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Changes in expression of nociceptin/orphanin FQ and its receptor in spinal dorsal horn during electroacupuncture treatment for peripheral inflammatory pain in rats

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ARTICLE INFO

Article history:
Received 25 January 2007
Received in revised form
26 March 2007
Accepted 26 March 2007
Published on line 5 April 2007

Keywords:
Peripheral inflammatory pain
Electroacupuncture
Spinal cord
N/OFQ
NOP receptor

ABSTRACT

The neuropeptide nociceptin/orphanin FQ (N/OFQ), the endogenous agonist of the N/OFQ peptide receptor (NOP receptor), has been demonstrated to be involved in many physiological and pathological functions including pain modulation. It was reported that electroacupuncture (EA) had a potent analgesic effect on inflammatory pain by activating various endogenous transmitters such as the opioid peptides. In the present study, we investigated the effect of EA on peripheral inflammatory pain and the expression of N/OFQ and the NOP receptor in the spinal dorsal horn of rats, using a behavioral test, RT-PCR, immunohistochemistry and Western blot analysis techniques. The results showed: (1) EA had an accumulative analgesic effect on chronic inflammatory pain; (2) in the superficial layers of the spinal dorsal horn, the level of mRNA of the precursor protein for N/OFQ (preproN/OFQ, ppN/OFQ) was increased and the N/OFQ immunoreactivity was decreased after peripheral inflammation, and could be significantly increased by EA treatment; (3) both mRNA and protein levels of the NOP receptor in the spinal dorsal horn were significantly increased after chronic inflammatory pain and could be further enhanced by EA treatment. The present data demonstrated that EA could activate the endogenous N/OFQ–NOP receptor system, and this might underlie the effectiveness of EA in the treatment of inflammatory pain.

1. Introduction

Discovery of the opioid-receptor-like (NOP) receptor and its endogenous ligand nociceptin/orphanin FQ (N/OFQ) has led to a new understanding of the endogenous pain modulation system [19,26]. Numerous studies demonstrate that N/OFQ plays an important role in nociception although its action is different at the spinal and supraspinal level, and the consensus opinion is that N/OFQ acts in an antinociceptive manner in the spinal cord [31]. At the spinal level, the NOP receptor agonist exhibits antiallodynic activity in neuropathic rats, suggesting that the NOP receptor might be involved in the pathological condition [21]. Furthermore, both the NOP receptor and the precursor protein for N/OFQ (preproN/OFQ, ppN/OFQ) are expressed widely in the mammalian central nervous system (CNS), but particularly intense expression is seen in areas involved in pain control, such as the spinal cord dorsal horn [20], which suggested that N/OFQ and the NOP receptor might play a role

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2007.03.022

in the modulation of nociceptive signals. It was reported that the expression of NOP receptor mRNA was increased and no significant change was observed for ppN/OFQ mRNA expression in the spinal cord following peripheral nerve injury in rats [4]. However, much less is known about changes of expression of N/OFQ and its receptor in the spinal cord during different time courses of chronic inflammatory pain. The subcutaneous intraplantar injection of complete Freund's adjuvant (CFA) into the local area of an animal can cause severe inflammatory pain around the injected area [1,5,6]. This CFA-induced inflammatory pain has been widely used as a model in the field of pain research since it was reported [14,23,24].

Chronic pain is associated with sensitization of peripheral nociceptive receptors and hyperexcitability of the CNS (e.g. spinal dorsal horn neurons) in relation to the transmission and modulation of noxious messages [27,35] that give rise to behavioral hyperalgesia or allodynia [27,37]. Such pain is often intense and refractory to conventional analgesic therapy. In the past 30 years, electroacupuncture (EA) as an established adjuvant analgesic modality has been used extensively in clinical treatments. EA studies have also been performed on chronic pathological pain animal models, such as CFA-induced inflammatory pain, which showed potent analgesic effects [34]. However, contradictory or conflicting results were reported on the effects of EA in the treatment of CFA-induced persistent pain [10]. So, it is worthwhile to investigate the effect of EA on chronic inflammatory pain.

In addition, it was also well known that EA analgesia was mediated by endogenous opioids and other neurotransmitters in the nervous system. We recently showed that N/OFQ synthesis and N/OFQ peptide levels as well as NOP receptor mRNA expression were changed in the brain after EA treatment in neuropathic pain rats, demonstrating that N/OFQ and the NOP receptor were involved in EA analgesia [17,18]. However, the possible effects of EA on the expression of N/OFQ and its high-affinity NOP receptor in the spinal cord during the development and maintenance of chronic inflammatory pain are unknown. Therefore, as a member of the opioid family, it would be important to study the detailed role of N/OFQ and the NOP receptor in EA analgesia in order to understand the mechanism of acupuncture analgesia.

Thus, the aim of this present study was to (1) characterize the expression of N/OFQ and the NOP receptor in the spinal dorsal horn with respect to the development of thermal sensitivity during various phases (days 4, 10 and 14) of CFA-induced peripheral inflammation; (2) examine whether EA has an analgesic effect on the thermal hyperalgesia in the rat model of inflammatory pain; (3) investigate the changes of expression of N/OFQ and the NOP receptor after EA was administered at correspondingly selected time points in CFA-induced chronic inflammatory pain rats.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (weighing 200–220 g, from Experimental Animal Center, Shanghai Medical College, Fudan University, China) were allowed to acclimate for 1 week and maintained at a room temperature of 22 ± 2 °C and a 12 h light/dark cycle with free access to food and water. The animal protocols were conducted according to the Animals Care and Use Committee of Shanghai Medical College, Fudan University, and conformed to the ethical guidelines of the International Association for the Study of Pain [36].

2.2. Induction of chronic inflammation

Chronic inflammation was induced by a subcutaneous injection of 100 μl CFA (Sigma, St. Louis, MO), which was suspended in a 1:1 oil/saline emulsion and contained 40 μg *Mycobacterium tuberculosis*, in the plantar surface of the left hindpaw using a 25-gauge hypodermic needle [29] under brief isoflurane anesthesia. The inflammation, manifesting as redness, edema and hyper-responsiveness to noxious stimuli, was limited to the injected paw, appeared shortly after the injection, and lasted about 2 weeks. Hyperalgesia was determined by a decrease in paw withdrawal latency (PWL) to a noxious thermal stimulus. Control rats for all experiments were naive rats that did not receive any injection into the hindpaw.

2.3. EA administration

The detailed EA procedure has been described previously [8]. In brief, during EA treatment, the trunk of the rat was kept motionless while the head and four limbs kept freedom of movement in a specially designed holder, and a pair of stainless steel needles of 0.3 mm diameter was inserted into the unilateral acupuncture points 'Huan Tiao' (GB30, located near the hip joint, on the inferior borders of gluteus maximus and piriformis muscle; the inferior gluteal cutaneous nerve, the inferior nerve; deeper, the sciatic nerve) and 'Yang Ling Quan' (GB34, located near the knee joint, anterior and inferior to the small head of the fibula, in the peroneus longus and brevis muscle; where the common peroneal nerve bifurcates into the superficial and deep peroneal nerves) at a depth of 7 and 5 mm, respectively. The two pins 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, bidirectional asymmetric pulse, 0.6 ms pulse width) were selected. The intensity was adjusted to induce slight twitch of the hindlimb (≤1 mA), with the intensity lasting for 30 min. In order to exclude the possibility of analgesia induced by severe stress, such as animal fixation, sham EA group animals underwent the same manipulation as the EA group except without electrical stimulation. This form of sham EA showed little anti-hyperalgesia [8] and seemed to be an appropriate control for non-specific needling effects. EA was administered once every other day from the second day after CFA injection until the end of the experiment.

2.4. Behavioral test

Rats were tested for hindpaw thermal hyperalgesia by a method developed previously [9]. Briefly, the rats were placed under a clear plastic chamber on the glass surface of the Model

390 paw stimulator analgesia meter (IITC/Life Science Instruments, USA) and allowed to acclimatize for 30 min. The radiant light was focused on the plantar surface of each hind paw. The intensity of the thermal stimulus was adjusted to derive an average baseline PWL of approximately 10.0 s in naive animals. A cut-off time of 20 s for the stimulation was used to prevent tissue damage.

The mean PWL was established by averaging the latency of four tests with a 10-min interval between each test. The average PWL values obtained before CFA injection and 2, 4, 6, 8, 10, 12 and 14 days after CFA injection, correspond to 0, 1, 2, 3, 4, 5, 6 and 7 times of EA treatment, respectively. At each time point, eight animals of each group were used for every analysis.

2.5. *Reverse transcription-polymerase chain reaction (RT-PCR)*

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect the mRNA levels of ppN/OFQ and the NOP receptor. Total RNA was isolated from the lumbar enlargement of the spinal cord at 4, 10 and 14 days post-CFA injection, correspond to 2, 5 and 7 times of EA treatment using a trizol reagent and following the instructions of the manufacturer. At each time point, six animals of each group were used for every analysis. 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. One microgram of total RNA was digested with DNase RNase-free enzyme (Promega, Madison, WI, USA) to eliminate genomic DNA, and then converted to complementary DNA (cDNA) using 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) in 20 μl of the buffer containing 0.4 mM deoxy-nucleotide triphosphates, 2 U/ml RNase inhibitor, and 0.8 μg oligo(deoxythymidine)$_{15}$ primer. As a control, a sample without reverse transcriptase was incubated in every experiment for each cDNA. Oligonucleotide primers used for RT-PCR amplification were designed according to the published sequences. The primers for amplifying the ppN/OFQ cDNA were 5′-GTGACTCTGAGCAGCTCAGC-3′ and 5′-TTCTGGTTGGCCAACTTCCG-3′, which anneal to bases 224–243 and 460–479 [2]. Specific primers for the NOP receptor cDNA were 5′-GTTCAAGGACTGGGTGTTCAGCCAGGTAGT-3′ and 5′-TGCTGGCCGTGGTACTGTCTCAGAAGTCTT-3′, which anneal to bases 844–873 and 1101–1072 [32]. The expression of the housekeeping gene, β-actin, was used as an internal control. A cDNA mixture of β-actin prepared from the primer 5′-CACCATGTACCCTGGCATTG-3′ and an antisense primer 5′-TAACGCAACTAAGTCATAGT-3′ was used. The primers were synthesized and purified by Shanghai Institute of Biochemistry, Chinese Academy of Science. The PCR conditions for all cDNAs were as follows: 5 min at 94 °C to activate the Taq polymerase, followed by 29 cycles of 45 s at 94 °C, 45 s at 60 °C, and 1 min at 72 °C. A final elongation step at 72 °C for 10 min completed the PCR reaction. Ten microliters of each PCR production were electrophoresed in 1% agarose gel, visualized by ethidium bromide staining and scanned with an 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 the internal standard in the same condition. All of the results were expressed as ratios of the intensity of the ppN/OFQ/NOP cDNA bands to that of the β-actin band.

2.6. *Immunohistochemistry*

For the N/OFQ protein immunohistochemistry study, the time points of analysis were selected as 4, 10 and 14 days after CFA injection, correspond to 2, 5 and 7 times of EA treatment, respectively. At each time point, six animals of each group were used for every analysis. Rats were given an overdose of urethane (1.5 g/kg, i.p.) and surgery proceeded with sternotomy, transcardiac aortic needle cannulation, and perfusion with 200 ml of normal saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Spinal cords were then removed and postfixed in fixative solution for 4 h at 4 °C. After immersion in PB containing 30% sucrose for 24–48 h at 4 °C for cryoprotection, the L4/5/6 segments of spinal cord were sectioned at a 30 μm thickness on a cryostat and collected in a cryoprotectant solution (0.05 M PB, 30% sucrose, 30% ethylene glycol) and then stored at −20 °C until use. The L4/5/6 segments of the spinal cord were confirmed by identification of the lumbar enlargement and nerve roots. Free-floating tissue sections were processed for N/OFQ protein immunocytochemistry by the avidin–biotin method. Sections were removed from storage at −20 °C and placed directly into 0.1 M PB for 10 min. 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). The sections were then incubated in rabbit anti-N/OFQ polyclonal antibody (1:1000, Phoenix Pharmaceuticals Inc.) 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, USA) for 1 h at room temperature, washed three times in 1% NGST and incubated for 1 h in avidin–biotin-peroxidase complex (1:200, Vector Laboratories) at room temperature. Finally, the sections were washed three times in 0.01 M PBS, and the N/OFQ-like immunoreactive product was visualized by catalysis of 3,3-diaminobenzidine (DAB) by horseradish peroxidase in the presence of 0.01% H_2O_2. The sections were then mounted, air dried, dehydrated in alcohol in a graded manner, cleared in xylenes and coverslipped. 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. The N/OFQ immunoreactive cells were observed and analyzed using a Leica Q500IW image analysis system. Five sections from the L4, L5 or L6 segments of the spinal cord of each rat were randomly selected. The superficial laminae (laminae I and II) on the side of the spinal cord ipsilateral to the site of inflammation were taken and the average of the number of positive cells in five slices was defined as the number of N/OFQ immunoreactive cells. The investigator responsible for image analysis was blind to the experimental condition of each rat.

2.7. Western blotting

For determination of NOP receptor protein levels, protein extracts were obtained from the L4/5/6 segments of the spinal cord according to the following protocol. The time points of analysis were selected as 4, 10 and 14 days after CFA injection, correspond to 2, 5 and 7 times of EA treatment, respectively. At each time point, six animals of each group were used for every analysis. Rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and spinal cord tissues were collected in dry ice and stored at −70 °C until assayed. 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 (50 μg of total protein) were dissolved with an 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) and separated on a 10% gel via 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 an anti-NOP receptor (KOR-3 (H-85)) rabbit polyclonal antibody (1:1000, Santa Cruz Biotechnology, USA) and a β-actin (Actin H-196) rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology, USA) to control for equal protein loading, 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. Immunoblots were developed by enhanced chemiluminescence ECL (Amersham Biosciences, UK) and visualized on 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).

2.8. Data analysis

Experimental data were presented as mean ± S.E.M. and were analyzed by the Statistical Package for the Social Sciences (SPSS) statistical software. The results were analyzed by one-way analysis of variance (ANOVA) and the significance of difference was determined by the Newman–Keuls test. When only two treatment groups were being compared, Student's *t*-test was used. $P < 0.05$ was considered statistically significant in all cases.

3. Results

3.1. Accumulative analgesic effect of repetitive EA on CFA-induced thermal hyperalgesia

As illustrated in Fig. 1, before CFA injection, the overall mean baseline PWL to radiant heat stimuli was similar in all groups of rats, and there was no significant difference in PWL. Following injection of 100 μl CFA into the left hind paw, animals showed evident signs of inflammation, which were assessed by localized erythema and an increase in the swelling of the paw. Its PWL significantly decreased compared to that of the pre-CFA level. EA treatment ($n = 8$) could significantly increase the PWL, an accumulative anti-hyperalgesic effect, from the fifth time post-EA treatment ($P < 0.01$ to $P < 0.001$), while sham EA showed little anti-hyperalgesia. There were no apparent changes in PWL on the right side (intact side) in all groups tested (data not shown).

3.2. Time course of ppN/OFQ mRNA expression and the number of N/OFQ-like immunoreactive (N/OFQ-LI) neurons during CFA-induced inflammation and EA treatment

Quantification of mRNA at the lumbar spinal cord by RT-PCR revealed a significant increase in mRNA expression for ppN/OFQ in CFA-injected and EA-treated rats. As shown in Fig. 2A and B, an expected 250 bp PCR product was obtained from all groups. Increased expression of ppN/OFQ mRNA at the ipsilateral lumbar spinal cord was seen at day 4 following peripheral administration of CFA, and remained elevated during the whole phase (days 10 and 14) of inflammation, which was further enhanced by EA treatment ($P < 0.05$). However, sham EA treatment did not have any effect on the level of ppN/OFQ mRNA at the above same time points.

As shown in Fig. 3, immunohistochemical examination revealed that N/OFQ-LI signals were mainly limited to the superficial layers of the spinal dorsal horn. CFA-induced chronic inflammation elicited a dramatic decrease of N/OFQ immunoreactivity in the ipsilateral spinal dorsal horn from 4 days after CFA injection. After EA treatment, the number of N/OFQ-LI neurons was significantly increased compared with that of inflammatory pain rats ($P < 0.01$). However, sham EA treatment could not induce immunoreactivity changes in N/OFQ at any time point of inflammation. There was no

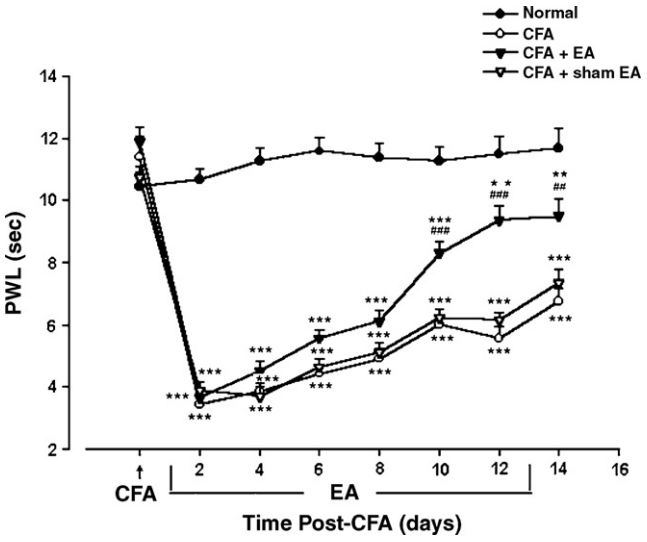

Fig. 1 – Effect of accumulative EA treatment on PWL in inflammatory pain rats. EA was administered once every other day from the second day after CFA injection until the end of the experiment. All points were expressed as mean ± S.E.M. ($n = 8$ in each group at each time point). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. normal group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. CFA group.

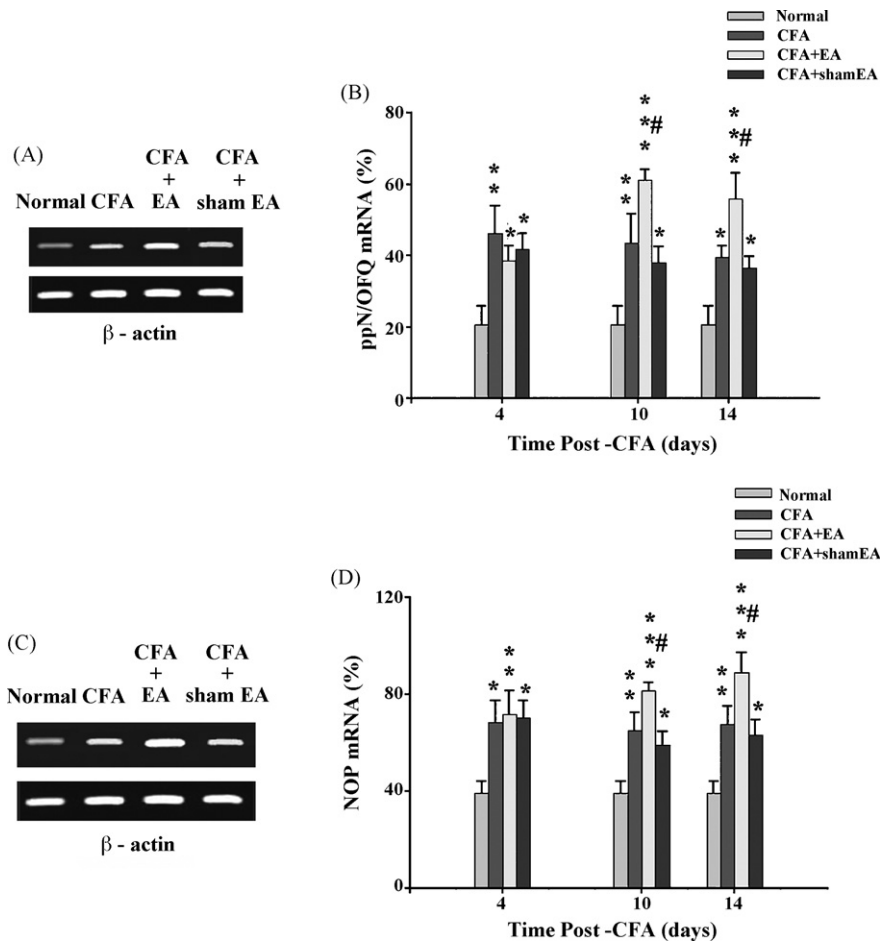

Fig. 2 – Changes in the mRNA levels of ppN/OFQ and the NOP receptor in the ipsilateral spinal dorsal horn detected by RT-PCR after CFA-induced inflammatory pain and EA treatment. EA was administered once every other day from the second day after CFA injection until the end of the experiment. Expected size PCR products were acquired corresponding to ppN/OFQ (A) and the NOP receptor (C). (A and C) The time point of 10 days after CFA injection (five times of EA treatment). (B and D) The results of all time points were quantified and demonstrated. The mRNA level was expressed as a ratio to β-actin. Data were represented as mean ± S.E.M. ($n = 6$ in each group at each time point). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. normal group; $^{\#}p < 0.05$ vs. CFA group.

significant change in the N/OFQ protein or mRNA levels of the contralateral side at any time of inflammation (data not shown).

3.3. *Time course of NOP receptor expression during CFA-induced inflammation and EA treatment*

Quantification of mRNA by RT-PCR and protein by Western blotting showed increased expression of the NOP receptor at the ipsilateral lumbar spinal cord of the CFA-injected and EA-treated rats. As shown in Fig. 2C and D, an expected 258 bp PCR product for NOP receptor mRNA was obtained from all groups. Increased expression of NOP receptor mRNA in CFA-injected rats was significant during the whole phase (days 4, 10 and 14) of inflammation, which was further enhanced by EA treatment ($P < 0.05$).

Fig. 4 shows that a single protein band of the expected size (~40 kDa) for the NOP receptor was detected in the Western blot with the NOP receptor-specific primary antibody. Increased expression of NOP receptor protein in CFA-injected rats significantly remained elevated during the different phases (days 4, 10 and 14) of inflammation compared with that of normal rats, which was further enhanced by EA treatment ($P < 0.05$). Sham EA treatment could not induce changes in the mRNA and protein levels of the NOP receptor at the above same time points. There was no significant change in the NOP receptor protein or mRNA levels of the contralateral side at any time point of inflammation (data not shown).

4. Discussion

In clinical practice, chronic inflammatory pain is one of the most common types of pathological pain. The target for analgesic therapy is a major focus in current mechanical research. Injection of CFA into a rat's hindpaw provides a very convenient animal model that has been widely used to study the mechanism of nociception.

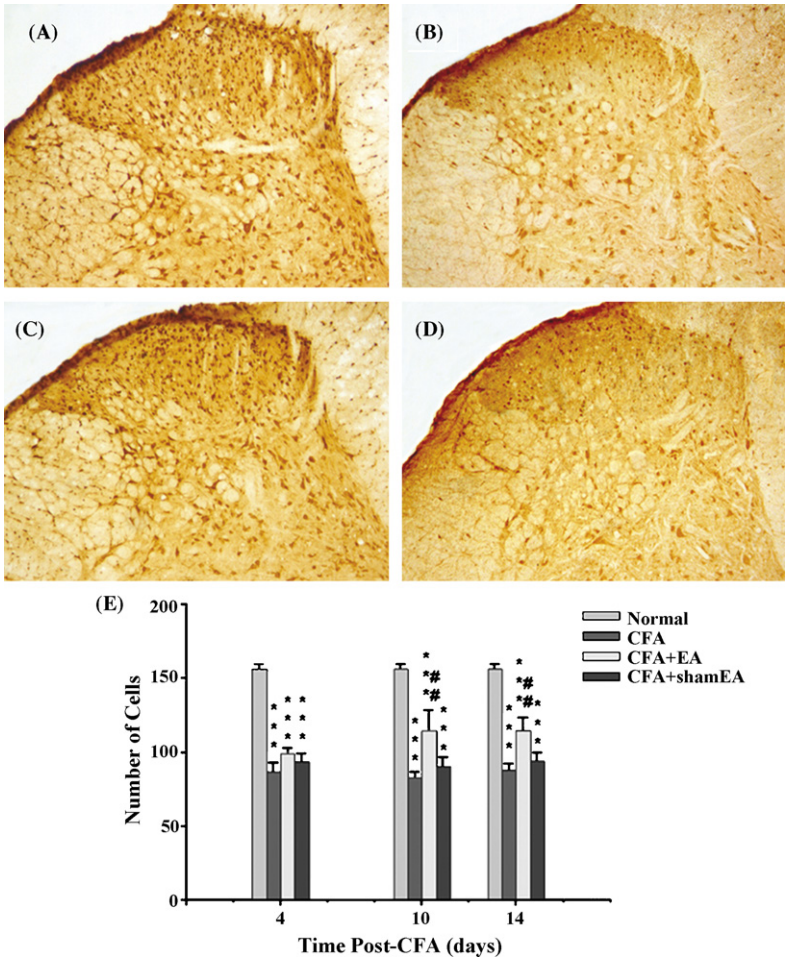

Fig. 3 – Changes in the expression of N/OFQ in the ipsilateral spinal dorsal horn detected by immunohistochemistry after CFA-induced inflammatory pain and EA treatment. EA was administered once every other day from the second day after CFA injection until the end of the experiment. Images were shown for N/OFQ immunostaining in the spinal dorsal horn of the normal group (A), CFA group (B), EA treatment group (C) and sham EA treatment group (D). (A–D) The time point of 10 days after CFA injection (five times of EA treatment). N/OFQ-LI signals were mainly limited to the superficial layers of the spinal dorsal horn. The results of all time points were quantified and demonstrated (E). Data were represented as mean ± S.E.M. (n = 6 in each group at each time point). $^{***}p < 0.001$ vs. normal group; $^{\#\#}p < 0.01$ vs. CFA group. Scale bar = 200 μm.

Many lines of evidence indicate that the spinal cord is an equally important CNS area for nociceptive processing and its modulation by N/OFQ and classical opioids. In particular, the superficial layers of the spinal cord dorsal horn, where thin and unmyelinated primary afferent nerve fibers terminate, represent an important structure for nociceptive processing. Our present study confirmed that N/OFQ and the NOP receptor were highly expressed in the spinal cord as previously described in normal rats [25], suggesting that the endogenous N/OFQ–NOP receptor system was involved in modulating pain transmission.

Persistent pain is associated with long lasting alterations of the nervous system [12]. Therefore, one of special interests is evaluating the effects of chronic pain on the changes of endogenous N/OFQ and NOP receptor levels in the spinal cord associated with pain regulatory mechanisms. The animals were studied 2 weeks after injection of CFA, at a time-course where marked signs of hyperalgesia were present. Our results revealed that the expression of ppN/OFQ mRNA was increased, while the number of N/OFQ-LI neurons was decreased. The reason might be that the N/OFQ protein synthesis could not be compensated for by the increasing release of N/OFQ in chronic inflammatory pain, which needs to be further studied by a push–pull perfusion technique to monitor the changes in spinal cord content of N/OFQ *in vivo*. The present study also showed that the mRNA and protein expression of the NOP receptor was increased, which lasted for 2 weeks and paralleled behavioral hypersensitivity. The changes of endogenous N/OFQ and NOP receptor levels suggested that the N/OFQ–NOP receptor system was activated during persistent inflammatory pain and was involved in regulating the sensation and perception of nociception, at least in inflammatory conditions. In agreement with our findings, there was evidence suggesting the important role for

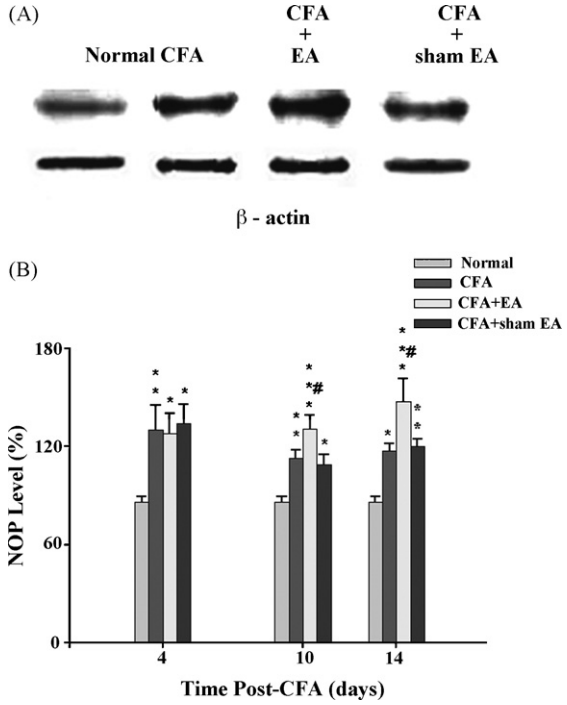

Fig. 4 – Changes in the expression of the NOP receptor detected by Western blot in the ipsilateral spinal dorsal horn after CFA-induced inflammatory pain and EA treatment. EA was administered once every other day from the second day after CFA injection until the end of the experiment. Western blot analysis detected expected protein bands correspond to the NOP receptor (A). The time point of 10 days after CFA injection (five times of EA treatment). (B) The immunoblot results of all time points were quantified and demonstrated. The protein level was expressed as a ratio to β-actin. Data were represented as mean ± S.E.M. (n = 6 in each group at each time point). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. normal group; $^{\#}p < 0.05$ vs. CFA group.

the endogenous N/OFQ–NOP receptor system in the modulation of persistent nociception. It has been shown that there is an up-regulation of NOP receptor mRNA expression in the spinal cord of neuropathic rats [4], and an increase in nociceptin binding sites 4 days after injection of CFA, a model of persistent peripheral inflammation, was observed [13], reflecting a possible function of the NOP receptor in the nociceptive processing which led to pathological pain. Furthermore, recent studies have shown an increase in sensitivity to inflammatory hyperalgesia in mice lacking the nociceptin precursor polypeptide or the nociceptin receptor, which indicated that endogenous N/OFQ significantly contributed to physiological analgesia under inflammation conditions [7]. However, contradictory or conflicting reports were shown that only a small non-significant change in the ppN/OFQ mRNA expression of the spinal cord occurred at any time after the chronic constriction injury of the sciatic nerve [4]; in a model of carrageenan-induced inflammation, N/OFQ-LI in the dorsal spinal cord significantly increased [28]. Causes of these conflicting results are still unclear. Different time points selected and different models of hyperalgesia may be involved, so further investigation would be necessary.

Electrical stimulation has been widely used in both experimental research on animal models and clinical treatments because it can be controlled and quantified easily, and thus it is repeatable. For Sham EA control, as one of the most commonly used control treatments, acupuncture needles were also inserted into acupoints, but without electrical current. This sham procedure produced little anti-hyperalgesia and was used successfully in many studies [34], just as in our study. In the present study, GB30 and GB34 were chosen based on Traditional Chinese Medicine (TCM) meridian theory [22], and its successful use in our previous studies, showing significantly transient anti-hyperalgesia [8]. In our present data, it was shown that repetitive EA at GB30 and GB34 could significantly increase PWL, meaning an accumulative anti-hyperalgesic effect, which coincided with clinical practice that the therapeutic effect of acupuncture lasted from days to weeks in patients with nociceptive pain [3]. The mechanisms underlying EA analgesia are very complicated. Our previous behavioral study showed that [Nphe^{1}]N/OFQ(1-13)NH_2, a selective antagonist of the NOP receptor, blockade on the inhibitory effect of a single session of EA stimulation on thermal hyperalgesia in CFA induced inflammatory pain supported the possible involvement of the spinal N/OFQ–NOP system, as a member of the opioid family, in EA analgesia. We also observed the effect of a single session of EA stimulation on the thermal hyperalgesia that was diminished 1 h after EA administration [8]. So we speculated that a single session of EA stimulation produced transient antinociception via release of N/OFQ. In order to determine whether the mechanisms for a single session of EA or repetitive EA treatments were similar, we are observing the effect of [Nphe^{1}]N/OFQ(1-13)NH_2 on repetitive EA. Furthermore, other published literature reported that repetitive EA treatments relieved analgesia by changing central neurotransmission in the pain control system distinct from the endogenous opioid system [16]. Therefore besides the N/OFQ–NOP system, which the neurotransmission system is involved, needs to be further studied. However, a recent study using female rats and the hot plate test demonstrated that repetitive administration of EA at 'Zu San Li' (ST36) and 'San Yin Jiao' (SP6) could not attenuate thermal hyperalgesia in the CFA model [10]. The discrepancy may be due to the sexual difference and/or the testing method. It is known that sexual hormones may have significant effects on pain [15].

In addition, another main finding of the present study was that EA treatment significantly enhanced the expression of N/OFQ and the NOP receptor in the spinal dorsal horn compared with those of inflammatory pain rats, implicating that the effect of EA on N/OFQ and NOP receptor levels was one of the underlying mechanisms by which EA attenuated hyperalgesia induced by CFA. Behavioral studies showed that EA analgesia was decreased by intrathecal injection of an N/OFQ-antibody [30] and NOP receptor antagonist [8], suggesting that the significant anti-hyperalgesia produced by EA was the result of the activation of the endogenous N/OFQ (for the NOP receptor) system at the spinal level during persistent pain. Furthermore, the pain inhibitory role through NOP or N/OFQ-ergic neurons in the dorsal horn of the spinal cord could be attributed to the

action on the second-order neurons for polymodal substance P fibers [11]. Given that acupuncture reduces electrophysiological spinal neuron response to noxious stimuli [33], we postulate that EA treatment induces increasing N/OFQ and NOP receptor levels in the spinal cord, which may play an in vivo inhibitory role on the second-order neurons for primary polymodal substance P-ergic fibers in the spinal cord and result in suppression of hyperalgesia in rats with persistent peripheral inflammation. However, further detailed studies are important for a better understanding of the involvement of the N/OFQ–NOP receptor system in the spinal cord in EA analgesia.

In summary, our experiments indicated that the N/OFQ–NOP receptor system could play an important role in the modulation of nociceptive signals at the spinal level, thereby showing its involvement during the inflammatory pain condition; EA might exert an analgesic effect on inflammatory pain partly through increasing N/OFQ and NOP receptor levels in the spinal cord. These findings suggested that the spinal N/OFQ–NOP system might be involved in EA analgesia, which will deepen our realization of the mechanism of EA analgesia and provide a rational basis for enhancing the EA analgesic effect.

Acknowledgements

This project was financially supported by the National Key Basic Research Program (No. 2005CB523306) and the Science Foundation of Shanghai Municipal Commission of Science and Technology (No. 02DZ19150-1).

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