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Changes of expression of glial cell line-derived neurotrophic factor and its receptor in dorsal root ganglions and spinal dorsal horn during electroacupuncture treatment in neuropathic pain rats

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Abstract

Injury to the nervous system occasionally leads to intense and persistent neuropathic pain, which is resistant to conventional analgesic methods. It was reported that electroacupuncture (EA) had potent analgesic effect on neuropathic pain by activating various endogenous transmitters such as the opioid peptides. Glial cell line-derived neurotrophic factor (GDNF) has been hypothesized to play an important role in modulation of nociceptive signals especially during neuropathic pain state. Using immunohistochemistry, Western blot, and RT-PCR analysis techniques, the present study observed the effects of EA on the expression of GDNF and GDNF family receptor α-1 (GFRα-1, the high-affinity receptor of GDNF) in neuropathic pain rats. The results showed that both protein and mRNA levels of GDNF and GFRα-1 in the dorsal root ganglions (DRG), as well as GDNF protein in the spinal dorsal horn, were significantly increased after chronic constriction injury (CCI) of the rats' sciatic nerve and could be further enhanced by EA treatment. The present data demonstrated that EA could activate endogenous GDNF and GFRα-1 system of neuropathic pain rats and this might underlie the effectiveness of EA in the treatment of neuropathic pain.

Keywords: GDNF; GFRα-1; Electroacupuncture; Neuropathic pain

GDNF was discovered as a potent neurotrophic factor for midbrain dopaminergic neurons by Lin et al. [13], and was shown to be a member of transforming growth factor-β (TGF-β) superfamily. The biological action of GDNF is mediated by a two-component receptor complex consisting of a glycosylphosphatidylinositol-linked cell surface molecule, the GDNF family receptor GFRα-1 (originally named GDNFR-α), which acts as a ligand-binding domain and the receptor protein tyrosine kinase Ret, which acts as the signal transducing domain. GDNF is thought to bind preferentially to GFRα-1 and GDNF fails to exert its biological effect in the absence of GFRα-1. Besides its potent survival-promoting effects on diverse groups of neurons, GDNF has been proved by previous studies to play an important role in modulation of nociceptive signals especially during neuropathic pain state [1,3,6,19].

Neuropathic pain arises as a debilitating consequence of injury to the nervous system, which is characterized by combination of spontaneous burning pain, hyperalgesia and allodynia. Such pain is often intense and refractory to conventional analgesic therapy. EA has long been used to relieve pain. In clinic, EA is an established adjuvant analgesic modality for the treatment of chronic pain. It has been shown that EA had potent analgesic effect in neuropathic pain patients and rat models [4,9,11], and it was also well known that EA analgesia was mediated by endogenous opioids and other bioactive substances in the nervous system. However, the mechanism of EA analgesia on neuropathic pain has not been fully understood, since EA has comprehensive modulating effects on the nervous system. It was reported that EA could enhance the expression of GDNF in the rat's brain that had been lesioned by medial forebrain bundle transection, indicating

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the involvement of GDNF in the therapeutic effect of EA on Parkinson's disease [12]. However, the possible effects of EA on the expression of GDNF and its high-affinity receptor GFRα-1 in neuropathic pain rats are unknown. Therefore, the present study was aimed to investigate changes of expression of GDNF and GFRα-1 in DRG and the spinal dorsal horn after EA was administered on CCI-induced neuropathic pain rats.

Experiments were performed on adult male Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College of Fudan University, China) weighing 200–220 g. Prior to experimental manipulation, rats were allowed to acclimate for 1 week and maintained on a 12-h light:12-h dark cycle with free access to food and water. All rats in the study were used strictly in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals in order to minimize the number of animals used and their suffering.

The hyperalgesic state was induced by CCI of the sciatic nerve with four loose ligatures as previously described [2]. Briefly, under isoflurane anesthesia, the left sciatic nerve was exposed at the level of middle of the thigh by blunt dissection through biceps femoris and four 4-0 chronic gut sutures were each tied loosely with a square knot around the sciatic nerve. In every animal, an identical dissection was performed on the right side, except that the sciatic nerve was not ligated. All animals postoperatively displayed normal feeding and drinking.

According to our previous study [14], 'Huan-Tiao' and 'Yang-Ling-Quan' acupoints were selected during EA treatment. A pair of stainless steel needles of 0.3 mm diameter was inserted with a depth of 5 mm into the unilateral acupuncture points 'Huan-Tiao' (GB-30, located near the hip joint, on the inferior borders of muscle gluteus maximus and muscle piriformis; the inferior gluteal cutaneous nerve, the inferior nerve; deeper, the sciatic nerve) and 'Yang-Ling-Quan' (GB-34, located near the knee joint, anterior and inferior to the small head of the fibula, in muscle peroneus longus and brevis, where the common peroneal nerve bifurcates into the superficial and deep peroneal nerves). The two needles were connected with the output terminals of an electroacupuncture apparatus (Model G 6805-2, Shanghai Medical Electronic Apparatus Company, China). Alternating strings of dense-sparse frequencies (60 Hz for 1.05 s and 2 Hz for 2.85 s alternately) were selected. The intensity was adjusted to induce slight twitch of the hindlimb ($\leq$1 mA), with the intensity lasting for 30 min. EA was administered once every other day from the seventh day after CCI surgery until the end of the experiment.

The changes of expression of GDNF and GFRα-1 were examined by Western blot, reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemisty analysis. The time points of analysis were selected as 2, 3, and 4 weeks after CCI surgery, corresponding to 1, 2, and 3 weeks of EA treatment, respectively. At each time point, six animals of each group were used for every analysis.

Western blot analysis was performed according to the following protocol. Rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4–L6 DRGs were collected in dry ice and stored at −70 °C until assayed. Each assay sample consisted of the pooled unilateral L4–L6 DRGs from one rat. Each sample was weighed and homogenized in 1.5 ml of sample buffer (0.01 M Tris–HCl buffer (pH 7.6) containing 0.25 M sucrose, 0.1 M NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonylfluoride) at 4 °C. Supernatant after 12,000 rpm centrifugation for 10 min was used for Western blotting. Samples (30 μg of total protein) were dissolved with equal volume of loading buffer (0.1 M Tris–HCl buffer (pH 6.8) containing 0.2 M DTT, 4% SDS, 20% glycerol and 0.1% bromophenol blue), separated on 10% SDS–PAGE and then electrotransferred at 100 V to Immun-Blot PVDF membrane for 1 h at 4 °C. Membranes were blocked in TBST containing 5% non-fat dried milk overnight at 4 °C before incubation for 2 h at room temperature with anti-GDNF polyclonal antibody (1:1000, Santa Cluz Inc., USA) or anti-GFRα-1 polyclonal antibody (1:1000, Sigma, USA) diluted in TBST containing 5% BSA. Blots were washed extensively in TBST and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) in TBST/1.25% BSA for 1 h at room temperature. The signal was detected by an enhanced chemiluminescence method (ECL kit, Amersham), and exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). The intensity of the selected bands was captured and analyzed using GeneSnap Image Analysis Software (Syngene, UK).

Reverse transcription-polymerase chain reaction was performed to detect the mRNA level of GDNF and GFRa-1. Rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4–L6 DRGs were collected in dry ice. Total RNA extraction was performed using the trizol reagent, following the instructions of the manufacturer. RNA was further purified using the RNeasy kit according to the RNA clean-up protocol, and eluted in 50 μl of RNase-free distilled H_2O. The amount of RNA was measured spectrophotometrically. 1 μg of total RNA was used for the synthesis of the first strand of cDNA using the SuperScript × reverse transcriptase. Briefly, RNA, oligo (dT)18 primers (0.5 μg/μl) were first denatured for 5 min at 65 °C, chilled on ice for 1 min, and then incubated for 50 min at 42 °C, 15 min at 70 °C in 20 μl of a reaction mixture containing 10× first-strand buffer, 10 mM dNTP mix, 0.1 M DTT and 50 units of SuperScript II reverse transcriptase. The sequences of primers were as follows: GDNF forward: 5-TTTGTCGTACATTGTCTCGG-3 41–61, reverse: 5-GACTCTAAGATGAAGTTATGG-3 504–523 (L15305); GFRa-1 forward: 5-ATTGGCACAGTCATGACTCCCAAC-3 1178–1201, reverse: 5-GAGGAGCAGCCATTGATTTTGTGG-3 1599–1622 (U59486) [17]; β-actin forward: 5-CACCATGTACCCTGGCATTG-3, reverse: 5-TAACGCAACTAAGTCATAGT-3. The primers were synthesized and purified by Shanghai Institute of Biochemistry, Chinese Academy of Science. One microliter of cDNA