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Electro-acupuncture induced NGF, BDNF and NT-3 expression in spared L6 dorsal root ganglion in cats subjected to removal of adjacent ganglia

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Abstract

This study evaluated the effect of electro-acupuncture (EA) on the NGF, BDNF and NT-3 expression in spared L6 dorsal root ganglion (DRG) in cats subjected to bilateral removal of L1-L5 and L7-S2 DRG, using immunostaining, *in situ* hybridization and RT-PCR. The positive products of NGF, NT-3 protein and mRNA in the small and large neurons of spared L6 DRG in EA side increased greatly more than that of control side, while the increased BDNF was only noted in small and medium-sized neurons. RT-PCR demonstrated that the mRNA level for three factors was not influenced by EA in intact DRG, when a significant increase was seen in the spared L6 DRG of EA side. As it has been well known that DRG neurons project to the spinal cord wherein morphological plasticity has been present after DRG removal, the present results might have some bearing to the observed phenomenon.

Keywords: NGF; BDNF; NT-3; Dorsal root ganglion; EA; Spinal cord plasticity

1. Introduction

Neuroplasticity in the central nervous system (CNS) was firstly demonstrated by Liu and Chambers (1958) who proposed that after surgically removing a series of dorsal root ganglia (DRG), axonal sprouting from the central processes of the spared ganglion occurred in Lamina II. This pioneering work prompted numerous researchers to look into the mechanism on the axonal regeneration and synaptic reorganization of neurons following traumatic lesions in the mammalian spinal cord (Leong and Lund, 1973; Guth, 1974; Steward, 1989; He, 1994; Mendell et al., 2001; Siddall and Loeser, 2001; Wolpaw and Tennissen, 2001).

Arising from some recent related work is the significant finding that failure of neurons to spontaneously regenerate after injury in the adult CNS might be attributed to the nonpermissive nature of the CNS environment (Aubert et al., 1995), like the lack of growth-promoting molecules (Varon and Conner, 1994) and the presence of inhibitory molecules (Fitch and Silver, 1997). Moreover, providing a growth supportive environment by the administration of neurotrophic factors (NTFs) (Lindsay et al., 1994) has been partially successful in inducing axonal regeneration within the adult mammalian CNS.

Though a good number of studies have investigated the roles of neurotrophic factors in preventing neuronal death or promoting anatomical reorganization after spinal cord injury (Huang and Reichardt, 2001; Kim et al., 2001; Liu et al., 2002), the involvement of endogenous neurotrophic factors in the dynamic modulation of local circuitry remains to be elucidated. Acupuncture, an ancient craft originating in China more than 3000 years ago, has been shown to promote functional recovery in spinal cord injury (Li et al., 1985; He, 1994).

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Electro-acupuncture (EA) is similar to the traditional acupuncture but where in EA, electrical stimuli are delivered instead of manual twist stimuli in the traditional acupuncture. It has been known that EA can increase the number of axonal terminal derived from spared DRG, indicating the effect of EA in meliorating synaptic reconstruction. Previous researches had demonstrated a correlation between the increase of some neurotrophic factors like nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5) and the development, survival and maintenance of neuronal function in both the peripheral and the central nervous system (Isackson, 1995; McAllister et al., 1999; Sendtner et al., 2000; Huang and Reichardt, 2001), in addition endogenous neurotrophic factors may be involved in neurite-outgrowth enhancement induced by dorsal root ganglionectomy (Xue et al., 1994). Moreover, our recent studies also determined some other neurochemicals expression in DRG after EA (Wang et al., 2005a,b, 2006). This experiment was therefore undertaken to observe the possible effects of EA in the expression of NGF, BDNF and NT-3 and their respective mRNA in the spared DRG neurons of cats after adjacent DRG removal. It is hoped that the results derived from immunohistochemistry, *in situ* hybridization and RT-PCR can provide some evidences for EA promoting the spinal cord plasticity, involved in the NGF, BDNF and NT-3 expressions.

2. Materials and methods

2.1. Animals

Twenty adult male cats, weighing 3–3.5 kg for each animal, were used in this study. The animals were provided by the Laboratory Animal Center of Kunming Medical College. Every effort was taken to reduce the number of animals and suffering during the experiments. Group I, consisting of 15 cats were used for immunohistochemistry, *in situ* hybridization and RT-PCR for each five, respectively. Animals Group II ($n = 5$) was also used to detect the mRNA expression changes in the normal cats after EA by RT-PCR. The animals were maintained under conditions of controlled light, temperature, food and water.

2.2. Surgical procedures

All experimental procedures were performed under anesthesia achieved with intraperitoneal injection of 3.5% sodium pentobarbital (1.3 ml/kg body weight). The DRG of cats in Group I associated with the first through the fifth lumbar (L1-L5) and the seventh lumbar through the second sacral (L7-S2) spinal nerves were first bilaterally removed, sparing the L6 DRG, then followed immediately with EA on the left side at acupuncture points. Group II were used as normal cats, and performed by EA as group I.

2.3. EA

On the day after surgery, all the cats were subjected immediately to unilateral electrical stimulation at acupuncture points (Fig. 1) (*xuewei*) *zusanli-xuanzhong*, and *futu-sanyinjiao*, both of which are known to lie in L6 dermatome (Xue et al., 1994). *Zusanli* (ST36) is located 1.5 cm below the front of the fibula head; *xuanzhong* (GB39), 1.5 cm above the front of the lateral malleolus; *futu* (ST32), 2–3 cm above the lower end of the patella; and *sanyinjiao* (SP6), 1.5 cm above the posterior end of the medial malleolus. The pairs of acupoints were stimulated alternately at the frequency of 98 HZ for 30 min every day. During acupuncture, the electrodes were changed every 15 min. EA is similar to traditional acupuncture except that electrical stimuli are delivered instead of manual stimuli in the traditional method.

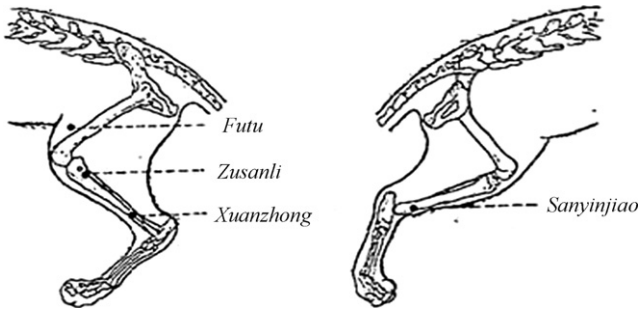

Fig. 1. Drawing showing *xuewei* is where EA was applied.

2.4. Immunohistochemistry

The cats were perfused under anesthesia on day 7 after EA. Their L6 DRG on both sides were removed and immersed in a 4% paraformaldehyde solution for 12 h, then placed in a 20% sucrosed phosphate buffered solution (PBS) over night. Serial longitudinal sections of the frozen DRG were sectioned into 20 μm slices. For an unbiased and accurate representation of the data, 5 sections were taken randomly, representing all the samples in one animal. The selected sections were processed for immunohistochemical demonstration of NGF, BDNF and NT-3, using specific NGF (rabbit anti-human 1:100), BDNF (rabbit anti-human 1:500) and NT-3 (rabbit anti-human 1:1500) antibodies using ABC (Avidin-Biotin Complex) method. Then the sections were stained by DAB (3,3′-diaminobenzidine). All antibodies, bought from Chemicon, have been identified by the Western-Blot method in our lab and shown to possess specificity for their own antigens. In addition, control experiments in which PBS was substituted for the primary antibody were performed to ascertain specificity of antibody staining.

2.5. In situ hybridization

The use of double-stranded cDNA as molecular probes for *in situ* hybridization to chromosomal preparations has been effective for localizing the position of particular genes like those of NGF, BDNF and NT-3. Slide-mounted DRG sections were warmed to room temperature (25 °C), postfixed in 4% paraformaldehyde (pH 7.4) for 5 min at 4 °C, rinsed in PBS, and treated with 0.25% acetic anhydride (in 0.1 M triethanoLaminae, pH 8.0) for 10 min at room temperature. After rinsing 2× in standard saline citrate (SSC) for 10 min and dehydration through a graded series of alcohols, the sections were delipidated in chloroform for 5 min at room temperature and subsequently re-hydrated to 95% ethanol in descending concentrations of alcohols and then air-dried. They were next washed in Tris (trihydroxymethyl aminomethane) at pH 9.4 and NaCl:$MgCl_2$ (20:1) for 2 min, then color-developed in Tris, pH 9.4 and NaCl:$MgCl_2$ (20:1) in the presence of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-2-indolyl phosphate (BCIP) for 5 h. The sections were finally rinsed several times and mounted on glass slides with Aqua Poly/Mount (Polysciences; Warrington, PA).

Controls for *in situ* hybridization: Preincubation with RNAase was used to assess the nonspecific binding of the probes to the section. Moreover, to demonstrate the validity of the probes of three neurotrophic factors, the cRNA probes were substituted with double distilled water without RNAase or the unmarked probes. No positive reaction was observed in both control experiments.

2.6. Cell number counting

The distributions of the immunopositive neurons for NGF, BDNF, NT-3 and their respective mRNA in different sized DRG neurons on the acupunctured and non-acupunctured side were observed and photographed in an Olympus light microscope at 40×. In order to calculate the average number of immunoreactive cells, photographs of DRG were scanned and projected on the screen of a computer. Then the areas of the DRG and the number of positive neurons were respectively measured using several manufactured square grids (0.8 cm per square grid). Therefore the average number in the area designated as 100 grids was acquired so as to compare the number of positive neurons in equal areas in different sections. The average numbers of labeled neurons were recorded in Tables 1–3.