

Electroacupuncture Induced Spinal Plasticity is Linked to Multiple Gene Expressions in Dorsal Root Deafferented Rats

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Abstract The underlying mechanism for electroacupuncture (EA) associated functional improvement in patients suffering from spinal cord injury (SCI) is largely unknown. Collateral sprouting is one plausible factor, where the cord microenvironment may contribute greatly. The present study evaluated the effects of EA on collateral sprouting from spared dorsal root ganglion (DRG), sensory functional restorations, and differential gene expressions in spinal cord after partial DRG removal in the rat. Following EA, N1 waveform latencies for cortical somatosensory evoked potential significantly shortened. The densities of terminal sprouting from the spared DRG significantly increased on the EA versus the non-EA side. Microarray analysis revealed that several genes were upregulated on the acupunctured side at different time points; they were ciliary neurotrophic factor (CNTF) at 1 day post-operation (dpo), fibroblast growth factor (FGF)-1, insulin-like growth factor (IGF) 1 receptor, neuropeptide Y, and FGF-13 at 7 dpo, and CNTF and calcitonin gene-related polypeptide-alpha at 14 dpo, respectively. Meanwhile, five genes (CNTF, p75-like apoptosis-inducing death domain protein, IGF-1, transforming growth factor-beta 2, and FGF-4) were down-

regulated at 7 dpo. Furthermore, reverse transcriptase polymerase chain reaction results supported the gene chip analysis. It was concluded that the EA induced sensory functional restorations following partial DRG ganglionectomies could be brought about by intraspinal sprouting from the spared DRG, as well as multiple differential gene expressions in the spinal cord. The results could have clinical application in EA treatment of patients after spinal injury.

Keywords Electroacupuncture · Dorsal root ganglionectomies · Sprouting · Genes · Rat

Introduction

Despite the enormous efforts of researchers to seek functional restoration in patients with spinal cord injury, the results are dismal. This could be attributed to the failure of adult mammalian central neurons to regenerate their axons to reestablish functional connections after injury. The process is complicated by the complex mechanisms of plasticity following injury. Though the tasks to find effective ways to improve neurological functions after spinal cord injuries are arduous, the rewards of being able to see such patients live a normal life provide the motivation for a tireless search for the mechanism of neuroplasticity and for an effective therapy.

Of the numerous treatment strategies, electroacupuncture (EA) has been widely used and known for its benefits in the repair, reconstruction, synapse formation, and neural rehabilitation following neurological lesions (Turbes 1997; Horvat et al. 1997). Morphological studies showed that the removal of afferents from adjacent dorsal root ganglia (DRG) led to axonal sproutings from the sensory neurons in the spared DRG to innervate the denervated territory in the

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spinal dorsal horns (Liu and Chambers 1958; Rodin et al. 1982; Murray and Goldberger 1986). This is commonly known as spinal cord plasticity. Our electron microscopic investigations showed that EA could promote such plasticity, as it increased the numbers of synaptic terminals in both spinal lamina II and nucleus dorsalis after partial dorsal root ganglionectomies (Xiao et al. 1989; Wu and Xiao 1992). Other studies showed that spinal cord plasticity may be linked to the expression of cytokines, like some growth factors (Ou et al. 2001; Yun et al. 2002; Liang et al. 2002, 2003; Guo et al. 2004; Long et al. 2005; Wang et al. 2005, 2007; Liu et al. 2006), interleukin-6 (Chen et al. 2003), immediate early genes, *c-jun*, and *c-fos* (Lee and Beitz 1992; Guo et al. 1996a, b; Wang et al. 2006). However, the mechanisms of EA promoted spinal cord plasticity are largely unknown. To further understand this phenomenon, the present study applied EA treatment on the rat model of partial dorsal root ganglionectomies and studied (i) the recovery of sensory functions of the hindlimbs, (ii) intraspinal collateral sproutings, and (iii) the temporal expression of 60 selected genes.

Materials and Methods

Animals Preparation

Animals and Surgery

Forty young adult rats (weighing 280–320 g at the start of the experiments) provided by the Animal Experimental Center, Sichuan University, were used in this experiment. They were individually housed in a 12/12 h light/dark, quiet and nonstrong light vivarium with free access to food and water at least 3 days before the experiment. All experimental procedure related to the use and care of animals complied fully with the guidelines for the Care and Use of Laboratory Animals stipulated by the US National Institute of Health (1996). The rats were divided into five groups, as shown in Table 1. Every effort was made to reduce the number and suffering of the animals.

Bilateral dorsal root ganglionectomies were performed on each animal. They were anesthetized by intraperitoneal

injection of pentobarbital sodium solution (2%, 2.0 ml/kg body weight). For removal of DRG, hemilaminectomy was carried out at lumbar levels without opening the vertebral canal. In this procedure, the articular processes of the respective vertebrae were clipped off with a pair of strong rongeurs and the dura was cut with a pair of small scissors. The first to the fourth lumbar (L1–L4), and the sixth lumbar (L6) to the first sacral (S1) DRG were exposed and removed at their respective intervertebral foramina, leaving the L5 DRG intact. After surgery, the overlying skin and muscle were sutured, and proper postoperative care was given regularly.

EA Treatment

The left sides of all the operated rats received EA stimulation at acupoints Zusanli (St36), Xuanzhong (GB39), Futu (St32), and Sanyinjiao (Sp6). Their locations, as indicated in Fig. 1, are as follows: Zusanli is located 0.5 cm below the front of the fibula head; Xuanzhong, 1.0 cm above the front of the lateral malleolus; Futu, 1.5 cm above the lower end of the patella; and Sanyinjiao, 0.5 cm above the posterior end of the medial malleolus. The acupoints are known to lie in the dermatome of L5 and are similar to those described in our previous reports on cat (Wang et al. 2006, 2007). EA was administered regularly from the first day after injury. In this procedure, one pair of acupoints was stimulated at 98 pulses per minute for 15 min. The electrodes for the two points were interchanged and EA applied for another 15 min on the first day. The same procedure was applied to the other pair of acupoints on the second day. Subsequently, each pair of acupoints was stimulated on alternate days. Groups I, II, and III rats received EA treatment for 1, 7, and 14 days, respectively, for gene chip and reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Group IV and V rats received 90 days of EA stimulation and were used for recording the cortical somatosensory evoked potentials (CSEP), tracing the central pathways of the spared DRG, using horseradish peroxidase conjugated cholera toxin B subunit (CB-HRP; provided by Chinese Academy of Medical Sciences, Liang and Wan 1989).

Table 1 Grouping

Grouping	I	II	III	IV	V
Number of rats	8	8	8	8	8
Survival periods (dpo)	1	7	14	90	90
Disposition	Microarray and RT-PCR	Microarray and RT-PCR	Microarray and RT-PCR	CSEP recording	CB-HRP labeling

dpo Days postoperation

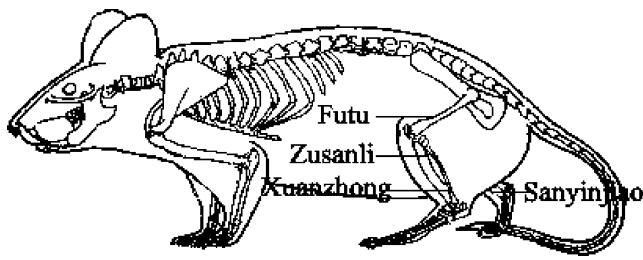


Figure 1 Drawing showing acupoints where EA was applied

CSEP

CSEP were recorded from bilateral electrical stimulation of the tibial nerve in eight rats (group IV). Recording was done in a warm and semidarkened room. Electrical pulses (0.2 ms duration) were delivered to the skin electrodes at the ankle and the stimulus intensity was adjusted slightly above the motor threshold. The reference electrode was placed at the lobe of the ear ipsilateral to the point of stimulation, as suggested previously (Tinazzi et al. 1997), and the ground electrode at the rostral extremity of the tail. The stimulation rate was 2.0 Hz, and CSEP were recorded in the P1–N1–P2 waveform.

CB-HRP Transganglionic Labeling and Histochemistry

After receiving 84 days of EA treatment, both the right and left sacrolumbar trunks in group V rats were exposed under deep anesthesia. A total of 10 μ l of 0.3% CB-HRP, which are known to label myelinated fibers, was injected slowly into the nerve trunk via a 10- μ l Hamilton syringe. The wound was closed and the rats left to recover with proper postoperative care. In the left period before sacrifice, the rats were still treated by EA stimulation as aforesaid.

Six days after CB-HRP injection, all the six rats were anesthetized as previously described and perfused via the left ventricle with 500 ml 0.1 mol/l phosphate buffered saline (pH 7.3) at room temperature, followed by 500 ml of 1.5% paraformaldehyde–1.25% glutaraldehyde in 0.1 mol/l phosphate buffered saline (pH 7.2–7.3, 4°C) for 30–40 min, and finally 100–200 ml of sucrosed phosphate buffer (pH 7.4). The L5 spinal segment in each rat was obtained and immersed in the 20% sucrosed phosphate buffer at 4°C till it sank to the bottom of the bottle.

The cord segment was then frozen, mounted, and sectioned transversely at a thickness of 30 μ m in a Leica CM900 freezing microtome. For an unbiased and accurate representation of the data, every five sections were taken. Sections were reacted with tetramethylbenzidine (Sigma, USA), dehydrated, cleared, and mounted. The density of the labeled terminals across the different laminae (I, III, IV, and V) was captured directly from the microscope, using a Leica DMIRB inversion fluorescence microscope. A box

(100 μ m²) was placed over the image at the superficial laminae of the dorsal horn in both sides of the same section to calculate the area. For the normalization, every photomicrograph (400 \times) of the same section was given a counting standardization. Then the mean numbers of the CB-HRP labeled central projective fibers in the spinal dorsal horn was calculated in every 10⁴ μ m².

Tissue Preparation

After receiving 1, 7, or 14 days of EA treatment, rats in groups I, II, and III were deeply anesthetized with pentobarbital sodium, as described earlier. Complete laminectomy of the entire vertebral column was rapidly performed, and the lumbar cord segments were immediately harvested and frozen in liquid nitrogen. Total RNAs were extracted using a TRIzol reagent (Invitrogen, USA) and following a procedure as previously described (Ikeda et al. 2002).

Microarray Analysis

As there are no available gene chips that can be used to analyze the gene expressing profile for neurite growth, we selected 60 genes which could be related to neuroplasticity according to literature review, as shown in Table 2. The microarray analysis (homemade 225 points array) was performed in CapitalBio Corp. (Beijing, China), and the details of the procedure have been described in previous studies (Guo et al. 2005; Patterson et al. 2006). Briefly, RNA was extracted from each cord sample individually using TRIzol reagent (Invitrogen, USA). An aliquot of 5 μ g total RNA was used to produce fluorescent dye (Cy5 and Cy3-dCTP)-labeled cDNA, and then hybridized to an array. Finally, arrays were scanned with a confocal LuxScanTM scanner (CapitalBio), and the data of obtained images were extracted with SpotData software (CapitalBio). Green (Cy3) represented the EA side samples and the red (Cy5) the non-EA side samples. The raw data were normalized using a space and intensity-dependant LOWESS program (Yang et al. 2002). For each test and control sample, two hybridizations were performed by using a reversal fluorescent strategy. Those genes whose alteration tendency kept consistent in both arrays and the mean expression ratios averaged above 1.5-fold were selected as differentially expressed genes. The description of this microarray study followed the Minimum Information About a Microarray Experiment guidelines (Brazma et al. 2001; Fig. 2).

RT-PCR Analysis

The concentrations of RNA samples were measured with a Nanodrop spectrophotometer (ND-1000). An equal amount of RNA (4 μ g) was used for each experiment. The 18S and

Table 2 The differential expression genes after EA stimulation at various time points

	Accession no.	Gene symbol	Clone description
1	M15481	<i>IGF-1</i>	<i>Insulin-like growth factor 1</i>
2	L29232	<i>IGF-1R</i>	<i>Insulin-like growth factor 1 receptor</i>
3	NM_031511	IGF-2	Insulin-like growth factor 2
4	NM_012756	IGF-2R	Insulin-like growth factor 2 receptor
5	NM_013166	<i>CNTF</i>	<i>Ciliary neurotrophic factor</i>
6	NM_001003929	CNTFR	Ciliary neurotrophic factor receptor
7	NM_012842	EGF	Epidermal growth factor
8	M37394	EGFR	Epidermal growth factor receptor
9	NM_019139	GDNF	Glial cell line derived neurotrophic factor
8	NM_012959	GFR α -1	Glial cell line derived neurotrophic factor receptor alpha-1
11	NM_012750	GFR α -2	Glial cell line derived neurotrophic factor receptor alpha-2
12	NM_023967	GFR α -4	Glial cell line derived neurotrophic factor receptor alpha-4
13	NM_012614	<i>NPY</i>	<i>Neuropeptide Y</i>
14	NM_017190	MAG	Myelin-associated glycoprotein
15	NM_017059	Bax	Bcl2-associated X protein
16	NM_016993	Bcl-2	B-cell leukemia/lymphoma-2
17	NM_017338	<i>CGRP-α</i>	<i>Calcitonin gene-related polypeptide-alpha</i>
18	M11596	<i>CGRP-β</i>	<i>Calcitonin gene-related polypeptide-beta</i>
19	NM_017195	GAP-43	Growth associated protein-43
20	NM_012671	TGF- α	Transforming growth factor-alpha
21	NM_021578	TGF- β 1	Transforming growth factor-beta 1
22	NM_031131	<i>TGF-β2</i>	<i>Transforming growth factor-beta 2</i>
23	NM_013174	TGF- β 3	Transforming growth factor-beta 3
24	NM_012775	TGF- β R1	Transforming growth factor-beta receptor 1
25	NM_031132	TGF- β R2	Transforming growth factor-beta receptor 2
26	NM_017256	TGF- β R3	Transforming growth factor-beta receptor 3
27	NM_012846	<i>FGF-1</i>	<i>Fibroblast growth factor-1</i>
28	NM_019305	FGF-2	Fibroblast growth factor-2
29	NM_130817	FGF-3	Fibroblast growth factor-3
30	AB079673	<i>FGF-4</i>	<i>Fibroblast growth factor-4</i>
31	NM_022211	FGF-5	Fibroblast growth factor-5
32	NM_131908	FGF-6	Fibroblast growth factor-6
33	NM_022182	FGF-7	Fibroblast growth factor-7
34	NM_133286	FGF-8	Fibroblast growth factor-8
35	D14839	FGF-9	Fibroblast growth factor-9
36	NM_012951	FGF-10	Fibroblast growth factor-10
37	NM_130816	FGF-11	Fibroblast growth factor-11
38	AF348446	FGF-12	Fibroblast growth factor-12
39	AF271786	<i>FGF-13</i>	<i>Fibroblast growth factor-13</i>
40	NM_022223	FGF-14	Fibroblast growth factor-14
41	NM_130753	FGF-15	Fibroblast growth factor-15
42	NM_021867	FGF-16	Fibroblast growth factor-16
43	NM_019198	FGF-17	Fibroblast growth factor-17
44	NM_019199	FGF-18	Fibroblast growth factor-18
45	NM_023961	FGF-20	Fibroblast growth factor-20
46	NM_130752	FGF-21	Fibroblast growth factor-21
47	NM_130751	FGF-22	Fibroblast growth factor-22
48	NM_130754	FGF-23	Fibroblast growth factor-23
49	NM_024146	FGFR1	Fibroblast growth factor receptor 1
50	L19104	FGFR2	Fibroblast growth factor receptor 2
51	AF277717	FGFR3	Fibroblast growth factor receptor 3
52	BC085772	FGFR4	Fibroblast growth factor receptor 4
53	NM_139259	<i>p75(PLAIDD)</i>	<i>p75-like apoptosis-inducing death domain protein</i>
54	X67108	BDNF	Brain derived neurotrophic factor
55	NM_031073	NT-3	Neurotrophin-3 (HDNF/NT-3)
56	NM_021589	Trk1	Trk precursor

Table 2 (continued)

	Accession no.	Gene symbol	Clone description
57	NM_012731	Trk2	Neural receptor protein-tyrosine kinase (trkB)
58	NM_019248	Trk3	Neural receptor protein-tyrosine kinase
59	AY028417	Netrin-1	Netrin-1
60	XM_343867	Netrin-3	Netrin-3

The table showed the checked genes of the 60 selected genes, and the italic genes were the ones which showed a significant up- or downregulated differential changes at various time points. That is, the mRNA value of EA/non-EA side were higher than 1.5 or lower than 1.5^{-1}
EA Electroacupuncture, *dpo* days post operation

28S ribosomal peaks were used to quantify RNA. RT-PCR was performed and β -actin was used as an internal control to ascertain changes of gene level in the gene chip analysis. Table 3 shows the primer sequences. For RNA amplification, the first strand cDNA synthesis was prepared from 4 μ g of total RNA, using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, USA). PCR was then carried out using the PCR Master Mix Kit (Fermentas, USA) for 35 cycles, consisting of denaturation at 94°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min. Gene primers were synthesized by TaKaRa Company (Japan). RT-PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide, and visualized using an ultraviolet gel imager (BIO-RAD, USA). The optical density (OD) of each product band, including the objective gene and β -actin was obtained, and the OD ratio between investigated genes and β -actin were calculated to semiquantify the objective gene level.

Statistical Analysis

SPSS 11.0 was used for statistical analysis. The results were expressed as mean \pm standard error of mean. Statistical significance ($p < 0.05$) of the microarray data was determined by the *t*-test. We accepted only those mRNA values when their value of EA/non-EA change was beyond 1.5 for upregulated genes or under 1.5^{-1} for downregulated genes.

One-way analysis of variance analysis was used, followed by the Fisher post hoc test for other data analysis.

Results

CSEP at Different Time Points

The mean latencies of the N1 wave components of the acquired curves for all rats at different time points are shown in Table 4. N1 wave latencies were in normal range before operation but were infinitely lengthened at 1 day postoperation (dpo; Fig. 3). There was significant difference in the values recorded from stimulations of the right and left hindlimbs ($p > 0.05$). Comparatively, following EA treatment, an obvious reduction ($p < 0.01$) at 7, 14, 21, 28, and 90 dpo on the EA side was noted, compared with that on the non-EA side. Although N1 wave was not recorded at 7 and 14 dpo on the non-EA side, it could be noted to exhibit a long latency at 21 dpo, which became shorter at 90 dpo, but did not return to the normal level (Fig. 3).

CB-HRP Labeling

CB-HRP labeled fibers appeared in laminae I, III, IV, and V of L5 cord segment but were notably absent in lamina II (Fig. 4). The numbers of labeled fibers on the EA side

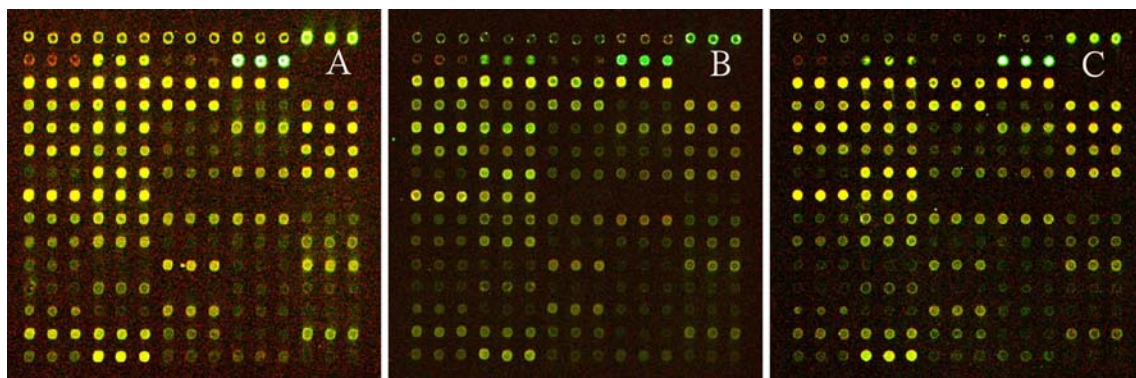


Figure 2 Microarray images of the different groups (a group I, b II, and c III) in the same array. Green (Cy3) represented the EA side samples and the red (Cy5) the non-EA side samples

Table 3 Primer names, sequences, and PCR conditions used for RT-PCR analysis

Gene	NCB nucleotide database mRNA	Primer sequence	Renaturation (°C)	Product size (bp)
β -actin	NM_131144	5' GTAAAGACCTCTATGCCAACA 3' 5' GGAATCATCGTACTCCTGCT 3'	52.5	500
CGRP	M34090	5' GTCACATAACCAGCAGGAGG 3' 5' ATTGATGGAAGTGGGAGGAT 3'	51	386
CNTF	NM_013166	5' AGAACCTCCAGGCTTACC 3' 5' CATCCATTTATTATACCACCC 3'	50	490
FGF-1	NM_012846	5' AGGGATGTGACAGTGAACG 3' 5' CAGCCAATGGTCAAGGGA 3'	51.5	644
FGF-4	NM_053809	5'TGCCTTTCTTTACCGACGAGTGT 3' 5' TCTTGGTCCGCCCGTTCTTAC 3'	51.5	124
FGF-13	AF271786	5' AGACGAGGACAGCACTTACA 3' 5' CAGACCCAGATAACCACCC 3'	52.5	242
GDNF	NM_019139	5' AAGATGAAGTTATGGGATGTCG 3' 5' AGTTCCTCCTTGGTTTCGTAG 3'	53	482
GFR α -1	U59486	5' TGATGTCCGCCGAGGTG 3' 5' TCAGGCTGAAGTTGGTTTCC 3'	52.5	141
IGF-1	X06043	5' TGGTGGACGCTCTTCAGTT 3' 5' GCAGGTGTTCCGATGTTTT 3'	50	376
IGF-1R	NM_052807	5' TCCGTGGCTGGAACTCTT 3' 5' TTGTTGATGGTGGTCTTCTCAC 3'	54	290
NPY	NM_012614	5' TGTGGACTGACCCTCGCTCTA 3' 5' GGGACAGGCAGACTGGTTTC 3'	50.5	350
TGF- β 2	NM_031131	5' CCCTCCGAACTGTCTGC 3' 5' GGCTCTTATTTGGGATGATGT 3'	54	478
P75	NM_012610	5' GACCAAGCGTGAGGAGGTAGA 3' 5' CAGCAACGGAGTCAGGAGTG 3'	53	421

were significantly more than those on the non-EA side (Table 5).

Identification of Total RNA

For measurement of the total RNA from each spinal cord sample, stringent quality controls were employed with formaldehyde denaturing gel electrophoresis (Fig. 5). All the bands were clear with the brightness ratio of 28S/18S ≥ 1 , the required purity quotient of RNA.

Microarray Analysis

Of the 60 genes selected and spotted on the 225 points array, ten were found to present differential expressions. Of these ten genes, five of them [fibroblast growth factor (FGF)-1, insulin-like growth factor 1 receptor (IGF-1R), neuropeptide Y (NPY), calcitonin gene-related polypeptide-alpha (CGRP- α), FGF-13] were upregulated by 1.5 times, and four of them [p75-like apoptosis-inducing death domain protein (P75), IGF-1, transforming growth factor-

Table 4 Showed NI CSEP latency in rats subjected to partial rhizotomy following EA (M \pm SEM)

Time point	Number of rats	Non-electroacupunctured side (ms)	Electroacupunctured side (ms)
Preoperation	8	14.32 \pm 0.39	13.82 \pm 0.45
1 dpo	8	–	–
7 dpo	8	–	24.07 \pm 0.42*
14 dpo	8	–	19.70 \pm 0.28*
21 dpo	8	24.40 \pm 0.75	17.00 \pm 0.46*
28 dpo	8	23.60 \pm 0.73	16.37 \pm 0.31*
90 dpo	8	17.74 \pm 0.58	14.95 \pm 0.22*

Numbers refer to mean latency (M) \pm standard error of mean (SEM)

* $p < 0.01$ compared with non-EA side

EA Electroacupuncture, dpo days postoperation

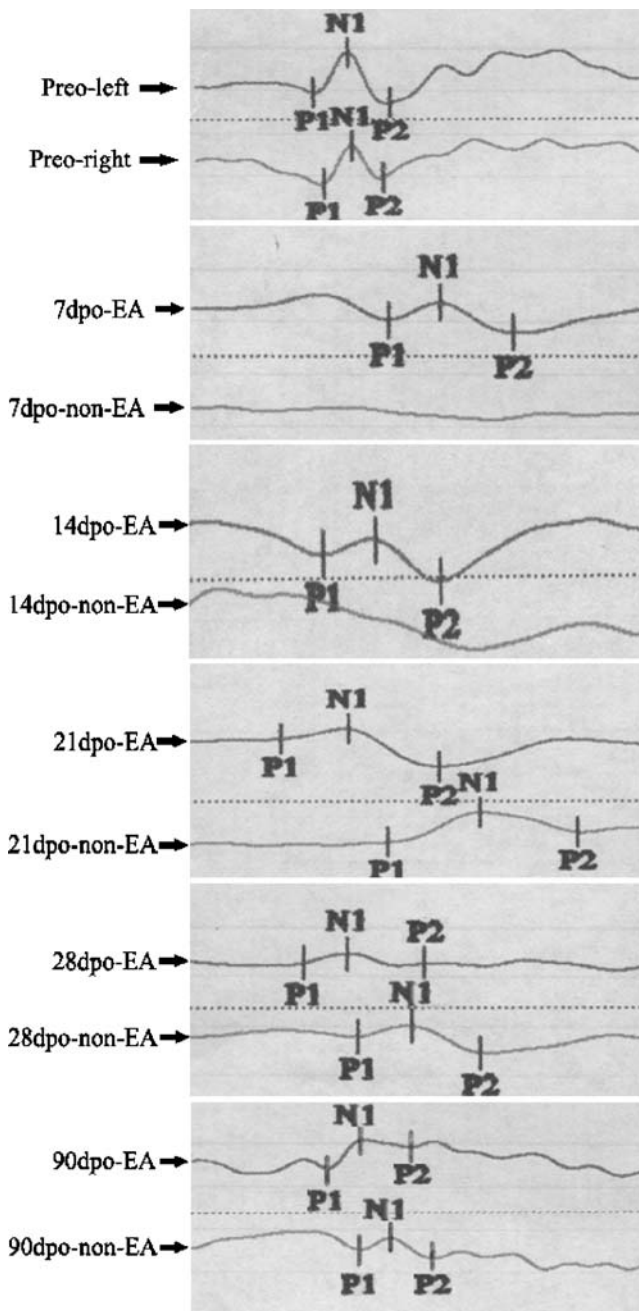


Figure 3 Tibial nerve CSEP at different time points. *preo* Preoperation, *dpo* days postoperation, *EA* electroacupuncture

beta 2 (TGF- β 2), FGF-4], downregulated by 1.5–1 times in the ratio of EA/non-EA side. One of them, known as ciliary neurotrophic factor (CNTF), was upregulated at 1 and 14 dpo but downregulated at 7 dpo (Table 6). In detail, only one upregulated gene (CNTF) showed a ratio of 1.92 at 1 dpo and two upregulated genes, the ratios of 1.68 (CNTF) and 1.54 (CGRP- α), at 14 dpo. However, at 7 dpo, the expressions of four genes (IGF-1R, NPY, FGF-1, FGF-13) were upregulated by 1.5 times while five genes (TGF- β 2,

FGF-4, IGF-1, P75, and CNTF) were downregulated by 1.5⁻¹ times. These representative data showed that more genes displayed differential expressions after 7 days of EA stimulation, compared with those of the other two time points.

RT-PCR Analysis

Consistent with the results of microarray analysis, among the ten genes, five of them (FGF-13, IGF-1R, FGF-1, CGRP, NPY) were upregulated ($p < 0.05$) and four of them (P75, IGF-1, TGF- β 2, FGF-4) were downregulated ($p < 0.05$) after EA at all time points (Fig. 6a, b; Table 7). Only CNTF gene was upregulated at 1 dpo, while both CNTF and CGRP were upregulated at 14 dpo (Fig. 6a, c; Table 7). However, at 7 dpo, the expressions of four genes (IGF-1R, NPY, FGF-1, FGF-13) were increased ($p < 0.05$), while those of another five genes (TGF- β 2, FGF-4, IGF-1, P75, and CNTF) were downregulated.

Discussion

The present study presented some evidences to show that EA could promote the sensory functions in rats subjected to partial dorsal root ganglionectomies. Intrasproutings of the spared DRG occurred earlier and were more intense when compared with the non-EA side. Moreover, acupuncture also affected the expressions of some genes.

Recovery of Sensory Functions

After partial dorsal root deafferentation, significant changes occurred in the rat CSEP recording. At 1 dpo, the CSEP on both sides disappeared, indicating interruption of the ascending sensory pathways (Nuwer 1990). However, N1 latencies on the EA side could be detected again at 7 dpo, while those on the non-EA side were not detected till 21 dpo, howbeit at a value lower than normal value. The earlier return of sensory function after EA could be clue to a facilitation of synaptic reorganization in the local circuitry, brought about by an increase in synaptic terminal numbers (Xiao et al. 1989; Wu and Xiao 1992; Dong et al. 1994) and increased intraspinal sproutings of the spared DRG, as demonstrated in this study by the use of CB-HRP, a tracer known for its transganglionic transport property.

There was significant increase ($p < 0.05$) in the number of intraspinal axons and terminals of the L5 DRG on the EA side at 90 dpo, compared to an earlier period. At the same time, the latency of N1 was much shorter ($p < 0.05$), providing an interesting and important correlation between intraspinal axonal sproutings and restoration of sensory function.

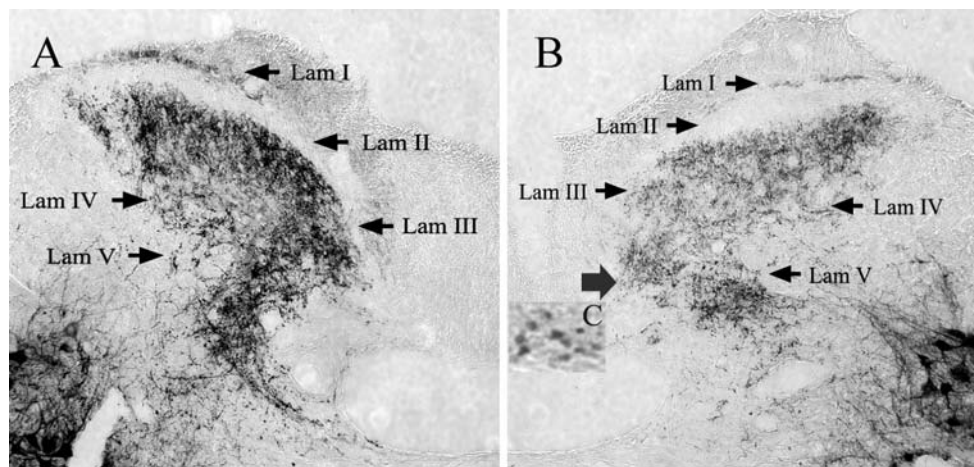


Figure 4 CB-HRP labeled fibers in the L5 cord segment following 90 days of EA treatment. **a** The labeled fibers on the EA side ($\times 100$) and **b** labeled fibers on the non-EA side ($\times 100$). **C** is the magnification ($\times 400$) of the small area indicated by *fat arrow*, including ten labeled fibers, and was considered as a counting standardization in this section. *Lam* Lamina

Changes in Gene Expressions Revealed by Microarrays and RT-PCR Analysis

CNTF

CNTF mRNA and protein had been localized to glial cells in the mammalian peripheral and the central nervous system (CNS; Sendtner et al. 1994). In contradistinction to an earlier report of upregulation of CNTF expression only in astrocytes after spinal cord injury (Oyesiku et al. 1997; Lee et al. 1998), the present study demonstrated different levels of CNTF expressions at different periods after acupuncture in DRG deafferented rats. After EA stimulation in the deafferented rats, CNTF was upregulated at 1 dpo, then downregulated at 7 dpo, and upregulated again at 14 dpo. Though the mechanisms are unknown, the results indicate strongly that CNTF gene and its dynamic state of expression might play a crucial role in the EA promoted spinal plasticity in the DRG deafferented rats.

IGF-1 and IGF-1R

Previous work showed that IGF-1 played a role in the CNS response to injury and was involved in CNS development as

Table 5 The density of CB-HRP labeled central projective fibers in the spinal dorsal horn following 90 days EA treatment (M \pm SEM)

Detected index	Non-EA side	EA-side
Density (/10 ⁴ μ m ²)	1563 \pm 87	2184 \pm 95*

Numbers refer to mean numbers (M) \pm standard error of mean (SEM)

* $p < 0.05$ compared with non-EA side

EA Electroacupuncture

well as growth of projection neurons, dendritic arborization, and synaptogenesis (Yamaguchi et al. 1990; Cheng and Mattson 1992; Sizonenko et al. 2001; Hung et al. 2007). IGF-1R controlled a wide variety of cellular functions. It was essential for normal growth, development and differentiation, and mediated signals for the suppression of apoptosis and promotion of mitogenesis (Romano 2003). As a tyrosine kinase receptor, IGF-1R was activated by the binding of its ligand (IGF-1) to the extracellular domain, which in turn caused the autophosphorylation necessary to send a potent mitogenic signal to the cell nucleus.

In the present study, the upregulation of IGF-1R gene indicated its ligand-IGF might be a very important factor for the recovery of injured spinal cord. The expression of IGF-1 was downregulated on the EA treated side in situ, suggesting an inhibitory role of EA in IGF expression.

Table 6 The changes of differential genes expression following EA at appropriate time points

Clone description	Ratio value (1 dpo)	Ratio value (7 dpo)	Ratio value (14 dpo)
CNTF	$\uparrow 1.9100$	$\downarrow 0.6100$	$\uparrow 1.6822$
FGF-13	–	$\uparrow 2.5218$	–
IGF-1R	–	$\uparrow 1.8050$	–
FGF-1	–	$\uparrow 1.6833$	–
CGRP- α	–	–	$\uparrow 1.5400$
NPY	–	$\uparrow 1.5290$	–
FGF-4	–	$\downarrow 0.5861$	–
TGF- $\beta 2$	–	$\downarrow 0.5420$	–
IGF-1	–	$\downarrow 0.4908$	–
P75	–	$\downarrow 0.4599$	–

$\uparrow \downarrow$ —The mRNA value of EA/non-EA side were higher than 1.5 or lower than 1.5⁻¹

EA Electroacupuncture, *dpo* days postoperation

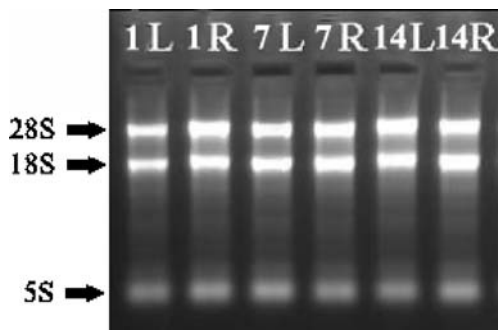


Figure 5 Results of formaldehyde denaturing gel electrophoresis at 1, 7, and 14 dpo on both sides of the spinal cord

Previous report demonstrated an increased expression of IGF-1 after spinal cord injury (Hammarberg et al. 1998). We had also shown that EA could cause a significant increase in IGF-1 expression in the spared DRG after adjacent dorsal root ganglionectomies (Liu et al. 2006). Therefore, the anterograde transport of IGF-1 from the spared dorsal root ganglia or the retrograde transport from spinal nerves might occur after EA stimulation, in order to compensate for the reduction of intrinsic IGF-1 in the spinal cord. If such be the case, the increased IGF-1R may provide the additional sites for the transported IGF. One cannot, of course, rule out the possibility that the increased IGF-1R sites could provide some sites for the attachment of some yet unknown factors.

FGF-1, 13, and 4

In the spinal cord of the DRG deafferented rat, the gene expression of FGF-1 and 13 were upregulated, while that of FGF-4 was downregulated on the EA side versus the non-EA side. This indicated the involvement of the FGF family in the spinal cord plasticity and that these three family members exhibited distinct cellular, temporal, and spatial expression maps.

FGFs mediate multiple developmental signals in vertebrates and are known as cell mitogens and differentiating factors with neuroprotective properties in the CNS (Johnston et al. 1996; Hossain et al. 1998). FGF-1, 13, and 4 are three important factors in the FGF family. The presence of FGF-1 immunoreactivity in dorsal column fibers and the anterograde transport of this factor in the ascending sensory fibers had been reported by Koshinaga et al. (1993). Niswander et al. (1993) had also demonstrated the expression of FGF-4 in limb bud structures that mediated limb bud outgrowth and patterning during the developing stage. Lastly, FGF-13 was known to be involved in the development and functional maintenance of the central and peripheral nervous systems (Hartung et al. 1997). All the three factors prevented the onset of both apoptotic and necrotic death in neurons otherwise “destined to die” following myocardial or cerebral hypoxic–ischemic injury. This could be attributed to the enhancing

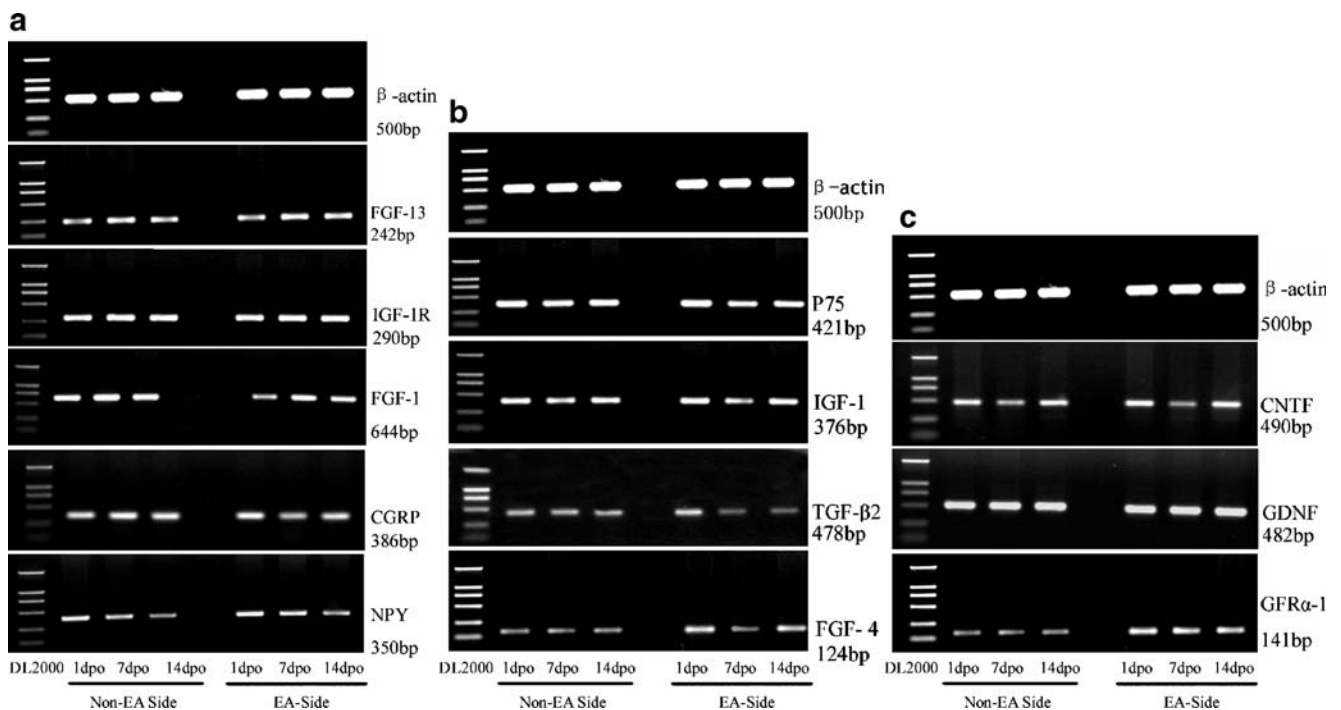


Figure 6 Similar to the results of microarray analysis, of the 12 genes, the expressions of five genes (a FGF-13, IGF-1R, FGF-1, CGRP, NPY) were upregulated ($p < 0.05$) and four genes (b P75, IGF-1, TGF- β 2, FGF-4) were downregulated ($p < 0.05$) in the ratio of EA/

non-EA at all the time points, while the CNTF gene c showed significant upregulation only at certain time points. There was no significant difference in the GDNF and its receptor GFR (6c) gene expressions

Table 7 The RT-PCR results of expression changes of the 12 genes in different time points (M±SEM)

Genes	Non-EA side			EA side		
	1 dpo	7 dpo	14 dpo	1 dpo	7 dpo	14 dpo
CNTF	1.00±0.31	0.95±0.53	1.02±0.43	1.51±0.25*	0.72±0.64**	1.40±0.33***
FGF-13	0.99±0.34	0.94±0.23	1.00±0.42	1.03±0.44	1.63±0.35**	1.10±0.54
IGF-1R	0.90±0.53	1.03±0.24	1.11±0.35	0.98±0.21	1.46±0.42**	1.12±0.24
FGF-1	1.09±0.21	1.02±0.31	0.99±0.25	1.00±0.19	1.57±0.40**	1.18±0.20
CGRP- α	1.01±0.32	1.03±0.24	0.97±0.33	1.04±0.29	1.62±0.45**	1.42±0.30***
NPY	0.97±0.33	1.00±0.26	0.98±0.46	1.01±0.27	1.58±0.47**	1.03±0.42
P75	0.98±0.42	1.04±0.46	0.99±0.34	1.02±0.36	0.62±0.24**	1.10±0.36
IGF-1	0.99±0.37	0.98±0.35	0.96±0.42	0.99±0.42	0.53±0.46**	1.12±0.41
TGF- β 2	1.02±0.28	1.00±0.31	1.01±0.29	1.07±0.26	0.54±0.34**	1.00±0.23
FGF-4	1.01±0.42	0.99±0.26	0.98±0.33	1.02±0.24	0.46±0.28**	0.99±0.43
GFR α -1	0.98±0.25	0.94±0.27	0.97±0.23	0.99±0.30	0.98±0.23	1.02±0.23
GDNF	1.00±0.34	1.03±0.12	0.99±0.36	1.04±0.10	1.03±0.44	1.05±0.15

Numbers refer to mean numbers (M)±standard error of mean (SEM)

* p <0.05 (compared with the non-EA side at 1 dpo); ** p <0.05 (compared with the non-EA side at 7 dpo); *** p <0.05 (compared with the non-EA side at 14 dpo)

EA Electroacupuncture, dpo days postoperation

of regional blood flow, amelioration of neurological deficits, intervention at the level of caspase-signaling cascades, and the restoration of the anti-apoptotic protein expression in central neurons (Russell et al. 2006; Fukuyama et al. 2007).

CGRP and NPY

In the present study, CGRP expression was upregulated in the spinal cord following EA stimulation at 14 dpo, accompanied by the recovery of motor and sensory functions. This suggested that the functional restoration could be attributed to an increase in CGRP. The increased CGRP might participate in both the primary afferent terminal sprouting and the plasticity of neuromuscular synapses. CGRP has been demonstrated to play a potential role in the pain-related synaptic plasticity in spinal dorsal horn neurons both in vivo (Bird et al. 2006) and in vitro (Fields et al. 1991). Increased intraspinal sprouting of CGRP containing primary afferent terminals had also been shown in the deafferented rat spinal cord (McNeill et al. 1991). Also, CGRP enhanced the release of native brain-derived neurotrophic factor from the trigeminal ganglion neurons (Buldyrev et al. 2006). It existed in motor nerve terminals and had an important effect on the sprouting of motor nerve terminals (Tsujimoto and Kuno 1988). In this study, changes in rat neuronal CGRP might be correlated with neuromuscular synapse remodeling and plasticity of axonal growth following EA stimulation (Tarabal et al. 1996).

NPY is a 36-amino-acid peptide that belongs to the pancreatic polypeptide family of structurally related peptides. Its synthesis by spinal neurons and vast distribution in the CNS had been reported (Lacroix et al. 1990), including

the DRG neurons (Wakisaka et al. 1992; Noguchi et al. 1993, Abdulla and Smith 1999). NPY could be released by discharging neurons (Emson and De Quidt 1984; Everitt et al. 1984) and played a role in regulating the transmission of nociceptive input by DRG neurons, particularly after peripheral nerve injury (Duggan et al. 1991; Ohara et al. 1994; Mark et al. 1998; Abdulla and Smith 1999). In this connection, exogenous NT-3 could downregulate the NPY expression in primary sensory neurons and mitigate the transganglionic NPY response following peripheral nerve injury (Ohara et al. 1995; Sterne et al. 1998). EA treatment after injury could increase the NT-3 expression level in the DRG where NPY receptors existed in some sensory neurons (Mantyh et al. 1994), but not in the spinal cord (Wang et al. 2007). It would then appear that much more NPY was associated with DRG in the normal status. EA treatment could possibly cause the retrograde transport of NPY from the spared DRG to the spinal cord, to bring about the improvement of sensory function.

p75NTR

The expression of p75 neurotrophin receptor (p75NTR) gene was downregulated in the spinal cord following EA treatment. p75NTR is mainly expressed during early neuronal development for controlling the survival and process formation of neurons. In the adult, p75(NTR) is reexpressed in various pathological conditions, including epilepsy, axotomy, and neurodegeneration. In fact, increased expression of neurotrophins usually accompanies the upregulation of p75NTR in many pathological conditions (Zhou et al. 2005). The increased neurotrophins may result in neurodegeneration in these conditions via activa-

tion of p75NTR. After sciatic nerve transection, apoptosis was prevented when fewer neurons expressed p75NTR (van Eden and Rinkens 1994; Zhou et al. 2005). Thus, suppression of p75NTR by neutralization or downregulation of its expression may prevent neurodegeneration in these pathological conditions. In the present study, EA induced decrease of p75 might contribute to the reduced nociception or injury-induced apoptosis.

TGF- β 2

TGF- β 2 expression was downregulated after EA stimulation. TGF- β , synthesized by neurons or glial cells, is widespread in the CNS. Its role may contribute to neuronal differentiation, neurotransmitter synthesis, synapse formation, neurite sprouting, and elongation of neurons (Ishihara et al. 1994; Kriegstein and Unsicker 1996; Kriegstein et al. 1998). Neutralization of endogenous TGF- β resulted in the protection of 50% of the spinal motoneurons that would otherwise die in the presence of TGF- β , unraveling TGF- β as a neuron survival antagonizing factor in vivo (Unsicker and Kriegstein 2002). This study showed that EA could downregulate the expression of TGF- β and may therefore contribute to the inhibition of apoptosis.

Cytokine Genes Showing No Change in their Expressions after EA Treatment

GDNF and GFR α

GFR α , the glycosylphosphatidylinositol-linked coreceptor for glial cell derived neurotrophic factor (GDNF), is the receptor component of GDNF associated with and activator of the tyrosine kinase receptor Ret. The role of GDNF as a potent trophic factor in promoting the growth and survival of spinal neurons has been reported (Henderson et al. 1994; Gouin et al. 1996; Watabe et al. 2000; Blesch and Tuszynski 2003). Both the protein and mRNA for GDNF and GFR α -1 in the DRG, as well as GDNF protein in the spinal dorsal horn, was significantly enhanced by EA treatment following peripheral nerve injury (Dong et al. 2005; Wang et al. 2005). The present results, however, could not demonstrate any EA-induced differential expression of GDNF and GFR α -1 genes in the spinal cord. It was possible that the GDNF synthesized in the spinal cord was transported to other regions (Coulpier and Ibanez 2004) while the GDNF synthesized in the DRG was anterogradely transported to the spinal cord after EA treatment.

Other Genes

The absence of differential expression of some other selected NTF genes, such as BDNF, NT-3, FGF-2, indicated that the

activity of these genes in the spinal cord was not affected by EA treatment. This was consistent with our previous report of lack of changes in the expression of these four factors in DRG deafferented cat spinal cord following EA stimulation (Wang et al. 2005, 2007).

Additionally, other selected cytokine genes, including netrin 1 and 3, Bcl2-associated X protein and B-cell leukemia/lymphoma-2, growth-associated protein-43, myelin-associated glycoprotein, etc. did not present significant differentiation after EA treatment. It was possible that these endogenous cytokines were not involved in the process of spinal cord plasticity.

To summarize, our study presented strong evidences that the EA induced changes in some gene expressions may be linked to the recovery of sensory functions. Microarray analysis are a useful tool to unravel the differential expression of genes and to determine their roles in collateral sproutings and functional restoration.

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