

Research report

A parametric study of electroacupuncture on persistent hyperalgesia and Fos protein expression in rats

Lixing Lao^{a,*}, Rui-Xin Zhang^{a,1}, Grant Zhang^a, Xiaoya Wang^a, Brian M. Berman^a, Ke Ren^b

^aCenter For Integrative Medicine, School of Medicine, University of Maryland, 3rd Floor, James Kernan Hospital Mansion, 2200 Kernan Drive, Baltimore, MD 21201, USA

^bDepartment of Biomedical Sciences, Dental School, University of Maryland, Baltimore, MD 21201, USA

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Abstract

We previously reported the anti-hyperalgesia of electroacupuncture (EA) on persistent inflammatory pain in an unrestrained, unседated, and conscious rat model. Using this model, induced by injecting complete Freund's adjuvant (CFA) into one hind paw, we systematically evaluated the anti-hyperalgesia of EA stimulation parameters (frequency, intensity, treatment duration, and pulse width). We assessed hyperalgesia by paw withdrawal latency (PWL) to a noxious thermal stimulus and found that 10- and 100-Hz EA frequencies at a current intensity of 3 mA produced the greatest anti-hyperalgesia, when compared to other parameters. Both frequencies significantly increased PWL in the early phases of hyperalgesia (2.5 and 24 h; $p < 0.05$), and 10 Hz EA also significantly increased PWL in later phases (5 to 7 days; $p < 0.05$). A sufficient but tolerable intensity of 3 mA was more effective than lower intensities (1–2 mA). A 20-min treatment produced better anti-hyperalgesia than longer and shorter (10 and 30 min) treatments. Acupoint specificity study demonstrated that GB30 produced significant EA anti-hyperalgesia, while Waiguan (TE5) and sham points, an abdominal point and a point at the opposite aspect of GB30, did not. The spinal Fos protein expression study demonstrated that the optimal EA selectively suppressed Fos expression in superficial laminae (I/II) and activated it in deeper laminae (III/IV) of the spinal dorsal horn. The results suggest that the EA anti-hyperalgesia is parameter-dependent and point-specific, and they provide important information for designing further clinical acupuncture research on persistent inflammatory pain.

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1. Introduction

Electroacupuncture (EA) has been investigated extensively with normal (uninjured) animals, but these studies are of limited clinical relevance. The EA stimulation used in those studies was so intense that the animals had to be restrained and may have been affected by stress-induced analgesia [43,61]. Moreover, the uninjured animal model does not resemble clinical pathological chronic pain conditions [17,18]. Chronic pain is associated with sensitization

of peripheral nociceptive receptors and hyperexcitability of the central nervous system (e.g. spinal dorsal horn neurons) in relation to the transmission and modulation of noxious messages [48,71] that give rise to behavioral hyperalgesia or allodynia [48,75]. Furthermore, it has been demonstrated that healthy and pathological conditions respond differently to EA. Previous studies with an uninjured animal model show only brief (20–60 min) acupuncture analgesia (AA) [4,19,51]. However, in a proportion of patients with nociceptive pain, acupuncture produces long-term (days, not minutes or hours) therapeutic pain relief [8].

In our own studies on the effects of EA, we have recently employed a persistent inflammatory pain rat model, induced by injecting complete Freund's adjuvant (CFA) unilaterally into a hind paw [31]. Using this model, we demonstrated that

* Corresponding author. Tel.: +1-410-448-6873; fax: +1-410-448-6875.

E-mail address: LLao@compmed.umm.edu (L. Lao).

¹ They made equal contributions.

EA induces prolonged (in days) anti-hyperalgesia and that EA treatment can be performed on conscious, unrestrained and unsedated animals [31]. A number of clinical trials have demonstrated that EA effects on chronic pain conditions such as lower back pain may be electro-frequency specific [18]. Previous studies [18,51] on uninjured animal models have shown that EA is parameter-dependent. However, a systematic evaluation characterizing the optimum EA parameter profile has not been previously reported for an animal model of chronic inflammatory pain. Our hypothesis was that optimizing the electro-parameters for various pain conditions would enhance the effectiveness of the treatment.

This acupuncture “dosage” response study was designed to evaluate the effect of various parameters of EA—pulse frequency, current intensity, treatment duration, and electrical pulse width—on a chronic inflammatory pain rat model using the paw withdrawal latency (PWL) test and the expression of spinal Fos protein, a marker of neuron activation. Acupuncture point specificity, or the specific effectiveness of acupoint Huantiao, also known as the 30th acupoint on the Gallbladder Meridian (GB30) [10], against inflammatory pain in this rat model, was also tested. Some of the results of this study have been previously published in abstract format [72].

2. Materials and methods

2.1. Animal preparation

Male Sprague–Dawley rats weighing 280–350 g (Harlan) were kept under controlled environmental conditions (22 ± 0.5 °C, relative humidity 40–60%, 7 am to 7 pm

alternate light–dark cycles, food and water ad libitum). The animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine. The ethical guidelines for the treatment of animals of the International Association for the Study of Pain were followed in these experiments.

Inflammation and hyperalgesia were induced by injecting CFA (Sigma, suspended in an 1:1 oil/saline emulsion, 0.1 ml, 50 µg *Mycobacterium tuberculosis*) subcutaneously into the plantar surface of one hind paw of the rat using a 25-gauge hypodermal needle. The inflammation, manifested as redness, edema, and hyper-responsiveness to noxious stimuli, was limited to the injected paw; it appeared within 2 h after the injection, peaked at 6–24 h, and lasted about 2 weeks. Hyperalgesia was determined by a decrease in PWL to a noxious thermal stimulus [20]. The effect of the hyperalgesia on the normal behavior of the CFA-inflamed rats appeared to be minimal, and they showed normal grooming behavior and levels of activity [20,31].

2.2. Experiment design

Rats were randomly divided into EA treatment with needle insertion into GB30, non-needle insertion sham control (non-invasive sham), needle insertion sham control (invasive sham) into GB30 without manipulation, and non-treatment control groups. EA treatment rats were divided into groups according to combinations of various EA parameters (Table 1). For each behavioral experiment, the EA treatment group consisted of seven to nine rats, and control consisted of one to two non-invasive sham rats, one to two invasive sham rats and one non-treatment rat. Data on the control rats in each experiment were pooled for

Table 1
Effects of EA at various parameters on hyperalgesia induced by peripheral inflammation ($n=7-9$)

Frequency (Hz)	Current (mA)	Duration (min)	Pulse (ms)	2.5 h	5 h	1 day	3 days	5 days	7 days
2	2	20	0.1						
	3	20	0.1						
10	1	20	2	$p < 0.05$	$p < 0.05$				
	1	20	1						
	1	20	0.1						
	2	20	0.1	$p < 0.05$					
	3	20	0.1	$p < 0.05$		$p < 0.05$		$p < 0.05$	$p < 0.05$
	3	10	0.1						
50	3	30	0.1						
	1	20	0.1						
	2	20	0.1	$p < 0.05$					
100	3	20	0.1	$p < 0.05$					
	1	20	2	$p < 0.05$					
100	1	20	1						
	1	20	0.1						
	2	20	0.1	$p < 0.05$					
	3	20	0.1	$p < 0.01$	$p < 0.01$	$p < 0.05$			
	3	10	0.1		$p < 0.05$	$p < 0.05$		$p < 0.05$	
	3	30	0.1						

$p < 0.05$, $p < 0.01$ vs. non-invasive sham group.

statistical analysis. Animal sample size was estimated at a power of 0.80 (1-beta) by a power analysis based on results of our previous experiments [31].

There were three main sets of experiments. Acupoint GB30 was used in the first and second experiments. In the first set, combinations of a range of electrical frequencies (2, 10, 50 or 100 Hz) and current intensities (1, 2, or 3 mA) were tested, while pulse width (0.1 ms) and treatment duration (20 min) were held constant (see Table 1). Three milliamperes was determined to be the maximum intensity that an unrestrained, unsedated animal can tolerate. In the first set of experiments, 10 and 100 Hz were identified as the optimal frequencies, and 3 mA as the most effective current intensity. In the second set of experiments, frequency (either 10 or 100 Hz) and intensity (3 mA) were held constant while various EA pulse widths (0.1, 1 and 2 ms) and treatment durations (10, 20, and 30 min) were tested. After optimal EA parameters were obtained, we conducted the third set of experiments on point specificity to test and compare the effect of EA at the following four points: (1) the relevant acupoint GB30 on the hind limb, (2) a sham point on the quadriceps at the opposite aspect of GB30 (OAGB30), (3) the acupoint Waiguan, also known as the 5th acupoint on the Triple Energizer Meridian (TE5) [10], on the forelimb, and (4) a sham point on the abdomen, 3 mm lateral to the umbilicus. The same EA parameters, 10 Hz/3 mA/0.1 ms pulse for 20 min, were applied at all four points.

2.3. Acupuncture treatment procedures

EA treatment was conducted following the procedures previously developed in our laboratory [31]. The equivalent of the human acupoint GB30 [10,42] was chosen based on traditional Chinese medicine (TCM) meridian theory [10,42] and on its successful use in our previous studies and studies by others [31,66,72,73]. In humans, GB30 is located at the junction of the lateral 1/3 and medial 2/3 of the distance between the greater trochanter and the hiatus of the sacrum; underneath are the sciatic nerve, inferior gluteal nerve and gluteal muscles [10]. The equivalent anatomical landmarks

were used to locate this point on the rat's hind limbs [31,66]. The animals were gently handled for 30 min each day for 2–3 days and habituated to the acupuncture treatment before the experiment. After cleaning the skin with alcohol swabs, disposable acupuncture needles (gauge #32, 0.5 in. in length) with electrodes soldered to their handles were swiftly inserted bilaterally, approximately 1/2 in. deep, into GB30 by one investigator while the other gently held the animal (Fig. 1A). The needles and the electrodes were stabilized with adhesive tape. The procedure typically lasted less than 20 s and caused little distress to the animal. The EA stimulation was delivered by an electrical stimulator (A300 Pulsemaster, World Precision Instruments) via an isolator (A360D Stimulus Isolator, World Precision Instruments) to convert electrical voltage into constant electrical current. The level of the current was indicated on the panel of the device. The electrical current was measured with an oscilloscope by converting the current to the voltage with a Dummy Load Resistor (World Precision Instruments). To maximize the anti-hyperalgesic effect and treat animals prophylactically, EA treatment was given twice, once right after administration of the CFA and again 2 h post-CFA (Fig. 2). While EA frequency was held constant (at 2, 10, 50 or 100 Hz), intensity was adjusted slowly (over the period of approximately 2 min) to the designated level (i.e. 1, 2, or 3 mA). Mild muscle twitching was observed at these current levels. During EA treatment, each rat was placed under an inverted clear plastic chamber (approximately 5" × 8" × 11") but was neither restrained nor given any anesthetic. The animals remained awake and still during treatment, and no signs of distress were observed (see Fig. 1B). The same treatment procedure was applied for each point tested in the point specificity experiments.

For non-invasive sham control, acupuncture needles were taped on the surface at GB30 and no electrical current was delivered. For invasive sham control, acupuncture needles were inserted into GB30, but no electrical stimulation or manual manipulation was applied. Non-treatment control consisted of CFA injection without treatment intervention.

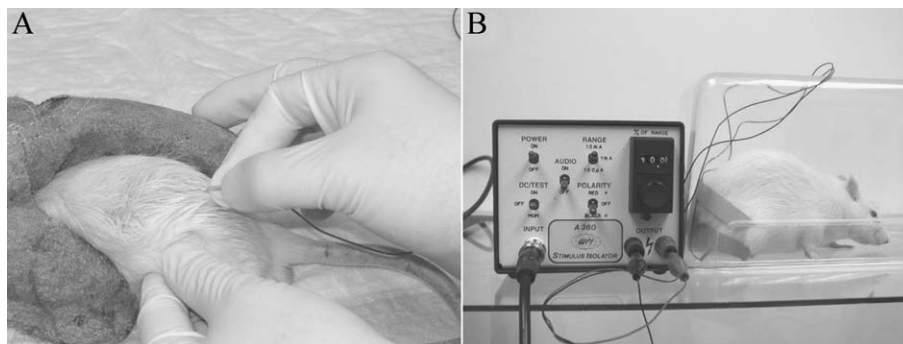


Fig. 1. Photographs showing the acupuncture procedure and EA treatment. Note that rat showed no signs of distress during EA.

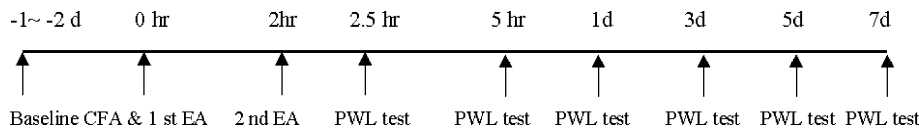


Fig. 2. CFA injection, EA treatment and behavioral test timeline.

2.4. Behavioral test

Rats were tested for PWL by a method previously developed [20,31]. The rats were placed under an inverted clear plastic chamber on the glass surface of the Paw Thermal Stimulator System (UCSD, San Diego) and allowed to acclimatize for 30 min before the test. A radiant heat stimulus was applied onto the plantar surface of each hind paw from underneath the glass floor with a projector lamp bulb (CXL/CXR, 8 V, 50 W). PWL to the nearest 0.1 s was automatically recorded when the rat withdrew its hind paw from the radiant heat stimulus. The intensity of the thermal stimulus was adjusted to derive an average baseline PWL of approximately 10.0 s in naive animals. A 20-s cut-off was used to prevent tissue damage [20,31].

Mean PWL was established by averaging the latency of four tests with a 5-min interval between each test. PWL was measured at pre-CFA and designated intervals post-CFA injection: 2.5, 5h, and 1, 3, 5, and 7 days. The investigator who performed the behavioral tests was blind to the treatment assignments.

2.5. Immunohistochemistry

For the Fos protein immunohistochemistry study, additional rats were divided into the following groups ($n=5$ per group): non-invasive sham control, selected EA treatment groups including an optimal EA parameter group determined by the behavioral tests, and a group of non-inflamed naive rats ($n=3$) treated with optimal EA parameters (Table 2). EA treatment was administered as in the behavioral study.

Fos expression experiments were conducted 2 h after CFA injection and acupuncture treatment [31], because Fos expresses in minutes and peaks in hours following stimulation [46]. Rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 100 ml of saline followed by 500 ml of 4% paraformaldehyde in 0.1 mol/l phosphate buffer at pH 7.4. The lumbar (L4-5) spinal cord was removed, immersed in the same fixative for 2 h and transferred to a 30% sucrose in phosphate buffer for overnight cryoprotection. Thirty micrometer-thick sections were cut with a cryostat at -20°C . Free-floating tissue sections were rinsed in phosphate-buffered saline (PBS) with 0.75% Triton X-100 for 1 h, in PBS with 1% normal goat serum (NGS) for 30 min, then incubated overnight with anti-Fos antibody (1:20,000, Ab-5, Oncogene) in PBS with 1% NGS. After primary antibody incubation, the tissue was exposed to a goat anti-rabbit biotinylated secondary IgG (Vector labs, Burlingame, CA) diluted at 1:400 in PBS for one hour and then to ABC complex (Vector labs) diluted at 1:100 in PBS for an additional hour at room temperature. The sections were visualized with the nickel-diaminobenzidine method (DAB substrate kit, Vector labs). Tissue sections were washed twice, for 20 min each time, in PBS between the antibody incubations. Finally, the sections were mounted on gelatin-coated slides, air-dried, dehydrated in graded alcohol, cleared in xylene, and coverslipped with DPX (Electron Microscopy Sciences).

The labeled Fos-like immunoreactive (FLI) neurons were identified by their densely stained nuclei and were counted under a light microscope (Nikon). The investigator responsible for counting the Fos immunoreactive neurons was blinded to the treatment assignment of each animal. The

Table 2

Effects of EA at various parameters on Fos expression, induced by peripheral inflammation, in the ipsilateral spinal cord ($n=3-5$)^a

Frequency (Hz)	Current (mA)	Duration (min)	Pulse (ms)	I-II M	I-II L	III-IV	V-VI	VII-IX	X
Non-invasive sham				37.42 ± 1.88	11.64 ± 0.92	12.04 ± 1.14	36.73 ± 3.89	3.81 ± 0.74	2.28 ± 0.47
10	3	20	0.1	22.87 ± 3.09**	19.2 ± 2.27*	21.3 ± 3.37*	45.35 ± 5.89	5.61 ± 1.62	2.35 ± 0.49
	3	30	0.1	32.51 ± 1.53	23.17 ± 1.44***	42.53 ± 3.67***	109.35 ± 10.92**	19.89 ± 3.32**	6.50 ± 0.81**
100	3	20	0.1	25.46 ± 1.81**	20.28 ± 1.31**	25.18 ± 1.62**	63.38 ± 4.66**	7.62 ± 0.84**	2.65 ± 0.16
	3	30	0.1	31.16 ± 2.38	20.47 ± 0.83***	31.32 ± 1.59***	75.40 ± 4.23***	12.01 ± 2.11**	5.16 ± 0.34***
10 ^b	3	20	0.1	14.61 ± 3.76**	16.69 ± 2.10	21.81 ± 2.31*	41.88 ± 6.4	6.38 ± 1.63	1.88 ± 0.23

The number of Fos-labeled neurons is presented as mean ± S.E.M.

^a * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. non-invasive sham group.

^b This group was given EA treatment without CFA injection.

following regions of spinal gray matter were delineated: laminae I–II medial, I–II lateral, III–IV, V–VI, VII–IX and X. Control sections without primary antiserum showed no FLI staining.

2.6. Statistical analysis

For the behavioral experiments, the results are presented as mean \pm S.E.M (Fig. 3, Table 2) or %change \pm S.E.M. (Figs. 4 and 5). The %changes were calculated as (post-CFA PWL-baseline PWL)/baseline PWL \times 100%. The actual PWL data were compared to the non-invasive sham control pool using analysis of variance (ANOVA) followed by the Dunnett's post hoc test. $p < 0.05$ was considered significant in all cases.

For immunohistochemistry experiments, 10 randomly selected L4–L5 sections from each animal were counted. The number of labeled Fos-immunoreactive neurons was averaged on either side of the spinal cord for the sections of each individual rat and then for the group. Statistical

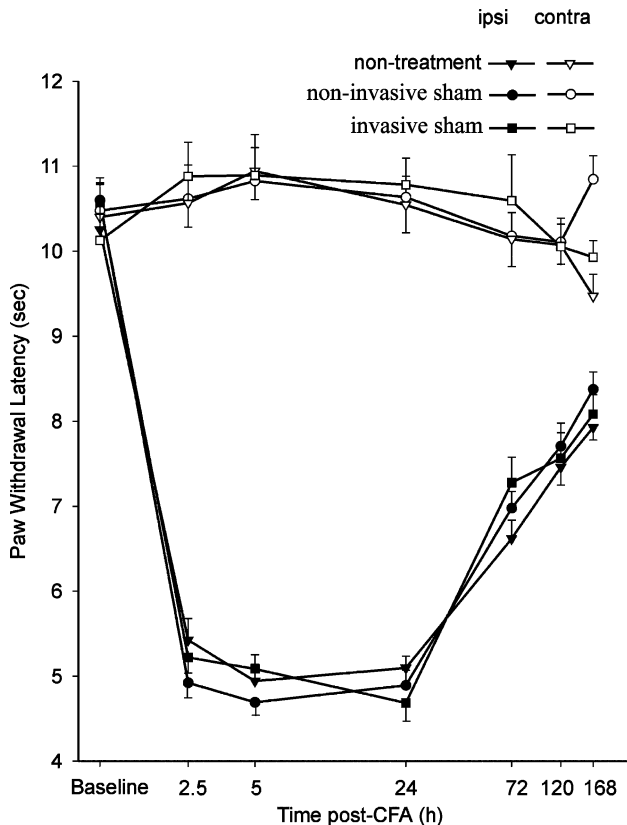


Fig. 3. Comparison of non-treatment to invasive sham and non-invasive sham treatment of inflammatory hyperalgesia. Injecting 0.1 ml of CFA into the left hind paw induced significant ipsilateral hyperalgesia. Invasive sham treatment (acupuncture needle inserted into GB30 without electrical current) had no effect on CFA-induced hyperalgesia in comparison to non-invasive sham (needle taped to skin at GB30) treatment or to non-treatment.

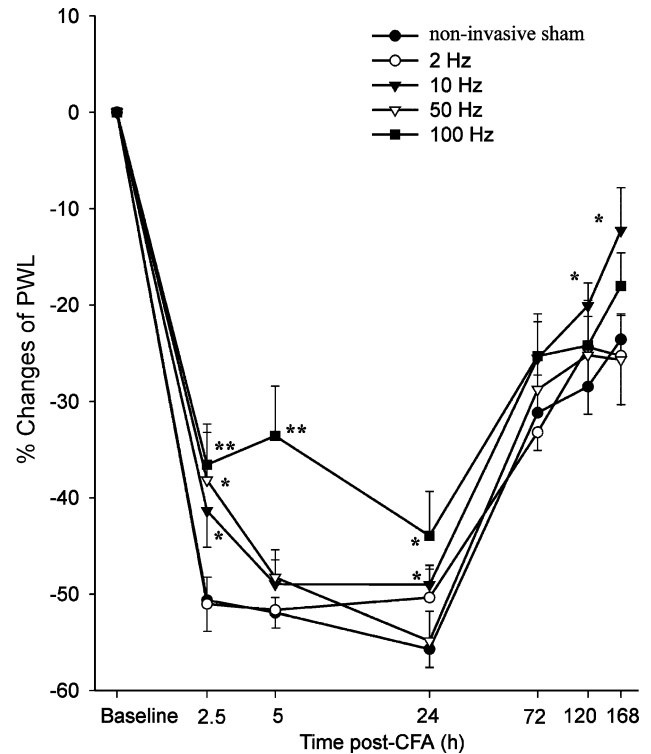


Fig. 4. Effects on withdrawal latency of the inflamed paw at various frequencies of 3 mA EA. At 3 mA, 10, 50 and 100 Hz inhibited inflammatory hyperalgesia, but 2 Hz did not. Note that 10 Hz/3 mA was effective in both early (2–24 h) and late (5–7 days) phases, while 100 and 50 Hz/3 mA were only effective during early phases. EA at 100 Hz/3 mA showed more powerful inhibitory effects than at 50 Hz/3 mA. Asterisks indicate significant differences compared to non-invasive sham. * $p < 0.05$; ** $p < 0.01$.

analysis was made using ANOVA. $p < 0.05$ was considered significant in all cases.

3. Results

3.1. Establishment of control groups in behavioral experiments

In order to ensure the reliability of the control groups, non-invasive sham control ($n = 23$) and invasive sham control groups ($n = 18$) were compared to a non-treatment group ($n = 12$; see Fig. 3). Following CFA injection into the hind paw, all three groups manifested similar courses of hyperalgesia during the 7-day observation period. No significant differences in PWL among non-invasive sham control, invasive sham control and non-treatment groups were observed ($p > 0.05$). There were also no significant PWL changes from baseline in the contralateral paws of these animals ($p > 0.05$). Because data showed no differences in PWL among these three groups, the non-invasive sham treatment group was used as the control for statistical analysis in all experiments (Fig. 3).

3.2. The effect of various EA parameters on PWL

Fig. 4 shows the effect of various EA frequencies—2, 10, 50, and 100 Hz—on PWL at a constant current intensity of 3 mA/0.1 ms for 20 min. EA treatment groups and the non-invasive sham control showed no significant PWL differences at the baseline. EA at 100 Hz produced marked and significantly longer PWL at 2.5, 5 h and 1 day ($p < 0.05$), while 10 Hz EA produced a less potent but prolonged effect, showing increased PWL at 2.5 h, 1, 5, and 7 days compared to non-invasive sham control ($p < 0.05$; Fig. 4, Table 1). EA at 50 Hz increased PWL only at 2.5 h, and 2 Hz EA had no effect ($p > 0.05$) at any time point (Fig. 4, Table 1). EA current intensity studies demonstrate that 2 mA EA at frequencies of 10, 50 and 100 Hz, but not 2 Hz, with 0.1 ms for 20 min, only briefly increased PWL at 2.5 h ($p < 0.05$), and 1 mA showed no effect on PWL at any time point (Table 1).

The optimal frequencies of 10 Hz and 100 Hz at 3 mA/0.1 ms were chosen for testing the effect of treatment duration. At 10 Hz EA, only the 20-min treatment produced a significant effect on PWL as stated above; 10 and 30 min had no effect on PWL at any time point (Table 1). At 100 Hz EA, 10-min stimulation significantly increased PWL at 5 h, 1 and 5 days ($p < 0.05$), and 20-min stimulation showed significant effects up to 1 day ($p < 0.05$; Table 1). However, 30-min stimulation produced no effect on PWL at any time point (Table 1).

In the pulse width experiments, only the EA current intensity of 1 mA was used, because these conscious animals could not tolerate higher intensity at pulse widths beyond 0.1 ms (e.g. 1 or 2 ms). At 1-mA intensity for 20 min, only the 2-ms pulse width at both 100 and 10 Hz significantly increased PWL from 2.5 to 5 h ($p < 0.05$; Table 1). No effect on PWL was found using narrower pulse widths such as 1 and 0.1 ms under this intensity at either higher (100 Hz) or lower (10 Hz) frequencies (Table 1).

3.3. The effect of point specificity

Based on the results of the EA parameter study, the optimal parameters of 10 Hz/3 mA/0.1 ms/20 min were chosen for the point specificity study. Fig. 5 compares the effect of 10 Hz EA at acupoints GB30 and TE5, sham point OAGB30, and an abdominal sham point to non-invasive sham control. The data shows that only treatment at GB30 significantly increased PWL (Fig. 5).

3.4. Effects of EA on inflammation-induced spinal Fos expression

A very limited number of FLI neurons were seen in the vehicle-injected control (data not shown). In EA-treated (3 mA/10 Hz), non-inflamed naive rats ($n = 3$), fewer FLI neurons were found in the medial half of laminae I–II (left: 14.61 ± 3.76 /section; right: 13.19 ± 1.16) but more FLI was seen in laminae III–VI on both sides of the spinal cord (left:

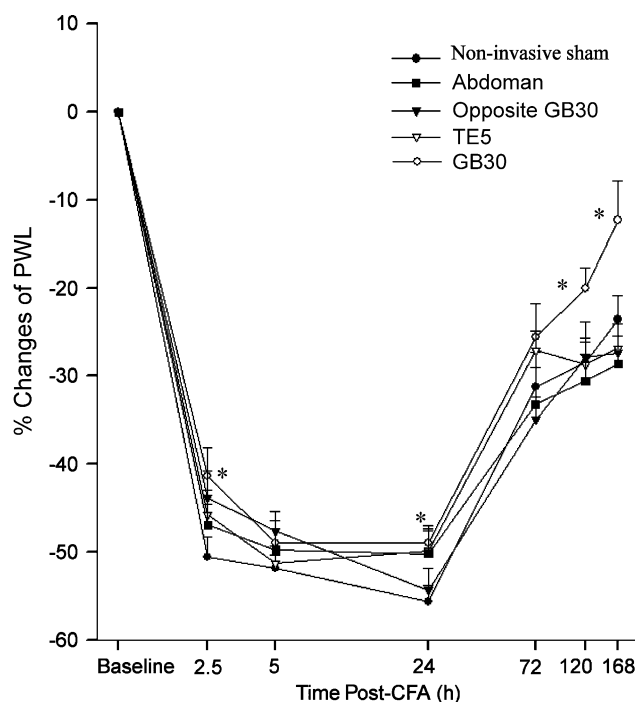


Fig. 5. Effects on withdrawal latency of the inflamed paw of 10 Hz/3 mA/0.1 ms/20 min EA at various points post-CFA. Note that EA at GB30 showed significant anti-hyperalgesia, while EA at other points showed limited anti-hyperalgesia. * $p < 0.05$.

21.81 ± 2.31 /section; right: 20.91 ± 0.67). In CFA-injected non-invasive sham control rats ($n = 5$), FLI was concentrated in the medial half of superficial laminae I–II, 37.42 ± 1.88) and the neck (laminae V–VI, 36.73 ± 3.80) of the dorsal horn of the L4–5 lumbar segments on the side ipsilateral to the inflamed hind paw (Table 2). The effects of the various EA parameters on Fos expression were apparent also mainly on the ipsilateral side as described below and illustrated in Table 2. Both 10 and 100 Hz at 3 mA/0.1 ms/20 min significantly suppressed Fos expression in the medial half of spinal laminae I–II (10 Hz: 22.87 ± 3.09 , $p = 0.01$; and 100 Hz: 25.4 ± 1.81 , $p < 0.01$) compared to non-invasive sham control (37.42 ± 1.88 ; Table 2) and significantly enhanced Fos expression in the lateral half of spinal laminae I–II, laminae III–IV and other regions. (Fig. 6, Table 2).

Unlike the 20-min treatments, 30 min EA at 10 Hz and at 100 Hz did not significantly suppress Fos expression in the medial half of spinal laminae I–II ($p > 0.05$), although it enhanced Fos expression in the lateral half of spinal laminae I–II, III–IX and X compared to control ($p < 0.05$; Table 2).

4. Discussion

4.1. EA parameter profile

Due to increasing concerns regarding the side effects of analgesics and non-steroidal anti-inflammatory drugs [6,53],

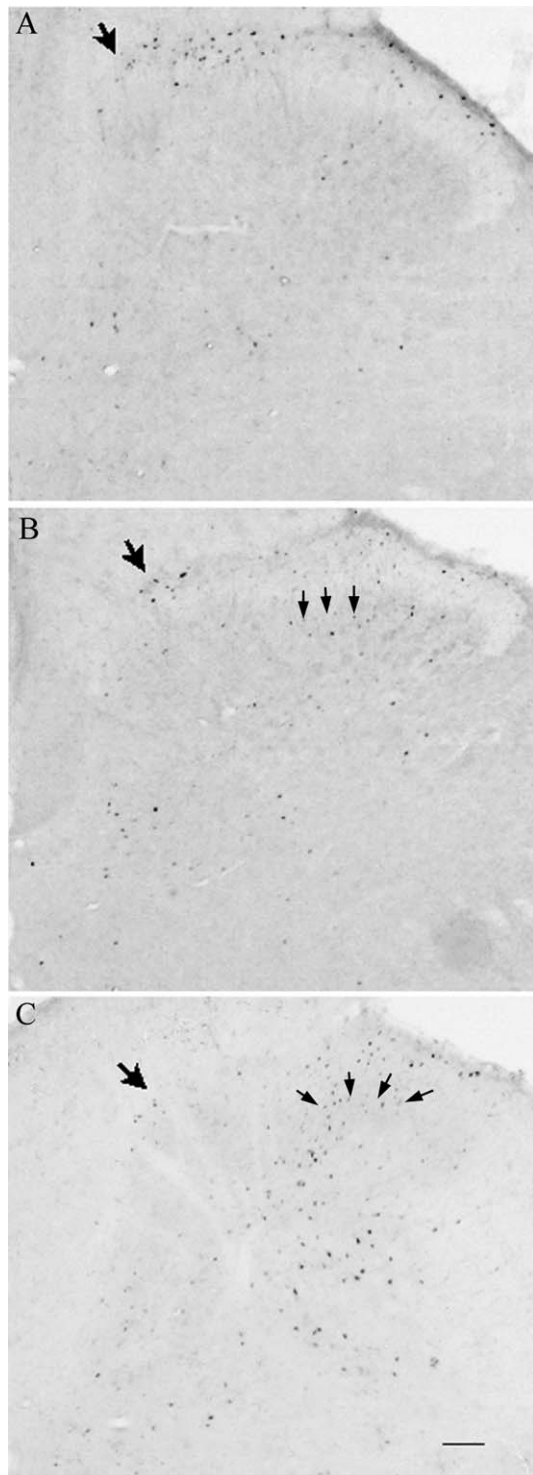


Fig. 6. Photomicrographs illustrating Fos immunoreactive neurons in transverse sections of the lumbar spinal cord 2 h after CFA injection into the left hind paw in a non-invasive sham rat (A) and in rats treated with 3 mA EA for 20 min at 10 Hz (B) or 100 Hz (C). Note that EA inhibited Fos protein expression in the medial part of superficial laminae (big arrows) and induced Fos protein expression in laminae III–IV (small arrows). Scale bar is 50 μ m.

non-invasive, side-effect free, non-pharmacological interventions such as acupuncture, EA or transcutaneous electrical nerve stimulation (TENS) have been widely used clinically as alternative or complimentary therapies for treating chronic pain [41]. The present study is an important initiative toward establishing optimum parameters for the clinical use of EA in chronic pain conditions. We found that the effect of EA is conditioned by multiple factors, including pulse frequency, intensity, pulse width, and treatment duration. We identified an optimal EA parameter profile, 10 Hz/3 mA/0.1 ms/20 min, under conditions which mimic the chronic pain seen clinically (pathological pain in an unseeded, unrestrained animal and multiple assessments at time points over a 7-day period). This profile establishes a valuable clinical reference, which will serve as a basis for both clinical studies and subsequent experiments to explore the mechanisms of EA anti-hyperalgesia.

4.2. EA frequency

The present study demonstrated that the anti-hyperalgesic effect of EA is frequency-dependent. At a given EA current intensity (3 mA), high frequency (100 Hz) EA produced the most potent anti-hyperalgesia in the early stages of CFA-induced hyperalgesia (2.5, 5 h, and 1 day), while low frequency (10 Hz) EA showed a prolonged inhibitory effect, reducing hyperalgesia not only within the first 24 h, but also at 5 and 7 day. This is consistent with previous findings of AA studies on uninjured animal models, in which higher EA frequencies (50–200 Hz) induced shorter lasting analgesia while lower EA frequencies (2–4 Hz) produced analgesia of longer duration [45]. However, it must be noted that the analgesia in these uninjured models lasted less than 1 h at all frequencies.

It has been demonstrated that persistent pain induces long-term neuroplasticity, which enhances neuronal responsiveness to external stimulation [31], and that the opioid system [18] and the hypothalamus–pituitary–adrenal glands (HPA) [26,57] are involved in acupuncture analgesia. Differences between the length of the EA analgesic effect in our inflammatory pain animal model and that of the uninjured animal models reported previously suggest that acupuncture may activate the central nervous system or endocrinology system differently in healthy and pathological conditions. This is consistent with reports that the analgesic effects of mu- and delta-opioid receptor agonists are potentiated during persistent inflammation [22,25]. We hypothesize that EA activates the endogenous pain inhibitory system, opioid system, and the HPA axis to a greater extent in the inflammatory model than in uninjured animals. This may explain clinical findings that the therapeutic effect of acupuncture lasts from days to months in patients with nociceptive pain [8].

It has been established that EA induces release of endogenous opioids, including endomorphin, endorphin, enkephalin and dynorphin [18]. Morphologically, it has

been demonstrated that endomorphin 2-like immunoreactive axon terminals in the spinal cord form synapses with profiles immunostained for the mu-opioid receptor [64]. Enkephalin and dynorphin are localized in the spinal cord neurons [7,21] and mu-, delta- and kappa-opioid receptors are distributed on both presynaptic primary afferent fibers and postsynaptic dorsal horn neurons [1,2,39]. Electrophysiologically, an agonist with mu-, delta-or kappa-opioid activity shows inhibitory actions on excitatory transmission in spinal dorsal horn neurons [15,54]. Further, it has been shown that the mu-opioid receptor agonist inhibits the nociceptive activities of the spinal neurokinin 1 immunoreactive neurons in superficial laminae, which can be depolarized by [Sar(9),Met(O(2))(11)]-substance P, a key neuropeptide for the transmission of noxious inputs [12]. The release of substance P is also blocked by activation of mu- and delta-opioid receptors [5,68]. Studies also showed that EA inhibits a tooth pulp stimulation-evoked increase in the release of immunoreactive SP [67] and that acupuncture reduces electrophysiological spinal neuron response to noxious stimuli [67]. Thus, EA may reduce the release of neurotransmitter (e.g. substance P) and inhibit the activity of noxious neurons through the release of endogenous spinal opioids.

In addition, it has been demonstrated that intracerebroventricular administration of opioid antagonists against mu, delta or kappa receptors blocked EA-produced analgesia, suggesting that supraspinal opioid receptors mediate EA analgesia [9]. The rostral ventromedial medulla (RVM) in the brainstem sends descending inhibitory fibers to the spinal dorsal horn with the most projections to the substantia gelatinosa [63]. Electrical [74] or chemical [23,74] stimulation of the RVM inhibits behavioral responses to noxious stimuli and also suppresses nociceptive response of neurons in the spinal dorsal horn. Unilateral lesion of the ipsilateral dorsolateral funiculus (DLF) blocks the antinociceptive effect of DELT, a delta-opioid receptor agonist, administered into the ventral medial medullary reticular formation in the formalin test [30]. Blockage of opioid receptors in RVM prevents TENS-produced anti-hyperalgesia [28]. These data suggest that EA may activate the supraspinal descending inhibitory system by means of the supraspinal opioid system, leading to anti-hyperalgesia. Hence, we hypothesize that the anti-hyperalgesia induced by both low (i.e. 10 Hz) and high (i.e. 100 Hz) EA frequency is mediated by both the spinal and the supraspinal opioid system [18].

It has been reported that low frequency EA (≤ 10 Hz) induces the release of endorphins and ACTH from the pituitary gland [26,38] and elevates the levels of peripheral cortisol [11,35]. It is known that glucocorticoid (e.g. cortisol) suppresses inflammation by shutting off the production of pro-inflammatory mediators (cytokines) peripherally at the sites of injury [47]. Our previous study [31] demonstrated that EA inhibits CFA-induced peripheral inflammation. Therefore, low frequency EA

may activate the HPA axis [26,45,57], which release corticosterone into the blood. The plasma corticosterone then in turn suppresses inflammation, which facilitates the recovery from the hyperalgesic state.

It is worth noting that in contrast to studies by other investigators, which show that low-frequency (2 Hz) EA stimulation produced analgesia in uninjured rats [44,51,62], our study on the inflammatory animal model showed no anti-hyperalgesia after 2 Hz EA. This discrepancy may be due to the following two factors: (1) we applied much weaker EA current intensity (2–3 mA), which was within the tolerance of an unsedated animal, while AA studies of uninjured animals often used far higher EA intensities (6 to 30 V, or 20–30 mA) and sometimes used anesthetized animals [34,44,51]; and (2) we used the hind paw withdrawal reflex to test the nocifensive response, while most previous studies used the tail flick reflex. Romita and Henry [50] reported that intense stimulation (4 Hz, 20–30 mA, 2-ms pulse for 20 min) at acupoints (GB-31, ST-32 and ST-36) produced a persistent inhibition of the tail flick reflex but had no effect on limb withdrawal reflexes. Since different neuronal circuits are involved in the tail flick and the limb withdrawal reflexes, these neuronal circuits may be modulated differently by low frequency EA.

4.3. EA current intensity

Our data also suggest that acupuncture anti-hyperalgesia is current intensity dependent: at levels between 1 and 3 mA, which are within the tolerance level of a conscious animal, the highest intensity, 3 mA, produced the best anti-hyperalgesia (Table 1). This finding is consistent with the classical acupuncture concept emphasizing that “de qi” (a sore, numb, distended, or heavy sensation felt by the patient at the needling site), induced by forceful manipulation, should be obtained to achieve the best results in an acupuncture treatment [10,42]. It should be noted that a relatively low EA current intensity (i.e. 3 mA) is sufficient to induce a therapeutic effect in the inflammatory pain model, whereas much higher EA intensities (e.g. 6 V or 20–30 mA) were required to induce effective analgesia in uninjured animals [34,51]. This phenomenon may be explained by the fact that tissue injury and inflammation induce an increased excitability in the pain modulatory circuitry [40,48,60], which may be sensitive to EA stimulation.

One pitfall of the EA study is potential distress and stress induced by the EA treatment procedure. Although the EA treatment procedure employed in the present study may have produced some degree of stress, the level of such stress was minimal compared to procedures in which the animals are restrained and treated at higher intensities [17,43]. Furthermore, little anti-hyperalgesia was observed in the control groups suggests that our experimental procedure did not confound the results.

4.4. Treatment duration and pulse width

To obtain maximum anti-hyperalgesia, we delivered EA stimulation twice, first prophylactically at the time of CFA injection and then 2 h post-CFA, after the CFA-induced inflammation had developed. We tested a single EA treatment, administered at the time of injection, but it produced no anti-hyperalgesia in this animal model (data not shown).

The present study demonstrated that, at a given optimal EA parameter, two 20-min EA stimulations produced either the longest therapeutic effect (10 Hz) or most potent short-term effect (100 Hz) when compared to two 10-min or two 30-min stimulations. This finding suggests that longer EA stimulation does not necessarily produce a better therapeutic effect. It is consistent with results of several previous studies by other investigators [36,51]. In a rabbit study investigating the effect of EA on adrenocortical hormone production, a 60-min EA treatment produced less ^{14}C uptake of corticosterone than a 30-min treatment [36]. Romita et al. [51] reported that 40 min of EA stimulation produced less inhibition of the tail withdrawal reflex from noxious radiant heat stimulation than 20 min of EA stimulation. We decided not to conduct 50 Hz for 10 or 30 min after little effect was observed at this frequency for a 20-min treatment.

The effect of EA pulse width has not been systematically evaluated, and there is no consensus in the literature on a standard and appropriate EA pulse width for AA research. A range of pulse widths from 0.1 to 2 ms has been reported, and widths vary from study to study [29,44,51,62]. The electrical pulse width is the length of a constant electrical stimulus. For example, within a given period of time and at the given frequency of 10 Hz EA, a 2-ms pulse width provides 20 times more electrical stimulus than a 0.1-ms pulse width. In the present study, we observed that EA stimulation at 2 ms/10 Hz produced short-term anti-hyperalgesia similar to that produced by 0.1 ms/100 Hz (Table 1). This demonstrates that the prolonged effects observed at 10 Hz/0.1 ms disappear when pulse width increased to 2 ms. It appears that increasing pulse width has the same effect as increasing electrical frequency, as both increase the amount of stimulation to the acupuncture point within a given time unit.

4.5. Acupuncture control and point specificity

Due to the nature of the intervention, determining a control group for an acupuncture study is often a challenge [52,69]. In most acupuncture studies, sham control has consisted of a needle inserted into a non-acupoint or non-relevant acupoint. Such controls in uninjured animal studies have produced contradictory results. Le Bar et al. [3,32] reported that sham acupuncture at a non-acupoint showed an analgesic effect and suggested that this effect may be associated with Diffuse Noxious Inhibitory Controls (DNIC), which produce non-specific analgesia. By contrast, findings of several other studies [24,34] showed that sham

needle insertion had little analgesic effect and could be used as a reliable control for AA studies. Acupuncture clinical trials using needle insertion sham control have also yielded inconclusive results [13,14,16,56], possibly due to non-specific effects produced in the sham control groups.

In the present study, we employed a variant sham control that consisted of an invasive sham control in which the needle was inserted, without manual manipulation or electrical stimulation, into the relevant point, GB30. We also used a non-invasive sham control in which the needle was not inserted into the point. Neither of these controls produced anti-hyperalgesia compared to the untreated group. Although the invasive sham treatment may produce some degree of effect, our study showed that it is insufficient to alter animal behavior, such as PWL, under conditions of inflammatory pain.

We selected the acupoint GB30 for the present study since GB30 is documented as a clinically effective point for treating inflammatory pain such as sciatic or arthritic pain of the lower limb, and its use for such conditions is based on traditional Chinese medical theory and meridian theory for “yang excess” conditions such as inflammation manifested in the “yang meridian” of the lower limb [10,42]. It has been successfully used in previous animal studies, including our own [31,66,72,73].

To test the specificity of GB30 in our persistent inflammatory pain animal model, we compared it to the acupoint TE5 and two non-specific points, OAGB30 and the abdominal point 3 mm from the umbilicus, using the optimal EA parameters determined in experiments 1 and 2. None of the non-specific points produced anti-hyperalgesia equal to that of the specific point GB30, which is consistent with results reported by other investigators [34,59]. For example, Lee and Beitz [34] reported that EA at point St 36, a specific point for analgesia, had significantly better analgesic effects than a nonacupoint control at the hamstring muscle of the hind limb adjacent to the spinal segment. Additionally, Takeshige et al. [59] demonstrated that analgesia was not induced by needling a nonacupoint control in the abdominal muscle (in a distal spinal segment), whereas it was induced by the specific analgesic point St36. We also observed that the distal acupoint TE5 showed little effect on the hyperalgesia of the hind paw. It should be noted that the TE5 point, located on the forelimb, and the inflammation, induced in the hind paw, were not at the same spinal segmental levels, while the GB30 and the inflammation were at the same lumbar spinal level. Since the stimulation of TE5 was ineffective against inflammatory hyperalgesia of the hind paw, these results suggest a convergence and interaction of inputs produced by EA and injury at the same spinal level, which is important for EA-produced attenuation of hyperalgesia.

In the present study, we also tested unilateral GB30 treatment, which showed less anti-hyperalgesia than bilateral GB30 treatment in this animal model (data not shown). Therefore, we used GB30 bilaterally in all experiments.

4.6. Spinal Fos protein expression

The data on spinal Fos protein expression show a positive correlation with the behavioral data. Both optimal EA parameters of 10 and 100 Hz EA not only produced anti-hyperalgesia in the behavioral studies, but also significantly suppressed Fos expression in the medial half of laminae I–II of the spinal cord where nociceptive primary afferents from the hind paw terminate. The data also show that these EA parameters enhanced Fos expression in deeper layers, such as laminae III–IV, where large myelinated fibers (A α and A β) terminate. These results confirm our previous findings and support our assertion that EA selectively inhibits and activates subpopulations of neurons in the spinal cord [31]. Our results are consistent with the findings reported by other investigators that both lower (3–4 Hz) and higher (90–100 Hz) frequency EA inhibit dorsal horn Fos expression induced by mechanical noxious stimulation or hind limb amputation [33,55] and nociceptive response [55]. It is noted that the EA treatment in non-inflamed naive rats resulted in more Fos expression in laminae III–IV than that in the superficial laminae (Table 2). A previous study has demonstrated that non-noxious stimuli such as walking on the rotating rod evoked significant Fos expression in the nucleus proprius of the spinal dorsal horn (laminae III/IV) [27]. The EA treatment in CFA-inflamed rats also induced significant Fos expression in laminae III–IV. Ma and Woolf [37] have shown that after CFA injection into the hind paw, touch stimuli induced significant Fos expression in the deeper laminae of the spinal cord dorsal horn compared to that in similarly stimulated naive rats. These data demonstrate that non-noxious stimuli induce Fos expression in deeper laminae of the spinal cord dorsal horn. Thus, the data that the EA treatment in non-inflamed naive rats resulted in Fos expression in laminae III–IV (Table 2) suggest that EA mainly activate large myelinated fibers. Since the EA treatment in non-inflamed naive rats also induced some Fos expression in laminae I–II, it seems likely that the EA in our study also activated some fine unmyelinated fibers (A δ and C). This is in accordance with a previous study that deletion of C-fibers by capsaicin treatment decreased the analgesic effects of EA [66]. Collectively, these data lead us to posit that the antihyperalgesic effect of EA mainly depends on the activation of large myelinated fibers and less on fine unmyelinated fibers. It is hypothesized that EA-activated spinal neurons convey acupuncture signals to the brain and activate the descending inhibitory system, which in turn inhibits both hyperalgesia and Fos expression in the medial part of laminae I–II in spinal cord [28,70]. Lee's study [33] indicates that opiates may mediate EA-induced suppression of Fos expression.

In correlation with the behavioral study, 30-min EA stimulation had no effect on Fos expression at the medial half of spinal laminae I–II. However, it induced Fos expression in the lateral half of spinal lamina I–II and all regions of laminae III–VI, VII–IX, and X. Research has

established that both the descending inhibitory and the descending facilitator systems modulate the transmission of noxious inputs at the spinal level [49,65]. We hypothesize that longer EA treatment may activate the descending facilitator system, attenuating pain inhibition in our inflammatory pain animal model.

It should be noted that one-third of the rats in our behavioral study and one-fifth in the Fos expression study did not respond (non-responder) to EA treatment (data were included in our statistical analysis). This finding is consistent with the findings of AA studies on uninjured animals reported by other investigators [45].

In conclusion, our EA “dosage response” study demonstrated that EA anti-hyperalgesia is parameter-dependent, 10 Hz (low dosage) being more beneficial than 100 Hz (high dosage) in the long-term treatment of an inflammatory condition, while 100 Hz has potent but short-term effects. The optimal parameters selectively inhibit and activate spinal dorsal horn neurons.

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References

- [1] C. Abbadie, M.C. Lombard, J.M. Besson, J.A. Trafton, A.I. Basbaum, Mu and delta opioid receptor-like immunoreactivity in the cervical spinal cord of the rat after dorsal rhizotomy or neonatal capsaicin: an analysis of pre- and postsynaptic receptor distributions, *Brain Res.* 930 (2002) 150–162.
- [2] D. Besse, M.C. Lombard, J.M. Zajac, B.P. Roques, J.M. Besson, Pre- and postsynaptic distribution of mu, delta and kappa opioid receptors in the superficial layers of the cervical dorsal horn of the rat spinal cord, *Brain Res.* 521 (1990) 15–22.
- [3] Z. Bing, L. Villanueva, D. Le Bars, Acupuncture and diffuse noxious inhibitory controls: naloxone-reversible depression of activities of trigeminal convergent neurons, *Neuroscience* 37 (1990) 809–818.
- [4] D.F. Bossut, D.J. Mayer, Electroacupuncture analgesia in naive rats: effects of brainstem and spinal cord lesions, and role of pituitary–adrenal axis, *Brain Res.* 549 (1991) 52–58.
- [5] S. Bourgoin, J.J. Benoliel, E. Collin, A. Mauborgne, M. Pohl, M. Hamon, F. Cesselin, Opioidergic control of the spinal release of neuropeptides. Possible significance for the analgesic effects of opioids, *Fundam. Clin. Pharmacol.* 8 (1994) 307–321.
- [6] M. Buffum, J.C. Buffum, Nonsteroidal anti-inflammatory drugs in the elderly, *Pain Manag. Nurs.* 1 (2000) 40–50.
- [7] L. Calza, M. Pozza, M. Zanni, C.U. Manzini, E. Manzini, T. Hokfelt, Peptide plasticity in primary sensory neurons and spinal cord during adjuvant-induced arthritis in the rat: an immunocytochemical and in situ hybridization study, *Neuroscience* 82 (1998) 575–589.
- [8] C. Carlsson, Acupuncture mechanisms for clinically relevant long-term effects—reconsideration and a hypothesis, *Acupunct. Med.* 20 (2002) 82–99.

- [9] X.H. Chen, E.B. Geller, M.W. Adler, Electrical stimulation at traditional acupuncture sites in periphery produces brain opioid-receptor-mediated antinociception in rats, *J. Pharmacol. Exp. Ther.* 277 (1996) 654–660.
- [10] X. Cheng (Ed.), *Chinese Acupuncture and Moxibustion*, Foreign Languages Press, Beijing, 1999, 590 pp.
- [11] R. Cheng, L. Mckibbin, R. Buddha, B. Pomeranz, Electroacupuncture elevates blood cortisol levels in naive horses: sham treatment has no effect, *Int. J. Neurosci.* 10 (1980) 95–97.
- [12] O. Cheunsuang, D. Maxwell, R. Morris, Spinal lamina I neurones that express neurokinin 1 receptors: II. Electrophysiological characteristics, responses to primary afferent stimulation and effects of a selective mu-opioid receptor agonist, *Neuroscience* 111 (2002) 423–434.
- [13] J.W. Dundee, R.G. Ghaly, K.M. Bill, W.N. Chestnutt, K.T. Fitzpatrick, A.G. Lynas, Effect of stimulation of the P6 antiemetic point on post-operative nausea and vomiting, *Br. J. Anaesth.* 63 (1989) 612–618.
- [14] M. Fink, E. Wolkstein, M. Karst, A. Gehrke, Acupuncture in chronic epicondylitis: a randomized controlled trial, *Rheumatology* 41 (2002) 205–209.
- [15] S.R. Glum, R.J. Miller, D.L. Hammond, Inhibitory actions of delta 1-, delta 2-, and mu-opioid receptor agonists on excitatory transmission in lamina II neurons of adult rat spinal cord, *J. Neurosci.* 14 (1994) 4965–4971.
- [16] G. Goddard, H. Karibe, C. McNeill, E. Villafuerte, Acupuncture and sham acupuncture reduce muscle pain in myofascial pain patients, *J. Orofac. Pain* 16 (2002) 71–76.
- [17] J.S. Han (Ed.), *The Neurochemical Basis of Pain Relief by Acupuncture*, Chinese Medical and Technology Press, Beijing, 1987, 597 pp.
- [18] J.S. Han, Acupuncture: neuropeptide release produced by electrical stimulation of different frequencies, *Trends Neurosci.* 26 (2003) 17–22.
- [19] Z. Han, Y.H. Jiang, Y. Wan, Y. Wang, J.K. Chang, J.S. Han, Endomorphin-1 mediates 2 Hz but not 100 Hz electroacupuncture analgesia in the rat, *Neurosci. Lett.* 274 (1999) 75–78.
- [20] K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia, *Pain* 32 (1988) 77–88.
- [21] T. Hokfelt, A. Ljungdahl, L. Terenius, R. Elde, G. Nilsson, Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P, *Proc. Natl. Acad. Sci. U. S. A.* 74 (1977) 3081–3085.
- [22] R.W. Hurley, D.L. Hammond, The analgesic effects of supraspinal mu and delta opioid receptor agonists are potentiated during persistent inflammation, *J. Neurosci.* 20 (2000) 1249–1259.
- [23] R.W. Hurley, P. Banfor, D.L. Hammond, Spinal pharmacology of antinociception produced by microinjection of mu or delta opioid receptor agonists in the ventromedial medulla of the rat, *Neuroscience* 118 (2003) 789–796.
- [24] B.G. Hwang, B.I. Min, J.H. Kim, H.S. Na, D.S. Park, Effects of electroacupuncture on the mechanical allodynia in the rat model of neuropathic pain, *Neurosci. Lett.* 320 (2002) 49–52.
- [25] J.L. Hylden, D.A. Thomas, M.J. Iadarola, R.L. Nahin, R. Dubner, Spinal opioid analgesic effects are enhanced in a model of unilateral inflammation/hyperalgesia: possible involvement of noradrenergic mechanisms, *Eur. J. Pharmacol.* 194 (1991) 135–143.
- [26] Y. Iguchi, M. Ozaki, S. Kishioka, S. Tamura, H. Yamamoto, The role of the pituitary in the development of electroacupuncture analgesia in rats, *Folia Pharm. Jpn.* 85 (1985) 453–465.
- [27] L. Jasmin, K.R. Gogas, S.C. Ahlgren, J.D. Levine, A.I. Basbaum, Walking evokes a distinctive pattern of Fos-like immunoreactivity in the caudal brainstem and spinal cord of the rat, *Neuroscience* 58 (1994) 275–286.
- [28] A. Kalra, M.O. Urban, K.A. Sluka, Blockade of opioid receptors in rostral ventral medulla prevents antihyperalgesia produced by transcutaneous electrical nerve stimulation (TENS), *J. Pharmacol. Exp. Ther.* 298 (2001) 257–263.
- [29] S.T. Koo, Y.I. Park, K.S. Lim, K. Chung, J.M. Chung, Acupuncture analgesia in a new rat model of ankle sprain pain, *Pain* 99 (2002) 423–431.
- [30] C.J. Kovelowski, M.H. Ossipov, V.J. Hruby, F. Porreca, Lesions of the dorsolateral funiculus block supraspinal opioid delta receptor mediated antinociception in the rat, *Pain* 83 (1999) 115–122.
- [31] L. Lao, G. Zhang, F. Wei, B.M. Berman, K. Ren, Electroacupuncture attenuates behavioral hyperalgesia and selectively reduces spinal Fos protein expression in rats with persistent inflammation, *J. Pain* 2 (2001) 111–117.
- [32] D. LeBars, L. Villanueva, J.C. Willer, D. Bouhassira, Diffuse noxious inhibitory control (DNIC) in man and animals, *Acupunct. Med.* 9 (1991) 47–56.
- [33] J.H. Lee, A.J. Beitz, Electroacupuncture modifies the expression of c-fos in the spinal cord induced by noxious stimulation, *Brain Res.* 577 (1992) 80–91.
- [34] J.H. Lee, A.J. Beitz, The distribution of brain-stem and spinal cord nuclei associated with different frequencies of electroacupuncture analgesia, *Pain* 52 (1993) 11–28.
- [35] S.C. Lee, S.J. Yin, M.L. Lee, W.J. Tsai, C.B. Sim, Effects of acupuncture on serum cortisol level and dopamine beta-hydroxylase activity in normal Chinese, *Am. J. Chin. Med.* 10 (1982) 62–69.
- [36] Y.-Y. Liao, K. Seto, H. Saito, M. Fujita, M. Kawakami, Effect of acupuncture on adrenocortical hormone production: I. Variation in the ability for adrenocortical hormone production in relation to the duration of acupuncture stimulation, *Am. J. Chin. Med.* 7 (1979) 362–371.
- [37] Q.P. Ma, C.J. Woolf, Basal and touch-evoked fos-like immunoreactivity during experimental inflammation in the rat, *Pain* 67 (1996) 307–316.
- [38] F. Malizia, G. Andreucci, D. Paolucci, F. Crescenzi, A. Fabbri, F. Fraioli, Electroacupuncture and peripheral beta-endorphin and ACTH levels, *Lancet* 2 (1979) 535–536.
- [39] A. Mansour, S. Burke, R.J. Pavlic, H. Akil, S.J. Watson, Immunohistochemical localization of the cloned kappa 1 receptor in the rat CNS and pituitary, *Neuroscience* 71 (1996) 671–690.
- [40] K. Miki, Q. Zhou, W. Guo, Y. Guan, R. Terayama, R. Dubner, K. Ren, Changes in gene expression and neuronal phenotype in brainstem pain modulatory circuitry after inflammation, *J. Neurophysiol.* 87 (2002) 750–760.
- [41] NIH consensus development conference on acupuncture, *JAMA* 280 (1998) 1518–1524.
- [42] J. O'Connor, D. Bensky (Eds.), *Acupuncture: A Comprehensive Text*, Eastland Press, Chicago, 1981, 741 pp.
- [43] B. Pomeranz, Relation of stress-induced analgesia to acupuncture analgesia, *Ann. N.Y. Acad. Sci.* 467 (1986) 444–447.
- [44] B. Pomeranz, L. Bibic, Electroacupuncture suppresses a nociceptive reflex: naltrexone prevents but does not reverse this effect, *Brain Res.* 452 (1988) 227–231.
- [45] B. Pomeranz, G. Stux, *Scientific Bases of Acupuncture*, Springer Verlag, Berlin, Germany, 1988.
- [46] R.W. Presley, D. Menetrey, J.D. Levine, A.I. Basbaum, Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord, *J. Neurosci.* 10 (1990) 323–335.
- [47] A. Ray, D.H. Zhang, M.D. Siegel, P. Ray, Regulation of interleukin-6 gene expression by steroids, *Ann. N.Y. Acad. Sci.* 762 (1995) 79–88.
- [48] K. Ren, R. Dubner, Inflammatory models of pain and hyperalgesia, *ILAR J.* 40 (1999) 111–118.
- [49] K. Ren, R. Dubner, Descending modulation in persistent pain: an update, *Pain* 100 (2002) 1–6.
- [50] V.V. Romita, J.L. Henry, Intense peripheral electrical stimulation differentially inhibits tail vs. limb withdrawal reflexes in the rat, *Brain Res.* 720 (1996) 45–53.
- [51] V.V. Romita, A. Suk, J.L. Henry, Parametric studies on electroacupuncture-like stimulation in a rat model: effects of intensity, frequency, and duration of stimulation on evoked antinociception, *Brain Res. Bull.* 42 (1997) 289–296.

- [52] D. Ryan, Toward improving the reliability of clinical acupuncture trials: arguments against the validity of “sham acupuncture” as controls, *Amer. J. Acupunct.* 27 (1999) 105–109.
- [53] J.M. Scheiman, The impact of nonsteroidal anti-inflammatory drug-induced gastropathy, *Am. J. Manag. Care* 7 (2001) S10–S14.
- [54] R.U. Stiller, B.D. Grubb, H.G. Schaible, Neurophysiological evidence for increased kappa opioidergic control of spinal cord neurons in rats with unilateral inflammation at the ankle, *Eur. J. Neurosci.* 5 (1993) 1520–1527.
- [55] W.Y. Sun, X.W. Li, Z.C. Luo, Y. Liu, Z.Q. Dun, Electroacupuncture at “huatuo Jiaji Point” inhibits the expression of Fos protein in rat spinal cord induced by traumatic pain, *Acupunct. Res.* 21 (1996) 60–64.
- [56] W. Takeda, J. Wessel, Acupuncture for the treatment of pain of osteoarthritic knees, *Arthritis Care Res.* 7 (1994) 118–122.
- [57] C. Takeshige, M. Tsuchiya, S.Y. Guo, T. Sato, Dopaminergic transmission in the hypothalamic arcuate nucleus to produce acupuncture analgesia in correlation with the pituitary gland, *Brain Res. Bull.* 26 (1991) 113–122.
- [59] C. Takeshige, T. Sato, T. Mera, T. Hisamitsu, J. Fang, Descending pain inhibitory system involved in acupuncture analgesia, *Brain Res. Bull.* 29 (1992) 617–634.
- [60] R. Terayama, Y. Guan, R. Dubner, K. Ren, Activity-induced plasticity in brain stem pain modulatory circuitry after inflammation, *Neuro-Report* 11 (2000) 1915–1919.
- [61] G.W. Terman, Y. Shavit, J.W. Lewis, J.T. Cannon, J.C. Liebeskind, Intrinsic mechanism of pain inhibition: activation by stress, *Science* 226 (1984) 1270–1277.
- [62] G.A. Ulett, S. Han, J.S. Han, Electroacupuncture: mechanisms and clinical application, *Biol. Psychiatry* 44 (1998) 129–138.
- [63] H. Wang, M.W. Wessendorf, μ - and δ -opioid receptor mRNAs are expressed in spinally projecting serotonergic and nonserotonergic neurons of the rostral ventral medulla, *J. Comp. Neurol.* 404 (1999) 183–196.
- [64] Q.P. Wang, J.E. Zadina, J.L. Guan, S. Shioda, Morphological evidence of endomorphin as an agonist for the mu-opioid receptor in the rat spinal cord, *Neurosci. Lett.* 341 (2003) 107–110.
- [65] F. Wei, R. Dubner, K. Ren, Nucleus reticularis gigantocellularis and nucleus raphe magnus in the brain stem exert opposite effects on behavioral hyperalgesia and spinal Fos protein expression after peripheral inflammation, *Pain* 80 (1999) 127–141.
- [66] R. Xu, X. Guan, C. Wang, Influence of capsaicin treating sciatic nerve on the pain threshold and the effect of acupuncture analgesia of rats, *Acupunct. Res.* 18 (1993) 280–284.
- [67] N. Yonehara, T. Sawada, H. Matsuura, R. Inoki, Influence of electroacupuncture on the release of substance P and the potential evoked by tooth pulp stimulation in the trigeminal nucleus caudalis of the rabbit, *Neurosci. Lett.* 142 (1992) 53–56.
- [68] V. Zachariou, B.D. Goldstein, Kappa-opioid receptor modulation of the release of substance P in the dorsal horn, *Brain Res.* 706 (1996) 80–88.
- [69] C. Zaslowski, C. Rogers, M. Garvey, D. Ryan, C.X. Yang, S.P. Zhang, Strategies to maintain the credibility of sham acupuncture used as a control treatment in clinical trials, *J. Altern. Complement. Med.* 3 (1997) 257–266.
- [70] R.X. Zhang, R. Wang, J.Y. Cheng, J.T. Qiao, Effects of descending inhibitory systems on the c-fos expression in the rat spinal cord during formalin-induced noxious stimulation, *Neuroscience* 58 (1994) 299–304.
- [71] R.X. Zhang, M.A. Ruda, J.T. Qiao, Pre-emptive intrathecal Mk-801, a non-competitive *N*-methyl-D-aspartate receptor antagonist, inhibits the up-regulation of spinal dynorphin mRNA and hyperalgesia in a rat model of chronic inflammation, *Neurosci. Lett.* 241 (1998) 57–60.
- [72] R.X. Zhang, G. Zhang, X. Wang, B. Berman, K. Ren, L. Lao, The effects of electroacupuncture (EA) on persistent hyperalgesia and inflammation-induced Fos protein expression in rats: a parametric study, *Soc. Neurosci. Abstr.* 27 (2001) 260.
- [73] L.X. Zhu, C.Y. Li, C.F. Ji, B. Yang, W.M. Li, The role of substance P and somatostatin in acupuncture and moxibustion-induced postsynaptic inhibition, *Acupunct. Res.* 18 (1993) 290–295.
- [74] M. Zhuo, G.F. Gebhart, Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat, *J. Neurophysiol.* 78 (1997) 746–758.
- [75] M. Zimmermann, T. Herdegen, Plasticity of the nervous system at the systematic, cellular and molecular levels: a mechanism of chronic pain and hyperalgesia, *Prog. Brain Res.* 110 (1996) 233–259.