dpo ($P<0.05$), and a significant decrease in level of NGF, PDGF, TGF-β1 mRNA at 1, 3, 7 and 14dpo ($P<0.05$), BDNF mRNA at 14dpo ($P<0.05$), NT-3 mRNA at 3dpo ($P<0.05$), TrkA mRNA at 1 and 7dpo ($P<0.05$), TrkC mRNA at 1 and 3dpo ($P<0.05$) and IGF-I mRNA at 3 and 14dpo ($P<0.05$) were found in CSS of rats after EA treatment (**Figure 1**).

In situ hybridization

NGF, BDNF, NT-3, PDGF, TGF-β1, CNTF, IGF-1, FGF-2, TrkA, TrkB and TrkC mRNA were detected in cytoplasm of most neurons and part of glia cells in the spinal cord (**Figure 2A-L**).

Immunohistochemical findings

Positive staining for NGF, BDNF, NT-3, PDGF, TGF-β1, CNTF, IGF-1, FGF-2, TrkA, TrkB and TrkC were seen in spinal neurons (**Figure 3A-L**).

CGRP IR was found that fibers of spinal lamina I, II, IV, V in sham group (**Figure 5A**) were more than that of SCT rats in the CSS (**Figure 5B**). EA treatment increased further the number of CGRP IR in the CSS (**Figure 5C**).

GAP-43 and Synaptophysin IR were found in the ventral horn of the sham operated rats, respectively (**Figure 5D**, **5G**). There were a significant increase in the number of GAP-43 and Synaptophysin IR following SCT (**Figure 5E**, **5H**). Moreover, EA promoted intense GAP-43 and Synaptophysin IR in the CSS (**Figures 5F**, **5I**) compared in SCT rats.

Corticospinal tracts tracing

In rostral and caudal of injured spinal cord of sham rats, labeled corticospinal tracts were

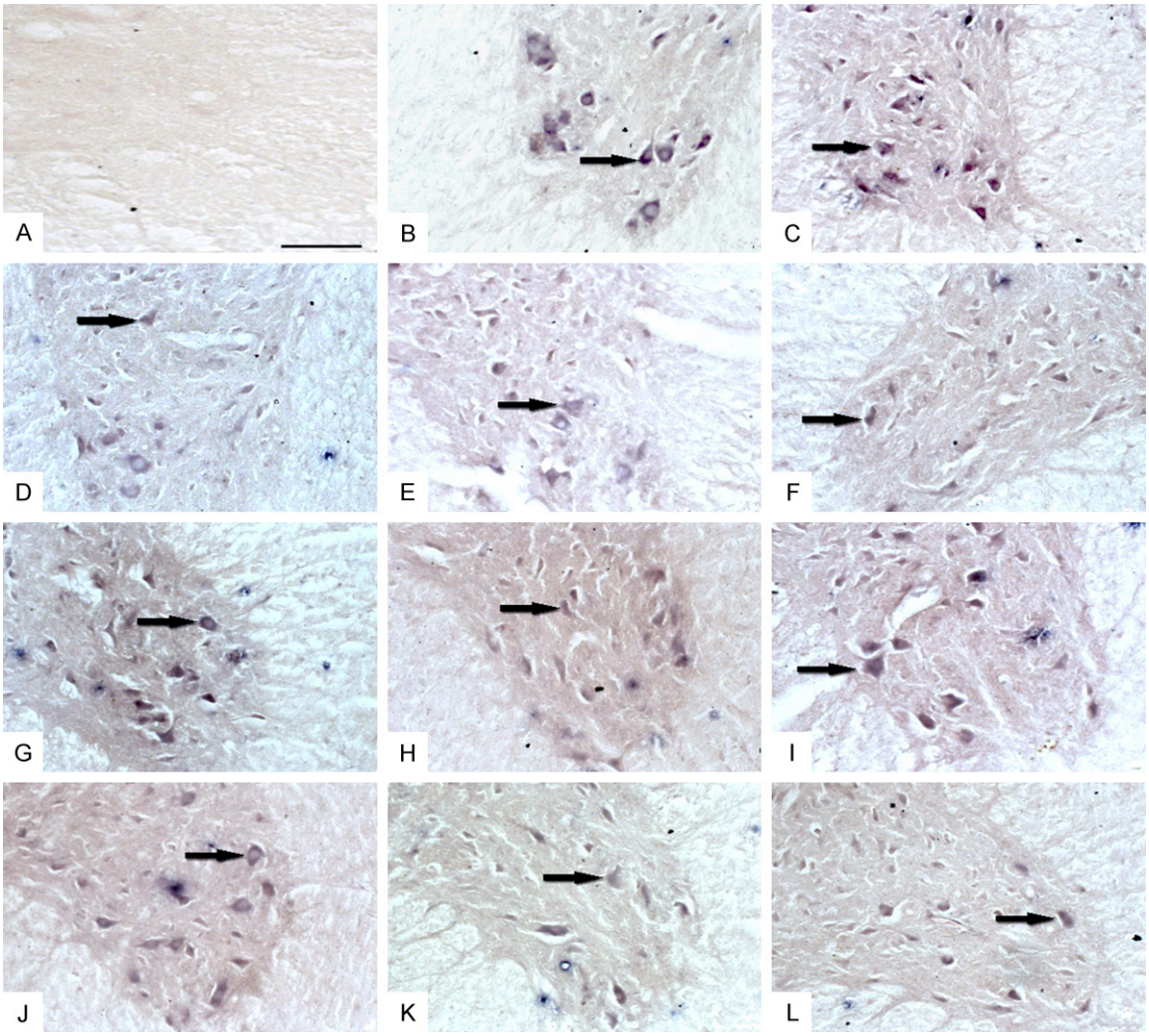

Figure 2. Location of mRNA for NTFs and tyrosine kinase receptors *in situ* hybridization. The arrows in (B-L) indicate the positive neurons or part of glia cells of NGF, BDNF, NT-3, PDGF, TGF-β1, CNTF, IGF-1, FGF-2, TrkA, TrkB and TrkC, respectively. Negative control was shown in (A) 200×, scale bar (shown in A) = 200 μm.

detected in the whole posterior funiculus, following injection of BDA into the sensorimotor cortex (**Figure 4A-D**). However, BDA labeled corticospinal tract only distributed in rostral but not caudal of injured spinal cord of both SCT and EA rats in the same regions (**Figure 4E**, **4F**).

Functional recordings

BBB evaluation in hindlimbs: The baseline BBB score of sham-operated rats was 21 which was much more than that of SCT rats. SCT rats showed flaccid paralysis of the hindlimbs in the first week after injury. And a gradual slight recovery of hindlimb locomotor functions was showed from 1-4 weeks after lesion. EA could effectively improve the locomotor functional recovery in hindlimbs, indicated by BBB scores. And the scores at 3 and 4 weeks after EA were significantly better ($P<0.05$) than those in the SCT rats (**Figure 6**).

CSEP

The mean latencies of P1 and N1 and the amplitude of P1-N1 of the acquired curves for all the rats in different time points are shown in **Table 4**. The latencies and the amplitude were in normal range before operation, but latencies of P1 and N1 were infinitely lengthened after SCT (no P1 and N1 were detected). However, waves of P1 and N1 could be recorded at 14, 21 and 28dpo in EA rats (bottom line), compared to SCT rats, although it is weaker than normal one (**Figure 7**).

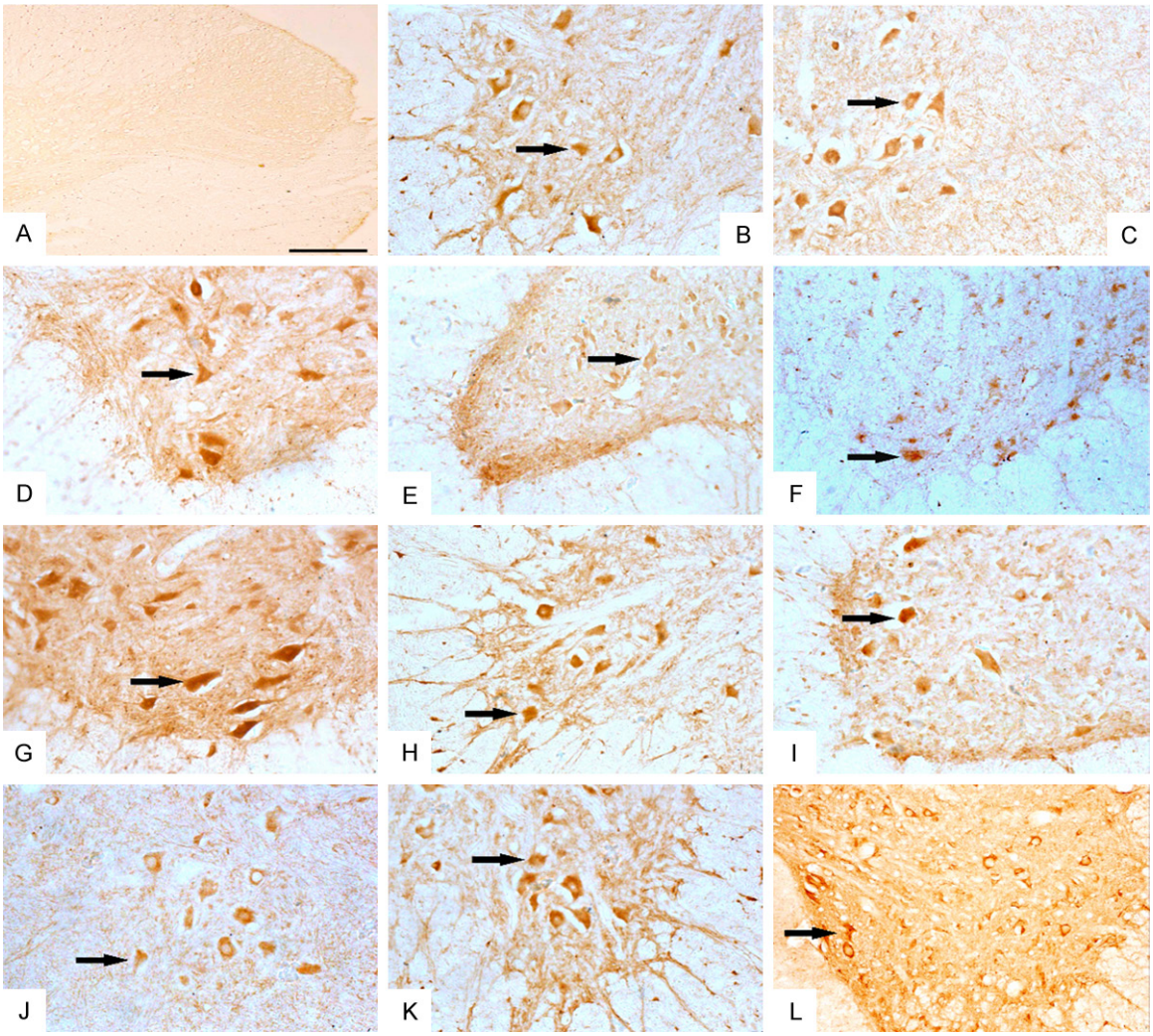

Figure 3. Positive immunostaining profiles for NTFs and tyrosine kinase receptors. Positive immunostaining profiles for NGF, BDNF, NT-3, PDGF, TGF-β1, CNTF, IGF-1, FGF-2, TrkA, TrkB and TrkC were showed in spinal motor neurons, respectively (B-L). The arrows indicate the positive motor neurons in spinal cord. Negative control was shown in (A). 200×, scale bar (shown in A) = 200 μm.

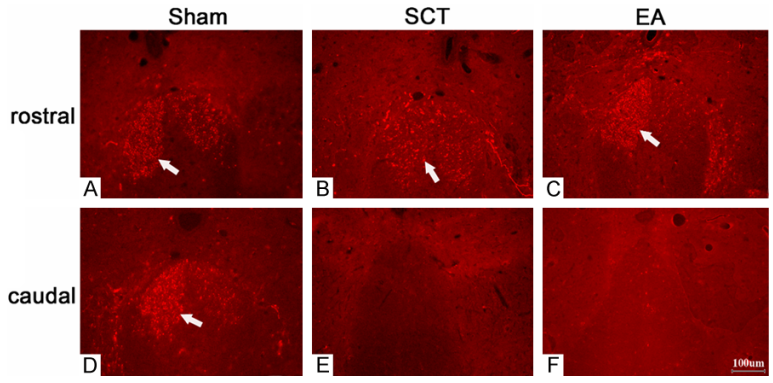

Figure 4. Distribution of Corticospinal tract of rostral and caudal to the lesion in different groups. BDA labeled corticospinal tracts were detected in the dorsal funiculus in the rostral to the lesion and sham operated rats in caudal spinal cord to the injury (A-D), but not in the CSS of SCT rats and EA rats (E, F). First line, second line represented the 200× magnification image of the rostral and caudal spinal cord to the injury, respectively. The BDA tracking in sham group, SCT group and EA group were showed in1st-3rd row. Scale bar (shown in F) = 100 μm.

Discussion

The present study aimed to determine the possible effects of EA on systematic regulation of multiple gene expressions in rats subjected to SCT. Changes in the expression of

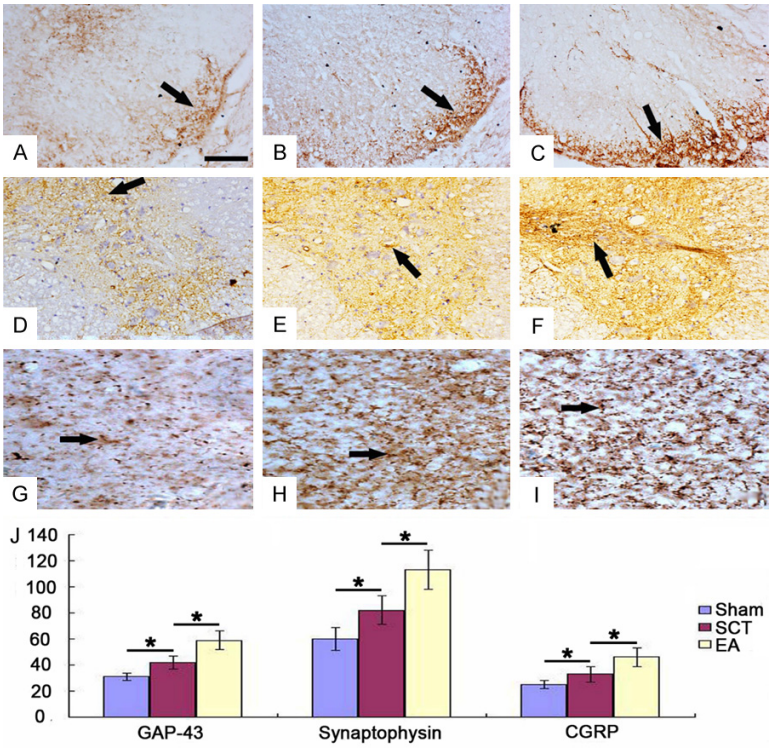

Figure 5. The changes of nerve fibers and synaptogenesis. CGRP IR was found in fibers of spinal lamina I, II in the sham operated rats (A), and they could be found in the CSS of SCT rats (B). EA increase significantly the number of CGRP IR in the CSS of transected cord rats (C). Both GAP-43 IR (D) and Synaptophysin IR (G) with weak staining were found in the gray matter of spinal cord of sham group, respectively. A significant increase in the number of GAP-43 (F) and Synaptophysin (I) IR were detected in the EA rats than the SCT rats (E, H), respectively. Quantitative analyses for the CGRP, GAP-43 and Synaptophysin IR were shown in (J). 200×, scale bar (shown in A) = 100 μm. **P*<0.05 vs. Sham group or SCT group.

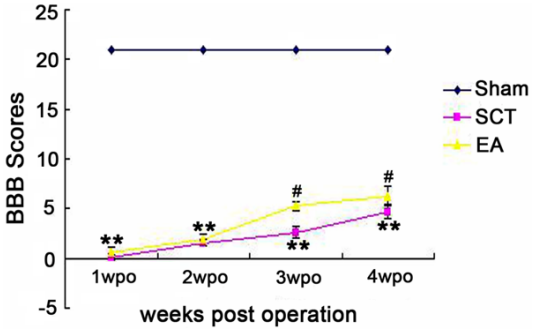

Figure 6. Evaluation of hindlimb locomotor functions. The baseline BBB score of sham-operated rats was 21. SCT rats showed flaccid paralysis of the hindlimbs in the first week after injury, and a gradual slight recovery of hindlimb locomotor functions from 2-4 weeks post operation was seen, while EA showed a significant improvement of BBB score compared with those in the SCT rats. ***P*<0.01 vs. sham group, #*P*<0.05 vs. SCT group.

NTFs and tyrosine kinase receptors were observed in the CSS. Parallel to the gene changes was the occurrence of partial nerve plasticity and recovery of functions.

Effect of EA on the NTF expressions

The study noted different changes in the expressions of NTFs and their receptors (TrkA, TrkB, TrkC) in the CSS. Some of the genes including CNTF, FGF-2 and TrkB were upregulated, while others like NGF, PDGF, TGF-β1, IGF-1, TrkA and TrkC were downregulated after EA treatment. These data showed that NTF and their receptors played different roles in the CSS of SCT rats with EA treatment. The present findings suggest that EA may induce neural plasticity through systematic upregulation of NTFs or their receptors. BDNF, combined with its functional receptor TrkB, showed the crucial role in inducing neurite outgrowth, synaptogenesis, and neuronal survival [47-49]. In addition, FGF-2 has been shown to promote the outgrowth of neurites from ventral spinal cord neurons [50] and induced by enriched environment enhanced motor function in chronic hypoxic-ischemic brain injury [51]. What's more, Furukawa S, et al. reported that FGF-2 may play roles in nerve regeneration in the injured spinal cord [52]. It's reported that CNTF was necessary for a sprouting of innervating motor axons [53]. N.M. Oyesiku, et al. also considered that CNTF was identified as a potent factor that induces growth of axons from sensory and motor neurons of the spinal cord [54]. These NTFs and their receptors could be important to the recovery of injured spinal cord [34, 54-56]. Our data provided the direct evidences to regulate genes map in injured spinal cord after EA. It is available to find new strategy by NTF synergistic administration for the treatment of SCI in the future clinic trial.

Table 4. The records of CSEP test (M ± SD)

Time point		P1 latency (ms)	N1 latency (ms)	P1-N1 amplitude (μv)
Sham operation		11.10±3.26	22.34±4.03	8.37±4.40
SCT	14dpo	-	-	---
	21dpo	-	-	---
	28dpo	-	-	---
EA	14dpo	54.40±14.10$^{\#}$	59.30±9.89$^{\#}$	5.37±0.58
	21dpo	25.5±10.06$^{\#,\&}$	28.00±7.06$^{\#,\&}$	5.37±0.58
	28dpo	22.17±5.37$^{\#,\&}$	26.33±4.41$^{\#,\&}$	6.06±0.32

Numbers refer to mean (M) ± standard deviation (SD). -, latencies of P1 and N1 infinitely lengthened. ---, no P1 and N1 amplitude. $^{\#}$P<0.05 compare to SCT 14dpo, SCT 21dpo, SCT 28dpo, respectively. $^{\&}$P<0.05 compared to EA 14dpo. EA, acupuncture. dpo, days post operation.

Significant decreases of NGF, PDGF, TGF-β1, IGF-1, TrkA and TrkC were detected in CSS after EA treatment. The implication of these changes keeps to be known. We analyzed that downregulated NTFs may function together with upregulated NTF like FGF-2, depending on systematic regulatory mechanism. In addition, PDGF and TGF-β1 could be induced by neuroinflammation [57], and their increase results in the proliferation of astrocytes, then leading to scar formation. It was therefore hypothesized that downregulation of PDGF and TGF-β1 after EA could be benefit to the recovery of injured spinal cord. Effect of EA on neuroplasticity might be result from the inhibition of neuroinflammation [58], which is associated to the systematic regulation of NTFs according to our findings.

In this study, we also found that EA treatment could increase the number of GAP-43, Synaptophysin and CGRP IR in CSS, whereas BDA-labeled corticospinal tract was not detected. CGRP has been well known as a marker for sensory axons transmitting pain sensation [59]. Increases in the number of CGRP positive fibers, GAP-43 and Synaptophysin IR (representing the regrowth of cone and synaptic formation) in the CSS suggests that nerve plasticity had occurred in the CSS by EA treatment, which may be available to reconstruct of local circuitry for further functional recovery [60-62].

After SCT, the rats showed flaccid paralysis of the hindlimbs immediately, suggesting that the SCT model was established successfully. Recovery of motor function was also found after EA treatment in SCT rats. The BBB scores in EA rats were higher than those in the SCT rats at 21 and 28dpo. These suggest that EA treatment may have a tendency to promote the recovery of hindlimb locomotor function after SCT. In addition, we found that the significant reduce N1 and P1 latencies at 2, 3 and 4 weeks post operation indicated partial recovery of sensory functions after EA, compared with those of SCT rats. The amplitude of P1-N1 has been detected again by CSEP recordings after EA treatment, even though they were still lower than normal levels. Of these, N1 and P1 recovered to near normal levels, whereas P1 was double that of the normal values at 28dpo. Hence, the results indicate significant improvement in sensory function has occurred in EA rats.

While endogenous NTFS expression has been launched after SCT, regeneration of corticospinal tracts was the result of multiple factors [63], coupled with the scar formation following injury; it was difficult to detect the labeled corticospinal tracts in CSS. In other words, the corticospinal tract could not pass through scars to re-establish local neural circuits after EA treatment for 28 days. Of course, the local neural circuits may be able to rebuild with longer EA treatment. As we did not find the regenerating corticospinal tracts in CSS at present, it is possible that there are some subcortical contributions to the functional recovery, by rearrangement of local spinal circuits and systematic regulation of NTFs in SCT rats subjected to EA treatment.

Conclusion

Our data showed that EA could cause differential expression of NTFs in the CSS, which could play a key role in neuroplasticity and functional recovery. It's suggested that EA may be a potential therapeutic strategy on SCI in the future clinic trial.

Disclosure of conflict of interest

None.

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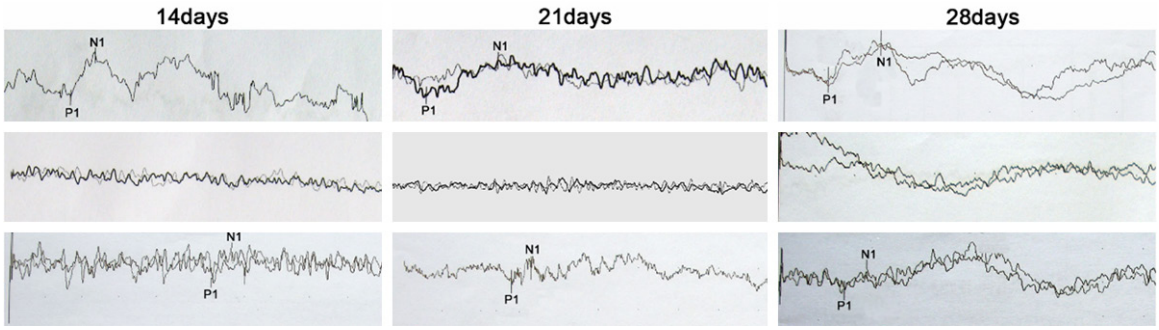

Figure 7. Evaluation of sensory function. The normal latencies of P1 and N1 could be recorded in the sham operated rats (above line), while the latencies of P1 and N1 were infinitely lengthened and they could not be recorded at 14, 21 and 28dpo after SCT (middle line). However, waves of P1 and N1 could be recorded at 14, 21 and 28dpo in EA rats (bottom line), compared to SCT rats, although it is weaker than normal one. Quantitative comparison on waves of P1 and N1 were shown in **Table 4**.

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