Effects of electroacupuncture on expression of somatostatin and preprosomatostatin mRNA in dorsal root ganglions and spinal dorsal horn in neuropathic pain rats

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Abstract

Somatostatin (SOM) is an endogenous non-opioid neuropeptide that has analgesic effect in rodents and human beings. Previous studies indicated that SOM might be involved in the modulating effects of electroacupuncture (EA). Using immunohistochemistry and RT-PCR, the present study observed the effects of EA on the expression of SOM peptide and preprosomatostatin (ppSOM) mRNA in a rat model of neuropathic pain induced by chronic constriction injury (CCI) to the sciatic nerve. No significant change was detected in the expression of SOM and ppSOM mRNA following CCI. However, EA could significantly enhance SOM expression in dorsal root ganglion (DRG) and spinal dorsal horn as well as ppSOM mRNA level in DRG of neuropathic pain rats. The present data demonstrated that EA could activate endogenous SOM of neuropathic pain rats and this might be one of the mechanisms that underlie the effectiveness of EA in the treatment of neuropathic pain.

Keywords: Somatostatin; Preprosomatostatin; Electroacupuncture; Neuropathic pain

Somatostatin (SOM) was first isolated from the ovine hypothalamus on the basis of its ability to inhibit growth hormone (GH) release from rat pituitary cells in culture [3]. SOM exhibits a widespread distribution throughout the central nervous system (CNS) where it acts as a neurotransmitter and neuromodulator. In vivo, SOM is synthesized as preprosomatostatin (ppSOM), a precursor peptide that is cleaved to release active SOM. Previous studies suggested that SOM might play a role in pain modulation. In DRG, a subgroup of nociceptive sensory neurons expresses SOM. These neurons extend their unmyelinated axons centrally to lamina II of the spinal cord [1]. SOM is released from these unmyelinated axons in the spinal cord following noxious stimulation of peripheral nerves and produces an inhibitory effect on nociceptive neurons [18,20]. The analgesic effect of somatostatin in various types of pain has been reported both in animal and human studies [4,6,17].

Peripheral neuropathic pain, resulting from nerve injury due to trauma or disease, is one of the most difficult challenges in pain management. EA has long been used to relieve pain. In clinic, EA is an established adjuvant analgesic modality for the treatment of chronic pain. Previous studies indicated that EA had potent analgesic effect on neuropathic pain both in patients and in rat models [7,12,14], and it was well known that EA analgesia was mediated by endogenous opioids and other bioactive substances in the nervous system [11,22]. However, the mechanism of EA analgesia on neuropathic pain has not been fully understood, since EA has comprehensive modulating effects on the nervous system. Previous studies suggested that SOM might be involved in the modulating effects of EA such as the inhibitive effect of EA on meal-stimulated acid secretion [13] and the therapeutic effect of EA on acute ischemic cerebrovascular diseases [23]. It has also been reported that SOM in nucleus raphe magnus (NRM) might be involved in EA analgesia [15] and microinjection of SOM into NRM [15] or intracerebroventricular (icv) injection of SOM [24] could enhance EA analgesia. However,
it is unclear whether SOM is involved in EA analgesia on neuropathic pain. Therefore, the present study was aimed to investigate the effects of EA on the expression of SOM and ppSOM mRNA in dorsal root ganglions and spinal dorsal horn in 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 12:12 h light–dark cycle with free access to food and water. All rats in the study were used strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals in order to minimize the number of animals used and their suffering.

To observe the effect of EA on thermal hyperalgesia of neuropathic pain rats, 42 rats were randomly divided into three groups: normal group, CCI group, CCI + sham-EA group and CCI + EA group. The normal group was 6 rats without CCI or EA treatment. The CCI group was 12 rats with CCI-induced neuropathic pain. In the CCI + EA group, EA was performed on 12 rats with neuropathic pain 1 week after CCI surgery, once every other day, until the end of the experiment. Paw withdrawal latency (PWL) was examined to determine the thermal hyperalgesia of neuropathic pain rats. On the days of EA treatment, PWL was examined before EA treatment to avoid the disturbance of immediate influence of EA on pain behavior. Another 84 rats (12 in normal group, 36 in CCI group and 36 in CCI + EA group) were used to examine the expression of SOM peptide and ppSOM mRNA by using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) analysis. The time points of analysis were selected as 2 weeks, 3 weeks and 4 weeks after CCI surgery, corresponding to 1 week, 2 weeks and 3 weeks of EA treatment, respectively. At each time point, 6 animals of each group were used for every analysis.

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 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.

For EA treatment, rats were placed in wood holders. The rat was bound moderately to the holder so that the movement of the rat’s body was restrained while the hind limbs could move freely. Rats were allowed to acclimate for 30 min before EA treatment. According to our previous study [8,16], ‘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 contralateral 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 (≤1 mA), with the intensity lasting for 30 min. As EA control, sham EA was performed, i.e. inserting a pair of stainless steel pins into the contralateral acupoints just like EA treatment but without electrical stimulation. EA and sham EA were administered once every other day from 1 week after CCI surgery until the end of the experiment.

The paw withdrawal latency (PWL) to radiant heat was examined as previously described [2] for evidence of thermal hyperalgesia in animals using the Model 336 combination unit for paw stimulation (IITC/Life Science Instruments, USA). The rats were placed beneath an inverted, clear plastic cage upon an elevated floor of window glass. After an adaptation period of 30 min, radiant heat (50 W, 8 V bulb) was applied to the plantar surface of each paw until the animal lifted its paw from the glass. The time from onset of radiant heat application to withdrawal of the rat’s hindpaw was defined as the PWL. The heat was maintained at a constant intensity, and a cut-off time of 20 s was imposed on the stimulus duration to prevent tissue damage.

For immunohistochemistry, rats were given an overdose of urethane (1.5 g/kg, i.p.) and perfused through the ascending aorta with 200 ml normal saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The L4/5/6 DRG and L4/5/6 segments of the spinal cord were then removed, postfixed in the fixative solution for 4 h at 4 °C, and immersed in 30% sucrose in PB for 24–48 h at 4 °C for cryoprotection. Frozen sections (30 μm) were cut and collected in cryoprotectant solution (0.05 M PB, 30% sucrose, 30% ethylene glycol) and then stored at −20 °C until use. Free-floating tissue sections were processed for SOM immunohistochemistry by the avidin-biotin method. Briefly, following three 15 min rinses in 0.01 M PBS, the sections were preincubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in 0.01 M PBS with 0.3% Triton-X 100 (NGST) and then incubated in rabbit anti-SOM polyclonal antibody (1:1000, Santa Cluz, Inc., USA) diluted in 1% NGST at 4 °C for 48 h. The incubated sections were washed three times in 1% NGST and incubated in biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:200, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The sections were then washed three times in 1% NGST and incubated for 1 h in avidin-biotin-peroxidase complex (1:200, Vector Laboratories) at room...
Table 1
Analgesic effect of EA on CCI-induced thermal hyperalgesia in rats

<table>
<thead>
<tr>
<th>Time Post-CCI</th>
<th>Normal (n = 6)</th>
<th>CCI (n = 12)</th>
<th>Sham-EA (n = 12)</th>
<th>CCI + EA (n = 12)</th>
</tr>
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<tbody>
<tr>
<td>1 week</td>
<td>11.861 ± 0.413</td>
<td>5.476 ± 0.291</td>
<td>5.374 ± 0.237</td>
<td>5.239 ± 0.332</td>
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<tr>
<td>2 weeks</td>
<td>12.534 ± 0.382</td>
<td>5.303 ± 0.227</td>
<td>5.586 ± 0.341</td>
<td>7.172 ± 0.332</td>
</tr>
<tr>
<td>3 weeks</td>
<td>12.161 ± 0.313</td>
<td>5.154 ± 0.451</td>
<td>5.754 ± 0.433</td>
<td>8.364 ± 0.620</td>
</tr>
<tr>
<td>4 weeks</td>
<td>12.734 ± 0.332</td>
<td>6.034 ± 0.345</td>
<td>6.182 ± 0.364</td>
<td>10.346 ± 0.545</td>
</tr>
</tbody>
</table>

Values are PWL (s) to radiant heat (mean ± S.E.M).

* P < 0.05.
** P < 0.01.
*** P < 0.001 vs. CCI + EA group.

temperature. Finally, the sections were washed three times in 0.01 M PBS, and immunoreactive products were visualized by catalysis of 3,3-diaminobenzidine (DAB) by horseradish peroxidase in the presence of 0.01% H$_2$O$_2$. The sections were then mounted, dehydrated and covered. To test the specificity of the primary antibody, controls were performed, including the substitution of normal rabbit sera for the primary antibody and omission of the primary antibody. None of these controls showed any sign of immunohistochemical reaction. For quantification, images of positive staining in the DRG sections were captured and analyzed using Leica Q500W image analysis system. The total number of neurons and the number of neurons with positive immunoreactivity were counted for each section and percentages of immunoreactive neurons...
Fig. 2. Effect of EA on the expression of SOM in ipsilateral spinal dorsal horn in neuropathic pain rats. Images were shown for SOM immunostaining in spinal dorsal horn of normal group (A), CCI group (B) and CCI + EA group (C). (A), (B) and (C) correspond to the time point of 3 weeks after CCI surgery (2 weeks of EA treatment). SOM-ir signals were mainly limited to the superficial layers of the spinal dorsal horn. The results of all time points were quantified and demonstrated (D). The optical density was expressed as a percentage to that of the normal group (100%). Data were represented as mean ± S.E.M. (n = 6 in each group at each time point). *P < 0.05, **P < 0.01, vs. CCI group. Scale bar = 100 μm.

were calculated. For each animal, nine sections were taken (three of each L4/5/6 DRG) from the unilateral DRG and the mean of the percentages of immunoreactive neurons was calculated. For quantification of spinal sections, the total density of positive signals in the spinal dorsal horn per section was measured. For each animal, 10 sections were randomly taken from the L4 to L6 spinal cord segments and the mean value was counted. The investigator responsible for image analysis was blind to the experimental condition of each rat.

For RT-PCR analysis, rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4/5/6 DRG 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 20 μl of RNase-free distilled H2O. The amount of RNA was measured spectrophotometrically. Total RNA (1 μg) 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 for ppSOM were as follows: forward: antisense: 5′-CTAACAGGA TGTGAA TGTCTTC-3′, sense: 5′-AGCTGTCCTGCGCTCCAGT-3′ (J00787) [9]; β-actin forward: 5′-CACCA TGTACCCTGGCATG-3′, reverse: 5′-TACGCAACTAGTCATAGT-3′. The primers were synthesized and purified by Shanghai Institute of Biochemistry, Chinese Academy of Science. 1 μl of cDNA was added to 49 μl of PCR mix containing 5× PCR buffer, 18 pmol/l concentrations of each primer, 2.5 mM of dNTP, and three units of Pfu DNA polymerase. PCR reaction was performed as follows: 12 min at 94 °C to activate the Taq polymerase, followed by 30 cycles of 45 s at 94 °C, 45 s at 58 °C, and 1 min at 72 °C. A final elongation step at 72 °C for 10 min completed the PCR reaction. Each PCR production (10 μl) was electrophoresed in 1% agarose gel, visualized by ethidium bromide staining and scanned with ultraviolet transilluminator (GDS 8000, Gene Tools from Syngene Software, UK). The PCR quantitative method takes advantage of
the fact that β-actin was employed as internal standard in the same condition. All the results were expressed as ratios of the intensity of the ppSOM bands to that of β-actin band. Data are presented as mean ± S.E.M. and analyzed by SPSS 10.0. Repeated measures analysis of variance (ANOVA) followed by S-N-K test was used for post hoc analysis for differences between groups. \( P < 0.05 \) was considered statistically significant.

As shown in Table 1, CCI to the sciatic nerve produced severe thermal hyperalgesia in the rats’ ipsilateral hindpaws. Ipsilateral PWL of rats in the EA treatment group increased significantly compared with those of CCI group and sham EA group (\( P < 0.05 \) to \( P < 0.001 \)).

Expression of SOM was examined by immunohistochemistry. Some of the small-cell-diameter DRG neurons showed positive immunostaining of SOM (Fig. 1A–C). The percentages of SOM-immunoreactive (SOM-ir) neurons to total neurons were calculated and presented. The results showed that there was no significant change in the number of SOM-ir neurons after CCI-induced neuropathic pain. However, EA treatment could increase the number of SOM-ir neurons in ipsilateral DRG of neuropathic pain rats (\( P < 0.01 \), Fig. 1D).

Images from the sections of the spinal cord showed that SOM-ir signals were mainly limited to the superficial layers of the spinal dorsal horn (Fig. 2A–C). Density analysis revealed that expression of SOM in the ipsilateral spinal dorsal horn did not change significantly after CCI-induced neuropathic pain while could be enhanced by EA treatment (\( P < 0.05 \) to \( P < 0.01 \), Fig. 2D).

As shown in Fig. 3A, an expected 351-bp PCR product was obtained from all groups. Semi-quantitative analysis revealed that there was no significant change in ppSOM mRNA level after CCI-induced neuropathic pain while EA treatment resulted in a significantly enhanced mRNA level in ipsilateral DRG (\( P < 0.01 \) to \( P < 0.001 \), Fig. 3B).

There was no significant change in SOM expression or ppSOM mRNA level in DRG and spinal dorsal horn of the contralateral side (data not shown).

EA is a promising method for the treatment of neuropathic pain, which has been supported by both clinical trials and experimental researches [7,12,14]. Consistent with a series of previous studies, the present study proved that repeated EA had potent analgesic effect on neuropathic pain in rats. However, the mechanism of EA analgesia has not been fully understood. EA has modulating effect on the expression and release of various endogenous bioactive substances in the nervous system including monoamines, opioids, oxytocin and so on, which are important in the transmission and modulation of nociception. This is considered as one of the underlying mechanisms of acupuncture analgesia [10,22].

Although no apparent change of SOM expression was observed after CCI-induced neuropathic pain in rats, which was coincident with previous studies [11], the present study proved that EA could significantly enhance SOM expression in DRG and spinal dorsal horn as well as ppSOM mRNA level in DRG of neuropathic pain rats. The results indicated that endogenous SOM might play a role in EA analgesia on neuropathic pain.

Nociceptors in DRG, which are small-cell-diameter neurons, can be divided into two populations: some nociceptors contain substance P (SP) and also calcitonin-gene-related peptide (CGRP); a second population of nociceptors expresses SOM with or without CGRP. Nociceptors that contain SOM extend their axons centrally to lamina II of the spinal cord and SOM is released from these axons in the spinal cord following noxious stimulation of peripheral nerves and produces an inhibitory effect on nociceptive neurons [18,20]. SOM depresses the firing of spinal dorsal horn neurons activated by noxious stimulation, produces analgesia in animals and humans. The analgesic effects of SOM in various types of pain have been reported both in animal and human studies [4,6,17]. In clinic, octreotide (OCT, a synthetic octapeptide derivative of somatostatin) is used to treat opioid-resistant pain. However, on the other hand, there was also conflicting evidence that SOM might contribute to nociception. It was reported that intrathecal administration of anti-SOM antiserum inhibited responses to thermal stimuli in normal and adjuvant inflamed rats [19]. The role of SOM in nociceptive processing is thus uncertain. However, in the present study, we would preferentially hypothesize that the increased expression of SOM in DRG and spinal dorsal horn might be one of mechanisms of EA analgesia on neuropathic pain, since previous studies have proved that intrathecal OCT could significantly relieve thermal hyperalgesia of CCI-induced neuropathic pain rats [21].
Interestingly, it was reported that glial cell line-derived neurotrophic factor (GDNF) could enhance SOM content in DRG neurons and increase activity-induced release of SOM in the dorsal horn [5]. Our previous studies proved that EA could also enhance the expression of GDNF and its receptor GFRα-1 in DRG and spinal dorsal horn [8]. This might be one of the mechanisms that underlie the results of the present study.

Our experiment indicated that endogenous SOM in the nervous system might be involved in EA analgesia on neuropathic pain. This will deepen our realization of the mechanism of EA analgesia and provide a rational basis for enhancing EA analgesic effect.

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