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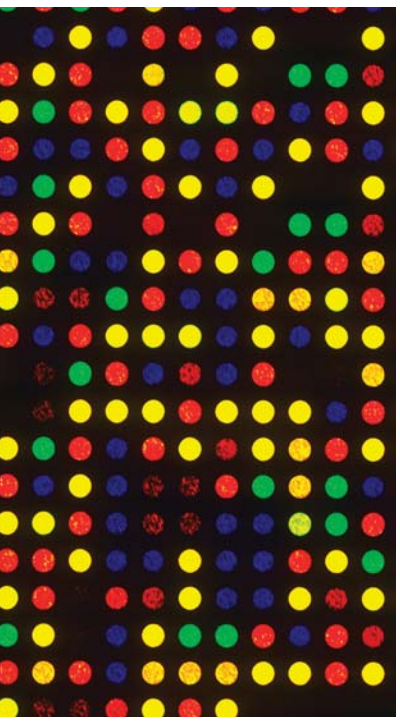
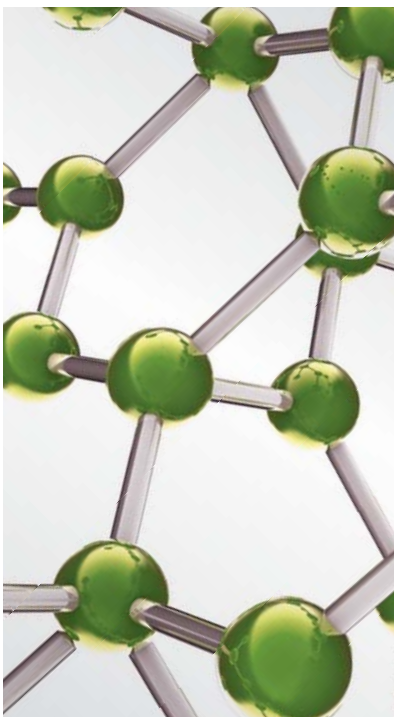
Neuroendocrine Mechanisms of Acupuncture

Guest Editors: Fengxia Liang, Rui Chen, and Edwin L. Cooper



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Editorial Board

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Editorial
Neuroendocrine Mechanisms of Acupuncture

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Acupuncture is currently gaining popularity worldwide as a "complementary" or "alternative therapy." The underlying mechanisms of acupuncture in general require further investigation to be delineated, although acupuncture therapy has been demonstrated to be effective in several clinical areas. Recently, there is a growing focus on the critical role of the brain and a need to explain how acupuncture affects endocrine function through the CNS (central nervous system). This special issue was developed to stimulate the continuing efforts in defining and promoting the neuroendocrine mechanism of acupuncture.

This special issue contains thirteen papers, of which five are related to analgesic effect of acupuncture, and two cover opiate addiction. There are single papers focusing on cardiac, Parkinson's disease, hot flashes, and hypertension. Another deals with brain-modulated effect of auricular acupressure. Finally, one explores the impairments of spatial memory.

"*Effects of electroacupuncture on N-methyl-D-aspartate receptor-related signaling pathway in the spinal cord of normal rats*" by H.-N. Kim provides evidence that calcium influx by N-methyl-D-aspartate receptor activation may play an important role in EA analgesia of normal rats through modulation of the phosphorylation of spinal phosphatidylinositol 3-kinase (PI3K) and cAMP response element-binding protein (CREB). "*Changes in cytokine expression after electroacupuncture in neuropathic rats*" by M. H. Cha revealed that EA reduced the levels of proinflammtory cytokines elevated after nerve injury in peripheral nerves and dorsal root ganglia (DRG). "*Effects of electroacupuncture at BL60 on formalin-induced pain in rats*" by K.-H. Chang showed the effect of EA in relieving inflammatory pain and the possible involved mechanism. Furthermore, "*Effect of electroacupuncture on activation of p38MAPK in spinal dorsal horn in rats with complete Freund's adjuvant-induced inflammatory pain*" by Y. Liang indicated that anti-inflammatory and analgesic effect of EA might be associated with its inhibition of spinal p38 MAPK activation and thereby provide a potential mechanism for the treatment of inflammatory pain by EA. On the other hand, "*Does acupuncture needling induce analgesic effects comparable to diffuse noxious inhibitory controls*?" by J. Schliessbach showed that acupuncture at low pain stimulus intensity did not produce a DNIC-like effect comparable to a classical, painful DNIC-test, indicating that the penetration of an acupuncture needle seems not to induce an analgesic effect mainly mediated by DNIC.

"*Acupuncture for the treatment of opiate addiction*" by J. G. Lin is a systematic review of randomized clinical trials which applied acupuncture for treating opiate addiction and analysed the possible mechanism underlying the effect of acupuncture. "*Electroacupuncture suppresses discrete cue-evoked heroin-seeking and Fos protein expression in the nucleus accumbens core in rats*" by S. Liu highlights the therapeutic benefit of EA in preventing relapse to drug addiction, through the results that EA stimulation reduced active responses elicited by discrete cues and attenuated Fos expression in the core but not the shell of the nucleus accumbens. "*Electroacupuncture at PC6 (Neiguan) improves extracellular signal-regulated kinase signaling pathways through the regulation of neuroendocrine cytokines in myocardial hypertrophic rats*" by J. Li revealed that EA could improve cardiac

function in rats with myocardial hypertrophy by modulating upstream neuroendocrine cytokines that regulate extracellular signal-regulated kinase (ERK) signaling. This proposes a mechanism underlying EA's effect in treating cardiac diseases.

The cortical and striatal gene expression profile of 100 Hz electro-acupuncture treatment in 6-hydroxydopamine-induced Parkinson's disease model by Li-Rong Huo applied high-throughput microarray analysis to analyze gene expressions. This study suggested that EA may induce recovery of homeostasis in the transcript network and many regulated functional clusters in the cortex and striatum; this characteristic underlies the mechanism of EA's effect in improving behavioral characteristics of PD rats. "*Acupuncture as treatment of hot flashes and the possible role of calcitonin gene-related peptide*" by A.-C. S. Holm discussed the role of CGRP involved in acupuncture as an alternative treatment for hot flashes, based on the evidence for connections between the opioid system and the release of CGRP. "*Neuroendocrine mechanisms of acupuncture in the treatment of hypertension*" by W. Zhou discussed current knowledge of acupuncture effects on central nervous system and how they contribute to regulation of acupuncture on the endocrine system. This approach provides a perspective on treating of hypertension. "*Brain-modulated effects of auricular acupressure on the regulation of autonomic function in healthy volunteers*" by X. Gao investigated the acute effect of ear acupressure on autonomic function, indicating that this approach of auricular acupressure was based on intensification of the related mechanism of blood pressure regulation. "*Acupuncture stimulation alleviates corticosterone-induced impairments of spatial memory and cholinergic neurons in rats*" by B. Lee demonstrated that stimulation of HT7 acupoint produced significant neuroprotective activity against the neuronal impairment and memory dysfunction by immune responses and gene expression.

Due to recent development not only in invasive methodology such as PET in human and animals and optogenetic technique, but also in molecular biology, the research of acupuncture at the whole organismic level and an in-depth analysis becomes more available. It is essential to focus on some critical factors which impact the effect of acupuncture, such as biophysical action of acupoints, combination of acupoints, and acupuncture method. A recent study proposed a neurophysiological mechanism to explain the beneficial effects of acupuncture based on the stimulated purinergic signalling by acupuncture. This potential provokes some scientists interested in acupuncture to investigate further in this rapidly expanding field. Finally, we should never forget the need for careful consideration of the role of placebo in this and other CAM analyses.

Of course, the selected topics and papers are not a comprehensive representation of the area of this special issue. Nonetheless, they represent the rich and many-faceted knowledge that we have the pleasure of sharing with the readers.

Acknowledgments

We would like to express appreciation to the authors for their excellent contributions and patience in assisting us. Finally, the fundamental work of all reviewers on these papers is also very greatly acknowledged.

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Research Article

Effects of Electroacupuncture at BL60 on Formalin-Induced Pain in Rats

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Acupuncture was used to treat symptoms of pain in the ancient orient. The present study was conducted to determine the effects of electroacupuncture (EA) at the BL60 acupoint on male Sprague-Dawley rats. Each rat received EA at BL60 acupoint before formalin injection. Behavioral responses were recorded using a video camera and c-Fos immunohistochemistry was performed thereafter. Treatment of EA at BL60 significantly inhibited flinching behavior and c-fos expression induced by formalin injection into the paw, compared to a control group. These results suggest that electroacupuncture at BL60 acupoint may be effective in relieving inflammatory pain.

1. Introduction

Acupuncture was used to treat symptoms of pain in the ancient orient. Currently, acupuncture has garnered increasing interest as a therapeutic method for treating pain. Recently, electroacupuncture, applying electrical stimulation, is being actively studied. Electroacupuncture was developed to resolve the problems of manual acupuncture, that is, the inconvenient twirling procedure and the difficulty of maintaining constant frequency [1].

The BL60 (Kunlun) acupoint has been shown to be involved in visual information processing [2]. Deadman et al. [3] reported that acupuncture stimulation at BL60 acupoint was effective in treating eye disorders as well as head disorders. Studies indicated that the BL60 acupoint has analgesic effects on chronic low back pain [4] as well as hind limb pain [5]. Li et al. [6] reported that bilateral EA treatment at both BL60 and ST36 acupoints was effective in alleviating inflammatory pain. However, there have been no reports that electroacupuncture at BL60 by itself has an analgesic effect on inflammatory pain.

Inflammatory pain is known to cause abnormality in the nervous system, thereby causing consistent pain [7]. Formalin has been widely used in experiments with animal models of inflammatory pain because of several strong points: (1) it induces behaviors that can be easily observable [7]; (2) the responses to the moderate and continuous pain can be measured [8, 9]; (3) anesthesia is not necessary, so that the behaviors of freely moving animals can be observed [8, 9]. The early phase of pain responses after formalin injection is due to the direct injury of tissues, reflecting nociceptive pain, while the late phase is due to peripheral inflammation and central sensitization [10].

One of the methods used to measure pain and analgesia in animal experiments is the immunohistochemical detection of the c-Fos protein encoded by c-fos, an oncogene. The c-fos gene is an immediate early gene and is rapidly expressed in neurons of the central nervous system when nociceptive stimuli are applied to a peripheral area; for this reason c-Fos is currently widely used as a marker of pain [11, 12].

The present study was performed to determine if electroacupuncture at the BL60 acupoint would alleviate

nociceptive pain in the early phase and inflammatory pain in the late phase of responses to formalin injection. In order to determine the effects of electroacupuncture on formalin-induced pain, a behavioral test and c-Fos immunohistochemical study were conducted.

2. Methods

2.1. Animals. Male Sprague-Dawley rats weighing about 250~300 g were used in this study. The animals were housed in cages equipped with the barrier system for SPF (specific pathogen free) animals. The system automatically maintained proper temperature (22 ± 2°C), humidity (50 ± 10%) and lighting (12 hr of light/dark). The beddings of the cages were regularly changed (twice a week). All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System.

2.2. Electroacupuncture. A stainless steel needle (diameter 0.25 mm; length 15 mm; Dongbang Acupuncture Inc., Boryeong, Republic of Korea) was used for electroacupuncture [13]. The needle was connected to a wire for better direct application of electric stimuli. The accurate application of electrical stimulation was confirmed in a preliminary study with the experimental animals. The acupoint selected for acupuncture was the BL60 acupoint, which is located at the ankle joint level between the external malleolus and tendo calcaneus in the hind limb [5].

The experiment was designed to determine the efficacy of pretreatment of electroacupuncture (acupuncture treatment before formalin injection) applied to the BL60 acupoint ipsilateral to formalin injection. For this purpose, the animals were divided into three groups: Group 1 was injected with formalin only (Formalin), Group 2 was treated with electrical stimulation by electroacupuncture before formalin injection (EA-For), and Group 3 was treated with needle insertion at the acupoint before formalin injection, but was not treated with real electrical stimulation (Sham-For).

All the animals received inhalation anesthesia of 2% enflurane (95% O_2/5% CO_2) prior to electroacupuncture and/or formalin injection. For the EA-For group, which was to receive electroacupuncture at the BL60 acupoint, electrical stimulation was applied using an electrical stimulator (A385, World Precision Instruments, Sarasota, FL, USA) and the Pulsemaster (A300, World Precision Instruments, Sarasota, FL, USA) at 1 Hz (2 ms pulses, 3 mA) for 10 min [14, 15]. The depth of insertion was 2-3 mm. The Sham-For group was kept for 10 minutes with the needle inserted at the BL60 acupoint but no electrical stimulation was applied. The Formalin group was kept under the inhalation anesthesia without any treatment before formalin injection. After electroacupuncture, the animals were kept awake for 10 min in order to acclimate to the test environment.

2.3. Behavioral Test. Formalin is an aqueous solution of formaldehyde (37%). A dilution of formalin in saline was used for the experiments herein: 50 μL of 5% formalin was used for injection, which is widely used to induce maximum pain response [16, 17], while avoiding adverse phenomena, such as ceiling effect, backward walking, and freezing [16, 17].

After electroacupuncture, the experimental animals received formalin injection beneath the left plantar skin using a 29 gauge insulin syringe. Then, the animals were immediately put in an observatory chamber (46 × 26 × 20 cm) and video-recorded for 60 min. The pain behaviors of the rats were analyzed by counting the flinching frequency of formalin-injected paws (number of flinches as 5 min passes) throughout the recording time. After video-recording, the rats were immediately subjected to c-Fos immunostaining.

2.4. c-Fos Immunohistochemistry. Normal rats as well as the three groups of rats above were used for c-Fos immunohistochemical study in order to observe changes in c-Fos immunoreactivity by comparing the experimental groups with normal rats. Under anesthesia with 25% urethane (1.25 g/kg, i.p.), the experimental animals were perfused through with 0.9% saline and 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.2), and the L5 spinal cord section was removed. The removed tissues were fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.2) for 4 hr at 4°C and then kept in 30% sucrose solution overnight at 4°C. The tissues were cut into 50 μm-thick slices using a Cryocut Microtome (Microm/HM500V, Walldorf, Germany). The total length of the L5 area was about 4000 μm, so that 80 slices of 50 μm thickness were obtained, every 5th slice of which was selected for study for a total of 16 slices. These 16 slices were subjected to free floating staining in the 24-well plates containing 1x phosphate-buffered saline. The tissues reacted in a solution of 30% methanol with 1% H_2O_2 for 30 minutes, followed by reactions in a solution with 3% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.3% Triton-X for 30 min; and then, the c-Fos antibody (c-Fos anti-rat polyclonal IgG, 1500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was treated as the primary antibody and the mixture was kept overnight at 4°C. Then, biotinylated anti-rabbit IgG (1 : 200, Vector, Burlingame, CA, USA) was treated as the secondary antibody and the mixture was allowed to react at room temperature for 2 hr, followed by treatment using the ABC kit (Vector, Burlingame, CA, USA) for 1 hr. 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) was used for staining and the degree of staining was checked using a microscope (BX40 microscope, Olympus, Tokyo, Japan).

The number of the c-Fos positive neurons was obtained by calculating the mean number of neurons in 4 slices out of the 16 slices that went through the immunohistochemical procedure. A microscope (BX40, Olympus, Tokyo, Japan) was used at a magnification of 10x to check the lamina on the ipsilateral site to the formalin injection, in order to distinguish between lamina I-II and III–VI areas [18]. The MetaMorph software (ver. 4.6, Universal Imaging, Downingtown, PA, USA) was used to count the stained neurons, along with a microscope (BX51, Olympus) mounted with a CCD

camera (Cool SNAP Photometrics, Roper Scientific, Tucson, AZ, USA) at a magnification of 10x.

2.5. Statistical Analysis. The SPSS 15.0 program (SPSS Inc., Chicago, IL, USA) was used to compare the pain behaviors and the number of the c-Fos positive neurons in each experiment. Data were presented as mean ± SEM, and statistical significance was given when P values were less than 0.05. As for the observation of behavioral responses, the flinching frequencies of each group, divided into the early phase and late phase responses, were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (2-sided) for post hoc analysis. As for the immunostaining, the spinal cords were classified into lamina I-II and III–VI, and the numbers of c-fos positive neurons were compared by the same statistical method mentioned above.

3. Results

3.1. Behavioral Test. After formalin injection into the plantar paw, rats typically showed vivid flinching behaviors. These responses were characterized into the early phase (short rise and decay) and late phase (long-lasting for about 1 hr) responses. Thus, we classified the flinching behaviors into two phases and compared the effects of electroacupuncture on each phase of the formalin-induced pain behaviors.

The frequencies of the flinching behavior in the early phase were as follows: Group 1 37.04 ± 16.76 in the group injected only with formalin (Formalin; $n = 12$); Group 2 33.85 ± 16.24 in the group treated with electroacupuncture before formalin injection (EA-For; $n = 17$); and Group 3 33.96 ± 10.28 in the group with needle insertion before formalin injection, but not treated with electrical stimulation (Sham-For; $n = 13$). As shown in Figure 1, flinching behaviors did not show any significant difference in the early phase ($F_{2,31} = 3.197$, $P > 0.05$).

The frequencies of the flinching behavior in the late phase were as follows: Group 1 329.88 ± 73.56 in the group injected only with formalin (Formalin; $n = 12$); Group 2 243.44 ± 109.33 in the group treated with electroacupuncture before formalin injection (EA-For; $n = 17$); and Group 3 291.42 ± 99.50 in the group with needle insertion before formalin injection, but not treated with electrical stimulation (Sham-For; $n = 13$). The EA-For group showed statistically significant decrease in pain response behaviors compared to the Formalin group ($F_{2,31} = 5.017$, $P < 0.05$; one-way ANOVA followed by Dunnett's multiple comparison) (Figure 1). These data showed that the pre-treatment with electroacupuncture at BL60 significantly inhibited flinching behavior, compared to the control group.

3.2. c-Fos Immunohistochemistry. The number of c-Fos positive neurons in the dorsal horn was counted separately for superficial layers (lamina I-II) and deep layers (III–VI). The representative photographs of the c-Fos positive neurons of the individual groups are shown in Figure 2. As shown in Figure 2, the EA-For group showed a remarkable decrease

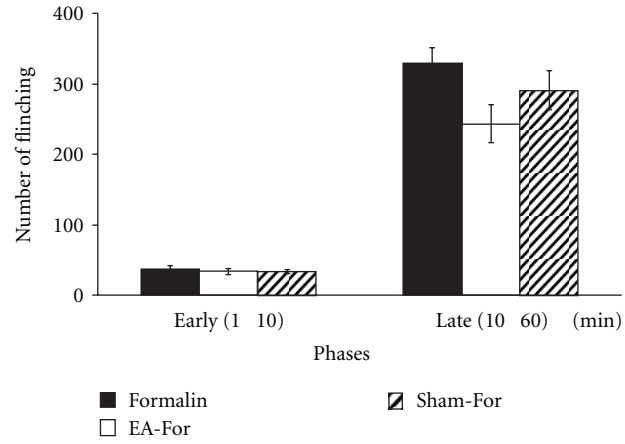

Figure 1: Effects of electroacupuncture on flinching numbers. Bar graphs were divided into early (0–10 min) and late phases (10–60 min). Experimental groups: Formalin formalin injection only group; EA-For electroacupuncture treatment at BL60 before formalin injection; Sham-For acupuncture needle insertion at BL60 but no electric stimulation before formalin injection. In the early phase, there were no significant differences between the groups. However, flinching numbers of the EA-For group were significantly decreased in the late phase. Each bar represents the group mean ± SEM (*$P < 0.05$).

in the number of c-Fos positive neurons, compared to the Formalin group.

In order to compare the level of c-Fos immunoreactivity in the Formalin group Group 1 with pain-free animals, a normal naïve animal group Group 0 was added to this study. The numbers of c-Fos positive neurons in lamina I-II were as follows: Group 0, 250 ± 30.68 in the group with no treatment (Normal; $n = 4$); Group 1, 2501 ± 255.54 in the group injected only with formalin (Formalin; $n = 7$); Group 2, 1163.12 ± 219.16 in the group treated with electroacupuncture before formalin injection (EA-For; $n = 8$); and Group 3, 1893 ± 132.91 in the group with needle insertion before formalin injection, but not treated with electrical stimulation (Sham-For; $n = 10$) (left in Figure 3).

For lamina III–VI, the numbers were 268.75 ± 45.38 (Normal; Group 0), 2143.57 ± 200.389 (Formalin; Group 1), 985.62 ± 195.79 (EA-For; Group 2), and 1762 ± 171.84 (Sham-For; Group 3), respectively (middle in Figure 3). The total numbers of the c-Fos positive neurons in lamina I through VI were 518.75 ± 71.97, 4645 ± 372.30, 2148.75 ± 406.42, and 3655 ± 274.78, respectively (right in Figure 3). When the Normal group Group 0 was compared to each group, the Formalin group Group 1 and the Sham-For group Group 3 showed a statistically significant increase in the number of the c-Fos-expressed neurons in each lamina (I-II: $F_{3,17} = 23.316$; III–VI: $F_{3,17} = 23.546$; I–VI: $F_{3,17} = 33.101$, $P < 0.05$; one-way ANOVA followed by Dunnett's multiple comparison). When the Formalin group Group 1, the EA-For group Group 2, and the Sham-For group Group 3 were compared, however, the EA-For group Group 2 showed a statistically significant decrease in the number of c-Fos positive neurons compared to the Formalin

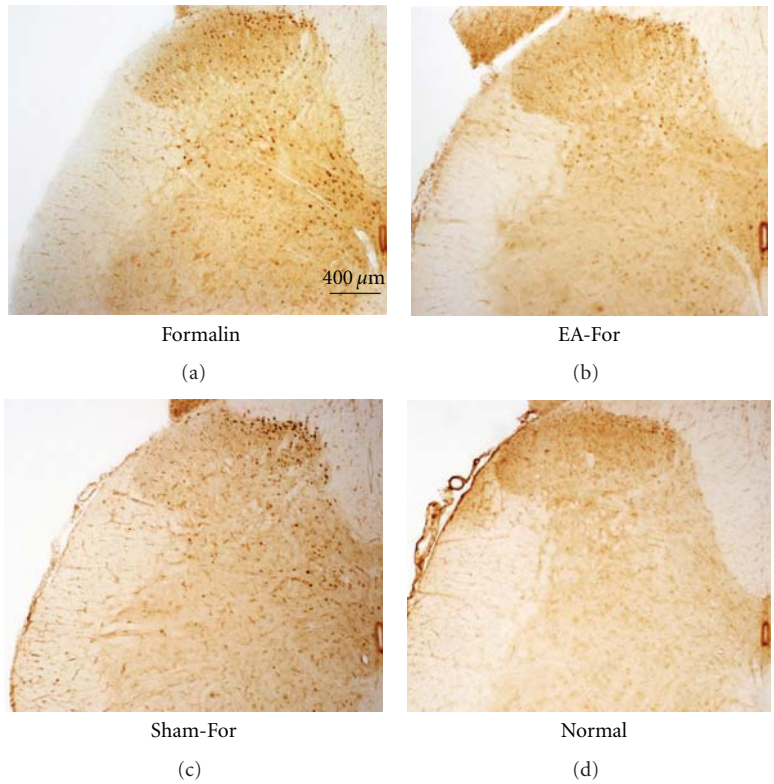

Figure 2: Representative photographs of c-Fos positive neurons in the spinal cord dorsal horn after electroacupuncture. (a) Formalin: formalin injection only group. (b) EA-For, electroacupuncture treatment at BL60 before formalin injection. (c) Sham-For, acupuncture needle insertion at BL60 but no electrical stimulation before formalin injection. (d) Normal: no treatment group.

group Group 1 in each lamina (I-II: $F_{2,14} = 14.711$; III–VI: $F_{2,14} = 15.647$; I–VI: $F_{2,14} = 21.340$, $P < 0.05$; one-way ANOVA followed by Dunnett's multiple comparison). These results showed that the pretreatment of EA at BL60 significantly inhibited c-Fos expression induced by formalin injection into the paw, compared to control group.

4. Discussion

Acupuncture has been used in Western medicine as well as Oriental medicine as an alternative treatment for pain-related disorders. In particular, electroacupuncture was developed to overcome the disadvantages of manual acupuncture, that is, the inconvenient twirling procedure and the difficulty of maintaining constant frequency.

Of inflammatory pain models, a formalin model was used in the present study in order to compare distinct biphasic nociceptive behavioral responses at both early and late phases after formalin injection. Electroacupuncture was pretreated before formalin injection in order to compare the effects of EA stimulation on both phases of formalin-induced pain but was not posttreated because the effects of electroacupuncture stimulation on both the early and late phases only would be unobservable if electroacupuncture was applied after formalin injection, even though post-treatment resembles a natural clinical condition.

In the present study, EA stimulation with low-frequency (1 Hz, 3 mA) at BL60 acupoint inhibited flinching behaviors in the late phase, but not in the early phase after formalin injection in rats. In electroacupuncture treatment, it has been shown that low-frequency stimulation produces prolonged analgesic effects relatively late in electroacupuncture; however, high-frequency stimulation produces short-lasting analgesic effects immediately after initiation of electroacupuncture [1]. Similarly, Lao et al. [18] showed that high frequency electroacupuncture produces the most potent anti-hyperalgesia in the early stage of complete Freund's adjuvant- (CFA-)induced hyperalgesia, while low-frequency electroacupuncture produces a prolonged inhibitory effect to reduce hyperalgesia.

As used in the present study, electrical stimulation with the intensity of 3 mA is known to be the maximum that conscious animals can withstand [18]. It was observed that the muscles around at the tip of the experimental animals' feet were trembling when electrical stimulation was applied with the intensity of 3 mA. It was reported that this intensity of electroacupuncture stimulation has a therapeutic effect in inflammatory pain models [18].

According to Chang et al. [19], analgesic effects were observed at both early and late phases when electroacupuncture (2, 10, 100 Hz frequency, 3 mA intensity, for 5 min) was applied bilaterally at the ST36 (Zusanli) acupoint in mice. Kim et al. [17] reported that bilateral electroacupuncture

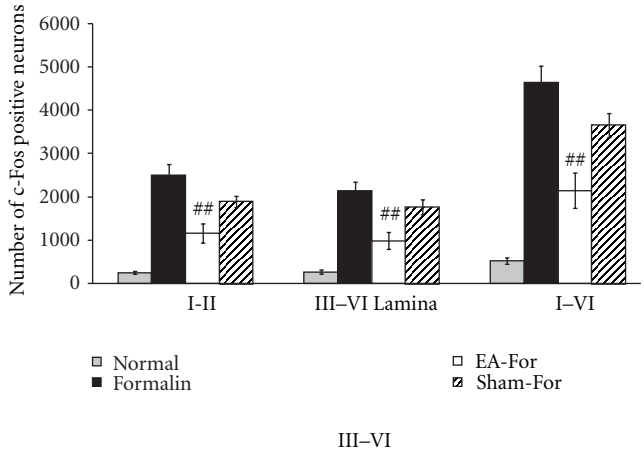

Figure 3: The number of c-Fos positive neurons in different groups. C-Fos positive cells were counted by MetaMorph software in lamina I-II and lamina III–VI at the L5 segment of the spinal cord ipsilateral to the site of formalin injection. Experimental groups were classified as follows: Normal: no treatment group; Formalin: formalin injection only group; EA-For, electroacupuncture treatment at BL60 before formalin injection; Sham-For, acupuncture needle insertion at BL60 but no electric stimulation before formalin injection. In each region, there was a significant decrease of c-Fos positive neurons when electroacupuncture was treated at BL60 compared to the Formalin group. On the other hand, in a comparison between Normal group and the other groups, there were notable increases in c-Fos positive neurons in the EA-For group and Sham-For group. Each bar represents the group mean ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$ for comparison between Normal group and all the other groups, one-way ANOVA followed by Dunnett's post hoc multiple comparison: $^{\#\#}P < 0.01$ for comparison between Formalin group and EA-For or Sham-For group, one-way ANOVA followed by Dunnett's post hoc multiple comparison).

(2 ms, 10 Hz, 3 mA, for 30 min) at both HE7 (Shenmen) and PE7 (Daling) acupoints produces analgesic effects lasting nearly 1 hour in both early and late phases of formalin-induced pain in rats. These results indicate that the pain-relieving effects of electroacupuncture persist after EA stimulation cessation.

However, our behavioral results herein are not fully consistent with other studies [17, 19] that observed analgesic effects of EA in both early and late phases. The discrepancy may be attributable to differences in the acupoints treated, parameters in electroacupuncture stimulation, laterality, or experimental animals.

However, aspirin [20], nonsteroidal anti-inflammatory drugs (NSIADs) such as indomethacin and naproxen [21], compound 48/80, a histamine and serotonin depleter [9], and spinal anesthesia [22] can reduce late phase but not early phase formalin-induced pain. In relation to these studies, our study suggests that electroacupuncture stimulation at BL60 acupoint may be effective in relieving persistent inflammatory pain rather than in relieving acute pain produced by immediate activation of nociceptors in formalin test models.

There have been numerous studies that observed the pain-relieving effects of acupuncture [13, 16, 17, 23–25]. To our knowledge, however, there are no reports except for Kim et al. [26] and Zou et al. [27] to study the pain-relieving effects of acupuncture at the BL60 acupoint. Disappointingly, Kim et al. [26] did not find any analgesic effect with acupuncture at BL60. According to Zou et al. [27], patients with lumbar intervertebral disc herniation treated with EA combined with acupoint-injection at L4 Jiaji (EX-B2), L5 Jiaji (EX-B2), Zhibian (BL54), Huantiao (GB30), Yanglingquan (GB34), Wizhong (BLA0), and Kunlun (BL60) showed significant improvement of pain, compared to a control group treated with electroacupuncture only. However, their study did not test the effects of electroacupuncture at BL60 acupoint itself. Therefore, our study may be the first to show the analgesic effects of electroacupuncture at BL60 acupoint in inflammatory pain.

Since Hunt et al. [28] reported c-Fos expression in response to peripheral nociceptive stimuli, c-Fos has been used as a neuronal marker of pain. Even though the role and significance of c-Fos expression in pain transmission is not fully understood [29], the remarkable correlation between the distribution of c-Fos positive neurons in the superficial layers (laminae I and II) of the lumbar spinal cord after formalin injection and the spinal distribution of pain-related afferent fibers innervating the plantar surface of the hind paw [30–32] suggests that the Fos protein may be expressed as a result of postsynaptic activation by pain-related afferents. In deep layers, few neurons receive direct pain-related afferents while most neurons receive convergent inputs from superficial layers [33], suggesting that Fos expression at the spinal cord reflects activation of second order neurons related to transmission of pain. Therefore, identification of c-Fos expression in the spinal dorsal horn, associated with paw flinching responses induced by formalin injection, can provide morphological anatomic evidence of pain and/or analgesia [34].

In relation to c-Fos expression in formalin-induced pain models, c-Fos seems to be expressed in superficial layers in relation to early phase pain and in deep layers in relation to late phase pain, while overall c-Fos expression appears to be concentrated in superficial lamina I-II [35]. However, in the present study, electroacupuncture treatment at BL60 acupoint reduced the number of c-Fos positive neurons in not only superficial layer laminae I-II but also in deep layer laminae III–VI of the spinal cord at the L4-5 level, compared to a formalin injection only group. This result is consistent with other studies which observed a reduction of c-Fos expression in both superficial and deep layers of the spinal cord dorsal horn in the electroacupuncture-treated groups of a carrageenan-induced pain rat model [16, 36] and a formalin model [17]. Furthermore, it is considered that the mechanism of pain suppression by electroacupuncture at BL-60 acupoint is due to its action at the spinal cord level, but not at the supra-spinal level. Also, the fact that there was a significant decrease in the number of c-Fos positive neurons in all parts of the lamina I-II and III–VI indicated that there is a close relationship between flinching responses and the number of c-Fos positive neurons during the treatment of electroacupuncture.

However, the mechanisms of analgesic effects of electroacupuncture treatment on formalin-induced pain are

uncertain. Our results showed significant decrease in flinching response after the pretreatment of electroacupuncture. In our accompanying study, however, there was no significant reduction in licking response after the pretreatment of electric acupuncture (data are not shown). The flinching response is regarded to be a simple flexor reflex which is mediated at the spinal level, while the lifting or licking response is considered to be a more complex reflex which is mediated at the supraspinal level [36, 37]. It has been shown that the number of neurons expressing c-Fos increases when pain-transmitting neurons are activated in the spinal cord [28–33]. Our study showed that the number of c-Fos positive neurons decreased after electroacupuncture stimulation. This suggests that electroacupuncture relieves pain by inhibiting the transmission of pain at the level of the spinal cord.

In the spinal cord, the transmission of pain may be regulated in different ways. For example, activation of descending pain inhibition system from the brain may reduce the transmission of pain [38–40]. According to the gate control theory of Melzack and Wall [41], on the other hand, fast-conducting somatosensory impulses by electroacupuncture stimulation arrive at the spinal cord dorsal horn to inhibit the activity of pain-transmitting neurons of the dorsal horn by blocking the input of pain information conducted from peripheral inputs [42–44]. Otherwise, electroacupuncture stimulation may induce diffuse noxious inhibitory controls (DNICs) in which analgesic effects by electroacupuncture treatment can be diffuse and unspecific, as the pain-relieving effect of electroacupuncture depends on the intensity of stimulation rather than the precise location thereof [45–47]. Nevertheless, the detailed mechanisms of the analgesic effects of electroacupuncture treatment still remain to be determined. In order to elucidate these mechanisms, further studies will be needed.

5. Conclusion

The effects of electroacupuncture treatment at the BL60 acupoint (BL60) were examined in experimental animals with inflammatory pain induced by formalin. Judging from the fact that there was suppression of flinching behavior responses and of c-Fos expression upon the treatment of electroacupuncture therein, the treatment of electroacupuncture at BL60 acupoint is very effective in alleviation of inflammatory pain.

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Review Article

Acupuncture for the Treatment of Opiate Addiction

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Acupuncture is an accepted treatment worldwide for various clinical conditions, and the effects of acupuncture on opiate addiction have been investigated in many clinical trials. The present review systematically analyzed data from randomized clinical trials published in Chinese and English since 1970. We found that the majority agreed on the efficacy of acupuncture as a strategy for the treatment of opiate addiction. However, some of the methods in several included trials have been criticized for their poor quality. This review summarizes the quality of the study design, the types of acupuncture applied, the commonly selected acupoints or sites of the body, the effectiveness of the treatment, and the possible mechanism underlying the effectiveness of acupuncture in these trials.

1. Introduction

Acupuncture, the practice of inserting thin solid needles into specific documented points of the body to treat many different disorders, has been practiced in China since 2500 BC [1]. Acupuncture is gaining popularity in Western countries as an alternative and complementary therapeutic intervention, and this therapeutic technique is also growing in popularity worldwide [2, 3]. Acupuncture is based on the principles of traditional oriental medicine and was developed according to the principle that human bodily functions are controlled by the "meridian" and "Qi and blood" systems. There are 365 designated acupuncture points located along these meridians that can be used for stimulation through needles to balance and harmonize the yin and yang by relieving blockages in the flow of Qi [4]. This method of healing has been used to promote balance in and improve the functions of the body's organs.

Acupuncture needles are either manipulated manually or via an electrical stimulator, that is, "electroacupuncture" (EA). New methods for stimulating the acupoints include applying electric current to skin electrodes over the points, directing a laser light onto the points, or using finger pressure to massage selected points (acupressure). In addition, many new points and entire "microsystems" of points have been described for specific body parts, for example, scalp acupuncture and ear acupuncture (auricular acupuncture). Acupuncture may be useful as an adjunct treatment in comprehensive management programs and might be efficacious in the treatment of pain [5] such as postoperative pain [6], benign prostate hyperplasia [7], nausea due to pregnancy, and postoperative and chemotherapy-induced nausea and vomiting [4]. Scalp acupuncture therapy appears to improve neurological deficits in patients with acute intracerebral hemorrhage [8]. Modern research is confirming the efficacy of auricular acupuncture for analgesia and anxiety-related diseases [9].

Acupuncture or EA stimulation typically elicits a composite of sensations termed "DeQi," manifesting as soreness, numbness, heaviness, and distention, which are believed to reflect the efficacy of the treatment [10].

In 1996, the World Health Organization (WHO) listed 64 medical problems that were considered suitable for acupuncture treatment, including the treatment of drug

abuse. There are 3 major advantages regarding the use of acupuncture to treat drug addiction. First, acupuncture therapy for opiate addiction is inexpensive, simple and has no side effects [11]. Second, acupuncture can be used for the prevention of opiate relapse [12]. Third, acupuncture therapy is safe for pregnant and parturient women [13].

The application of acupuncture to opiate addiction originated from a serendipitous observation by Dr. Wen in Hong Kong in 1972. Dr. Wen reported that acupuncture combined with electrical stimulation at 4 body points and 2 ear points relieved the symptoms of opioid withdrawal in persons with opiate addiction [14].

This method was later adopted in many clinical settings in Western countries, using a protocol developed in 1985 by the head of the US National Acupuncture Detoxification Association (NADA), Dr. M. Smith. The NADA protocol describes the insertion of 5 needles without the use of electrical stimulation bilaterally into the outer ear or auricle at points termed sympathetic, shenmen, kidney, lung, and liver. The NADA protocol advises that 5-point auricular acupuncture relieves withdrawal symptoms, prevents symptoms of craving, and increases patient participation rates in long-term treatment programs [15].

Auricular acupuncture is the most common form of acupuncture treatment for substance addiction in both the USA and the UK [16, 17]. In both countries, there are currently over 250 hospitals practicing acupuncture based on the NADA protocol [11].

A recent advance in this field was made by Dr. Han of Beijing's Peking University, whose 2005 protocol describes the placement of self-sticking electrodes to the skin over the acupoint followed by electrical stimulation to ameliorate opiate withdrawal signs and prevent relapse of heroin use. The device used for this purpose was named Han's acupoint nerve stimulator (HANS) [14].

1.1. Possible Mechanisms for the Effectiveness of Acupuncture on Opiate Addiction. The mesolimbic dopamine system originates in the ventral tegmental area (VTA) and projects to regions that include the nucleus accumbens and prefrontal cortex, which are believed to play a pivotal role in the development of opiate addiction [4]. Opiate abuse-induced changes in the levels of dopamine in the brain are associated with feelings of well-being and pleasure, providing positive reinforcement of continued opiate abuse [18, 19]. Conversely, withdrawal from chronic opiate administration reduces dopamine outflow in the nucleus accumbens [20, 21]. Opioid withdrawal causes dysphoria and significant distress, a state that addicts seek to avoid and one that can be a major motive for continuing opiate use (i.e., negative reinforcement) [22, 23].

Many studies in animals and humans have demonstrated that acupuncture causes multiple biological responses [24]. Manual acupuncture (MA) and EA are capable of triggering a chain of events that can be understood through controlled experiments. The best-known mechanism is via endogenous opiates and their receptors. Different kinds of endogenous opiates, such as β-endorphin, enkephalin, endomorphin, and dynorphin, reportedly act as frequency-dependent factors in EA. EA of low frequency (2 Hz) accelerated the release of β-endorphin and enkephalin in the CNS whereas EA of high frequency (100 Hz) accelerated the release of dynorphin [25–28].

Early works have demonstrated the involvement of κ opioid receptors in the mechanism underlying the effects of acupuncture on morphine addiction. In 1993, Han and Zhang reported the effectiveness of EA on morphine abstinence syndrome in a rat experimental model. The authors found that 100 Hz EA produced a statistically significant suppression of wet shakes, teeth chattering, escape attempts, weight loss, and penile licking ($P < 0.05$) whereas 2 Hz EA produced a mild but significant suppression in escape attempts and wet shakes [29]. These results suggest that 100 Hz EA was far more effective than 2 Hz EA in suppressing withdrawal syndrome. Further studies suggested that EA suppresses opiate withdrawal syndrome by activating κ opioid receptors and dynorphin release [29–33].

Additionally, acupuncture affects the reinforcing effects of morphine. The method of conditioned place preference (CPP) is a commonly used animal model of drug craving [34]. Wang et al. reported that morphine-induced place preference in rats is significantly suppressed by 2 Hz EA and 2/100 Hz, but not at 100 Hz [35]. However, Shi et al. showed that 100 Hz EA significantly attenuated morphine-induced CPP, and this effect was completely blocked by δ- and κ-opioid receptor antagonists, suggesting that the anticraving effects induced by 100 Hz EA are mediated by the activation of δ- and κ-opioid receptors [36].

In 2008, Yang et al. reviewed the possible mechanism underlying the effectiveness of acupuncture in the treatment of drug addiction and this review provided clear evidence for the biological effects underlying the use of acupuncture to treat drug abuse [4]. This review provided a hypothetical model of the effects of acupuncture on dopamine release in the nucleus accumbens. Regarding positive reinforcement, acupuncture treatment activates $GABA_B$ receptors on the dopamine cell body and activates presynaptic κ-opioid receptors in the nucleus accumbens through dynorphin neurons, resulting in decreased dopamine release. Regarding negative reinforcement, acupuncture treatment stimulates enkephalin neurons in the hypothalamus and interacts with μ-opioid receptors to inhibit VTA GABAergic interneurons and thus increases dopamine release in the nucleus accumbens [4].

Recent basic studies further support the above-mentioned theory and additionally suggest a role for brain-derived neurotrophic factor (BDNF) in this process. MA at Shenmen (HT7) points regulates the reinforcing effects of morphine via regulation of GABA receptors [37] and significantly suppresses morphine-induced increases in locomotor activity and Fos expression in the nucleus accumbens and striatum [38]. Further, results from several animal studies [39–41] showed that both 2 and 100 Hz EA facilitate the recovery of VTA dopaminergic neurons damaged by chronic morphine administration and upregulate BDNF protein levels in the VTA, suggesting

that the potential use of EA as a therapy for treating opiate addiction is associated with the activation of endogenous BDNF.

In summary, neurochemical and behavioral evidence have shown that acupuncture helps reduce the effects of positive and negative reinforcement involved in opiate addiction by modulating mesolimbic dopamine neurons. Moreover, several brain neurotransmitter systems involving opioids and GABA have been implicated in the modulation of dopamine release by acupuncture. However, many unanswered questions remain regarding the basic mechanisms of action of acupuncture. Future research could better determine the influence of acupuncture therapy on the regulation of dopamine and other neurotransmitters.

This paper provides an overview of trials that have investigated the clinical effectiveness of acupuncture in the treatment of opiate addiction. We here summarize the quality of the study design, types of acupuncture applied, commonly selected acupoints or sites of the body, and the effectiveness of the treatment in these trials.

2. Methods

2.1. Literature Search. In April 2011, a literature search was performed using the following English language databases: PubMed and EBSCOhost. The first search keyword used was "acupuncture" and the second keyword used was either "heroin" or "opiate".

2.2. Inclusion and Exclusion Criteria. We included studies that met the following criteria: (1) randomized control trials (RCTs) that adopted a double-blind, single-blind, or nonblind design and (2) participants met criteria for opiate/heroin dependence.

Exclusion criteria included (1) nonnumeric data, (2) comments and replies, and (3) animal study.

2.3. Data Extraction and Quality Assessment. Clinical trials on the treatment of opiate/heroin dependence were selected based on the predetermined inclusion and exclusion criteria. Data were extracted from study reports by one reviewer and were verified by a second reviewer. The following key information was extracted from each study: first author, publication year, study design, sample size, characteristics of participants, main acupoints/sites selected, outcome measures, results reported, and adverse events.

We assessed the quality of the studies using the Jadad scale [42], which rates studies for (1) randomization, (2) double blinding, (3) description of withdrawal, (4) description of randomization, and (5) description of blinding. Trials scoring 1 or 2 points were considered of low quality whereas trials scoring 3–5 points were considered of high quality.

3. Results

An initial search identified 184 published articles from PubMed and 55 published articles from EBSCOhost. Only 10 published articles met our inclusion criteria and these were systematically reviewed (Table 1). The other articles were excluded because they were not RCTs or included subjects without heroin or opiate addiction (Figure 1).

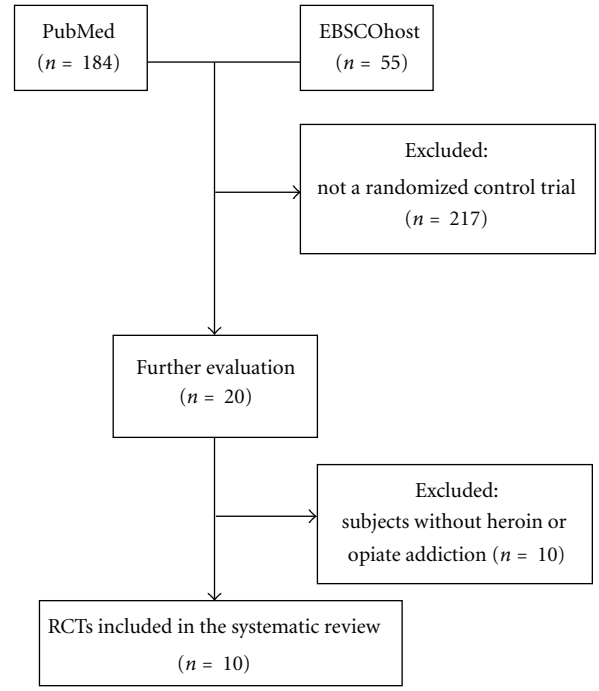

Figure 1: Flow diagram showing the number of studies included and excluded from the systematic review.

3.1. Types of Studies. Five studies mentioned the process of randomization. None of the studies mentioned the use of blinding on clinicians, subjects, or the raters of study outcomes. Four studies [45, 47, 48, 50, 51] were from Chinese journals.

3.2. Diagnostic Criteria and Characteristics of Participants. Five studies [47, 48, 50, 52] used the Diagnostic and Statistical Manual of Mental Disorders (DSM III, III-R, IV) criteria on opiate dependence, 1 study [47] used the Chinese Classification of Mental Disorders (CCMD II-R), 1 study [51] used the International Statistical Classification of Diseases and Related Health Problems (ICD-10) criteria on opiate dependence, and 4 studies [43, 44, 46, 49] did not mention the criteria used in diagnosing opiate dependence.

Ten studies involving 1034 subjects (including those in intervention groups and in control groups) were enrolled, of which 711 cases were from China, 200 were from the USA, 83 were from the UK, and 40 were from Iran. Forty participants were HIV positive.

3.3. Type of Intervention and Needling Method. Four studies [43, 44, 49, 52] used auricular acupuncture, 4 studies [46–48, 50] used body acupuncture with manual stimulation, 1 study [51] used body acupuncture with electrical stimulation, and 1 study [45] used HANS on the treatment group.

Table 1: Summary of studies included in the review.

Author (year)	Jadad score	No. of subjects (acup/control)	Mean age (male%)	Inclusion criteria	Intervention type	Treatment frequency (duration)	Treated acupoints	Type of control group	Outcome measure	Results reported	Adverse events
Washburn et al. (1993), [43] USA	1	100 (55/45)	(1) Standard treatment group: 40.5 (63%) (2) Sham treatment group: 40.4 (73%)	Self-reported with history of intravenous use of heroin confirmed by physical examination for signs of recent needle use	AA	Daily (21 days)	Sympathetic Shenmen Kidney Lung	Nonspecific points	(1) Attendance rate (2) Self reports of frequency of heroin use (3) Urinalysis	(1) $P < 0.05$ (2) $P < 0.01$ (3) NS	(1) Slight bleeding (2) Mild nausea (3) Mild dizziness
Wells et al. (1995), [44] USA	3	60/(31/29)	(1) Specific group: — (54.8%) (2) Nonspecific group: — (48.3%)	Only subjects for whom opiates were determined to be the primary drug and who met federal requirements for entry into methadone treatment	AA	Phase I: 5 days per week (2 weeks) Phase II: available on a daily basis (6 months)	Sympathetic Shenmen Kidney Lung Liver	Nonspecific points	(1) Attendance and retention rate (2) Self-reported symptoms (3) Cravings (4) Methadone dose (5) Urinalysis	(1) NS (2) NS (3) NS (4) NS (5) NS	NR
Zhang et al. (2000), [45] China	1	181 (121/60)	(1) Specific group: 27.1 (—) (2) Control group: 25.4 (—)	(1) DSM III for opiate dependence (2) Positive morphine in urine	HANS (The frequency was 2/100 HZ; the intensities were 12–16 mA on arms and 16–26 mA on legs)	Phase I: 4 times per day (3 days) Phase II: Twice a day (3 days) Phase III: Once a day (7 days)	Hegu (LI4) Laogone (PC8) Neiguan (PC6) Waiguan (SJ5) Zusanli (ST36) Sanyinjiao (SP6)	Electrodes were placed at the acupoints without any electrical stimulation	(1) Heart rate (2) Body weight (3) Sleeping time (4) Chilling (5) Pain (6) Anxiety (7) Catarrh (8) Craving	All 8 indices improved significantly ($P < 0.01$)	NR
Montazeri et al. (2002), [46] Iran	2	40 (20/20)	(1) Specific group: 32 (100%) (2) Control group: 31 (100%)	Self-reported with history of heroin or opium addiction less than 6 months	Body acupuncture with manual stimulation	Once per day for 3 days	Hegu (LI4) Neiguan (PC6) Zusanli (ST36) Shenmen (HT7) Taichong (LR3) Dazhui (DU14) Baihui (DU20)	ROD by naloxone	CINA	The acupuncture group had smaller increase in CINA score compared to control group ($P < 0.05$)	No adverse events

Table 1: Continued.

Author (year)	Jadad score	No. of subjects (acup/control)	Mean age (male%)	Inclusion criteria	Intervention type	Treatment frequency (duration)	Treated acupoints	Type of control group	Outcome measure	Results reported	Adverse events
Wu et al. (2003), [47] China	1	120 (30/30/30/30)	(1) Acupuncture group: — (—%) (2) Acupuncture plus opium group: — (—%) (3) Opium plus buprenorphine group: — (—%) (4) Opium plus HANS group: — (—%)	(1) CCMD II-R and DSM III-R for: opiate dependence (2) Positive morphine in urine	Body acupuncture with manual stimulation	Phase I: Twice a day (3 days) Phase II: Once a day (7 days)	Sishencong (EX-HN1) Neiguan (PC6) Hegu (LI4) Zusanli (ST36) Sanyinjiao (SP6)	(1) Opium plus buprenorphine therapies (2) Opium plus Han's therapies	(1) Opiate withdrawal scale (2) Craving degree with VAS	(1) Acupuncture group showed significant improvement in withdrawal syndrome after the 6th day ($P < 0.05$) (2) Acupuncture group showed significant improvement in craving degree after the 8th day ($P < 0.05$)	Mild dry mouth
Wen et al. (2005), [48] China	2	220 (111/109)	(1) Acupuncture group: 33.9 (77.5%) (2) Control group: 33.8 (78.0%)	(1) DSM IV for opiate dependence (2) Positive morphine in urine	Body acupuncture with manual stimulation	Once a day (10 days)	Hegu (LI4) Neiguan (PC6) Zusanli (ST36) Waiguan (SJ5) Shenmen (HT7) Sanyinjiao (SP6)	Oral administration of lofexidine hydrochloride	(1) Withdrawal symptom (2) Self-Hamilton anxiety scale	(1) Acupuncture group showed significant improvements in withdrawal syndrome before and after treatment ($P < 0.05$) (2) Acupuncture group showed significant improvement in the score of the self-Hamilton anxiety scale after the 4th day ($P < 0.001$)	NR
Margolin et al. (2005), [49] USA	1	40 (20/20)	(1) Five-needle NADA protocol group: 43.1 (65%) (2) Modified NADA protocol group: 42.6 (55%)	HIV-positive methadone-maintained patients	AA	5 days per week (8 weeks)	Sympathetic Shenmen Kidney Lung Liver	Month 1: Shenmen Month 2: Sympathetic Shenmen Lung	(1) Retention rate (2) Attendance rate (3) Drug use (4) Changes in depression and anxiety	(1) NS (2) NS (3) NS (4) NS	NR

Table 1: Continued.

Author (year)	Jadad score	No. of subjects (acup/control)	Mean age (male%)	Inclusion criteria	Intervention type	Treatment frequency (duration)	Treated acupoints	Type of control group	Outcome measure	Results reported	Adverse events
Zeng et al. (2005), [50] China	1	70 (35/35)	(1) Treatment group: 33.1 (83.9%) (2) Control group: 34.2 (80.8%)	(1) DSM III-R for opiate dependence (2) Positive morphine in urine (3) Had opioid withdrawal syndrome	Body acupuncture with manual stimulation	Once a day (10 days)	Baihui (GV20) Dazhui (GV 14) Shendao (GV11) Lingtai (GV10) Zhiyang (GV9) Mingmen (GV4) of the Du channel	Methadone 10-day decrescendo therapy	Scores of daily withdrawal symptoms	Acupuncture group showed significant improvement in withdrawal symptoms on the 1st, 2nd, 4th, 6th, 7th, 8th, 9th, and 10th days ($P < 0.05$ or $P < 0.01$)	NR
Mu et al. (2005), [51] China	2	120 (30/30/30/30)	(1) Acupuncture group I: 29.5 (40%) (2) Acupuncture group II: 29.7 (43.3%) (3) Simulation group: 28.6 (33.3%) (4) Control group: 31.7 (43.3%)	(1) ICD-10 for opiate dependence (2) Negative morphine in urine (3) Had opioid withdrawal syndrome	Body acupuncture with electrical stimulation (The frequency was 5 Hz; the intensity was 5 mA)	3 times a week (10 weeks)	(1) Acupuncture group I: Jiaji (EX-B2) Shenshu (BL23) (2) Acupuncture group II: Neiguan (PC6) Zusanli (ST36) Shenmen (HT7) Sanyinjiao (SP6)	(1) Simulation group: ST36, SP6 without electrical stimulation (2) Control group: no treatment	(1) Withdrawal symptom (2) Hamilton anxiety scale (HAMA) (3) Self-rating depression scale (SDS)	In the treatment of 4, 8, 10 weeks, acupuncture groups I and II showed significantly decreased withdrawal syndrome, HAMA, and SDS ($P < 0.01$)	NR
Bearn et al. (2009), [52] UK	3	83 (48/34)	(1) Acupuncture group: 36.2 (73%) (2) Control group: 35.7 (79%)	DSM IV for opiate dependence	AA	Once a day on weekdays (14 days)	Five points in the ear cartilage ridge area (acupoints not mentioned)	Application of oil to the ear followed by the attachment of 5 metal clips	(1) Withdrawal symptoms (2) Craving	(1) NS (2) NS	NR

Note. Code. NR: not reported; NS: not significant; AA: auricular acupuncture; HANS: Han's acupoint nerve stimulator; ROD: rapid opiate detoxification; CINA: clinical institute narcotic assessment; CCMD: Chinese Classification of Mental Disorders; DSM: The Diagnostic and Statistical Manual of Mental Disorders; ICD: International Statistical Classification of Diseases and Related Health Problems; VAS: visual analogue scale.

The reported courses of treatment in 6 studies [45–48, 50, 52] were between 1 to 2 weeks. The courses of the remaining studies were 3 days, 3 weeks, 8 weeks, and 6 months.

3.4. Outcome Measures and Effectiveness Assessment. In most of the reviewed studies, the outcome measures included attendance rate, craving scale, and opiate withdrawal symptoms. Seven studies [43, 45–48, 50, 51] provided evidence for acupuncture as treatment for opiate addiction whereas 3 studies [44, 49, 52] were against the use of acupuncture to treat opiate addiction.

3.5. Adverse Effects or Events. Most studies did not mention adverse effects or events, few studies described the monitoring of safety, and only 2 studies [43, 47] reported adverse events including slight bleeding, mild nausea, dizziness, and mild dry mouth.

3.6. Main Acupoints/Sites Selected. In the studies included in the review, several used a fixed set of acupoints or sites on their subjects and 1 study allowed some flexibility and needled additional points based on the symptom presentation of individual subjects. The 5 ear acupoints (sympathetic, shenmen, kidney, lung, and liver) were often used in the USA and UK. In China, the acupoints of Zusanli (ST36), Sanyinjiao (SP6), Hegu (LI4), and Neiguan (PC6) were most frequently used for the treatment of opiate addiction. A summary of the main acupoints or sites selected in the studies is presented in Table 2.

Table 2: Summary of main acupoints/sites selected in the reviewed studies.

Acupoints/sites	Frequency of appearance (N)	Percentage ($N/22 \times$ %)
Zusanli (ST36)	7	31.82
Sanyinjiao (SP6)	6	27.27
Hegu (LI4)	6	27.27
Neiguan (PC6)	5	22.72
Shenmen (HT7)	3	13.64
Laogone (PC8)	3	13.64
Sympathetic (ear)	3	13.64
Shenmen (ear)	3	13.64
Kidney (ear)	3	13.64
Lung (ear)	3	13.64
Liver (ear)	2	9.09
Waiguan (SJ5)	2	9.09
Baihui (GV20/DU20)	2	9.09
Dazhui (GV14/DU14)	2	9.09
Jiaji (EX-B2)	1	4.55
Shenshu (BL23)	1	4.55
Sishencong (EX-HN1)	1	4.55
Taichong (LR3)	1	4.55
Shendao (GV11)	1	4.55
Lingtai (GV10)	1	4.55
Zhiyang (GV9)	1	4.55
Mingmen (GV4)	1	4.55

Note. The sum was 22 for the percentage calculation.

3.7. Methodological Quality. Eight of the 10 trials [43, 45–51] reviewed in this paper were classified as having low quality according to Jadad's methodological quality assessment [42], scoring 2 or fewer points. The remaining 2 studies [44, 52] scored >2 points and were classified as having higher quality. All methodological quality scores are presented in Table 3.

4. Discussion

Although many studies have reported positive findings regarding the use of acupuncture to treat drug dependence, the evidence for its effectiveness has been inconclusive and difficult to interpret [53]. There are few randomized controlled clinical trials of acupuncture treatment for opiate addiction, and the methodological methods used in several clinical trials of acupuncture treatment for opiate dependence can be criticized for their poor quality. The quality issues include the following: small numbers of patients, no control subjects, lack of randomized assignment, lack of details regarding specific point locations for needle insertion, and no specification regarding the degree of blinding among research subjects.

In this paper, we classified trials as having low quality if they lacked double-blinding, description of withdrawal, and description of randomization. The majority of low-scoring trials displayed positive results regarding acupuncture treatment for opiate addiction. Further, acupuncture treatment showed potential for preventing relapse and reducing the severity of withdrawal symptoms.

Studies receiving a high methodological quality score produced interesting results. Two studies [44, 52] received high methodological quality scores but failed to report auricular acupuncture effectively. These 2 studies produced negative results, reporting that auricular acupuncture had no effect on withdrawal severity, craving, and attendance when provided as an adjunct to methadone treatment services.

Four studies [43, 44, 49, 52] used auricular acupuncture for the treatment of heroin addiction and 3 of these studies [44, 49, 52] did not report any clinical gains from acupuncture for the treatment of heroin addiction. Five studies [46–48, 50, 51] used body acupuncture with manual or electrical stimulation and all reported some clinical efficacy from the acupuncture for the treatment of heroin addiction. The single study that used HANS [45] for the treatment of heroin addiction reported a significant improvement in the severity of withdrawal syndrome.

Although most of the articles from China reviewed herein have favorable outcome, the type of intervention and needling methods were different between the studies from China and Western countries. Most studies from China used body acupuncture to treat opiate addiction whereas studies from the other countries used auricular acupuncture to treat opiate addiction. In addition, there are various differences

Table 3: Methodological quality scores.

	Washburn et al. [43]	Wells et al. [44]	Zhang et al. [45]	Montazeri et al. [46]	Wu et al. [47]	Wen et al. [48]	Margolin et al. [49]	Zeng et al. [50]	Mu et al. [51]	Bearn et al. [52]
(1) Was the study described as randomized?	V	V	V	V	V	V	V	V	V	V
(2) Was the randomization scheme described and appropriate?	x	V	x	V	x	V	x	x	V	V
(3) Was the study described as double-blind?	x	x	x	x	x	x	x	x	x	x
(4) Was the method of double-blinding appropriate?	x	x	x	x	x	x	x	x	x	x
(5) Was there a description of dropouts and withdrawals?	x	V	x	x	x	x	x	x	x	V
Results	1	3	1	2	1	2	1	1	2	3

V: yes = 1; x: no = 0; low quality, 0–2; high quality, 3–5.

in the auricular acupuncture system in different countries. These findings are intriguing considering that these body and auricular points exhibited different efficacies regarding the use of acupuncture to treat opiate addiction.

The most frequently used points or sites for the treatment of opiate addiction by acupuncturists are grouped below based on their locations: points on the extremities: Zusanli (ST36), Sanyinjiao (SP6), Hegu (LI4), and Neiguan (PC6); points and areas on the trunk: Jiaji (EX-B2), Shenshu (BL23), Sishencong (EX-HN1), Baihui (GV20), and Dazhui (GV14); and points on the ear: sympathetic, shenmen, kidney, and lung.

Adverse events associated with acupuncture are infrequently reported and only 2 studies reviewed herein [43, 47] reported adverse events. Ernst and White [54] determined the range of incidence of adverse events associated with acupuncture and found that those most commonly reported were needle pain (1–45%), tiredness (2–41%), and bleeding (0.03–38%), whereas fainting and syncope were uncommon (0–0.3%), pneumothorax was rare, but feelings of relaxation were very common (86%).

Acupuncture is based on the complex TCM theory that an energy (Qi) flows through meridians in each organ and most acupoints are located along one of these meridians. Because diseases are caused by an imbalance or disturbance of Qi, needling at these acupoints can harmonize Qi and cure diseases. Our experience suggests that better therapeutic acupuncture effects are obtained by doctors with several years, or even decades, of clinical training. Without sufficiently trained practitioners, specific therapeutic results may be masked by nonspecific and even placebo effects. Most modern acupuncture trials provide qualification details of the practitioners that performed the therapies. In several trials [44, 52], details of the practitioners' training were merely acceptable whereas other trials did not provide this information. Therefore, the results and conclusions of these trials do not totally represent clinical settings.

The weakness of this review is the lack of available high-quality data and the results should be interpreted with caution because of the lack of well-designed, high-quality randomized controlled studies. Many studies did not use standard treatment protocols, objective diagnostic criteria, standardized outcome measures, and effective assessment methods. The methodological quality and the description of the studies were poor in the majority of studies.

It is appropriate for a systematic review to calculate the results of each study identified by the study authors only when those studies are sufficiently comparable as to subjects, interventions, and outcomes, and similar enough in design. In addition, the effects of a study intervention on the consequent health or outcomes have to lie in the same direction or show homogeneity. Under these conditions, the individual estimates from each study can be combined to produce a pooled estimate of effect, which is usually more precise than the evidence provided by any of the individual studies. When these conditions cannot be met, it is difficult to interpret the combined findings from individual studies consisting of heterogeneous subjects, interventions, and outcomes.

Although the 10 studies identified by our systematic review shared the same design (randomized control trial), they differed in their inclusion criteria, mode of intervention, and outcome measures. In particular, although 5 outcome measures were used by more than one study (i.e., attendance rate, retention rate, urinalysis, cravings, and withdrawal symptoms), the operational definitions for these measures differed by duration and units of measure. This study heterogeneity prevented us from conducting a statistical analysis.

5. Conclusion

This review covered a wide body of Chinese and English research investigations into the use of acupuncture for the treatment of opiate dependence from the early 1970s up to 2011. After 35 years of active research by both Asian and Western scientists, this review cannot be used to establish the

efficacy of acupuncture in the treatment of opiate addiction because the majority of these studies were classified as having low quality. Although this review may provide a basis for clinicians and future research, future well-designed RCT studies are needed to confirm the efficacy of acupuncture in the treatment of opiate addiction.

Authors' Contribution

J. G. Lin and Y. Y. Chan contributed equally to this work as cofirst authors.

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Research Article

Electroacupuncture Suppresses Discrete Cue-Evoked Heroin-Seeking and Fos Protein Expression in the Nucleus Accumbens Core in Rats

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Relapse to drug seeking was studied using a rodent model of reinstatement induced by exposure to drug-related cues. Here, we used intravenous drug self-administration procedures in rats to further investigate the beneficial effects of electroacupuncture (EA) on heroin-seeking behavior in a reinstatement model of relapse. We trained Sprague-Dawley rats to nose-poke for i.v. heroin either daily for 4 h or 25 infusions for 14 consecutive days. Then the rats were abstinent from heroin for two weeks. 2 Hz EA stimulation was conducted once daily for 14 days during heroin abstinence. We tested these animals for contextual and discrete cue-induced reinstatement of active responses. We also applied immunohistochemistry to detect Fos-positive nuclei in the nucleus accumbens (NACc) core and shell after reinstatement test. We found that active responses elicited by both contextual cues and discrete cues were high in the rats trained with heroin than in saline controls. EA treatment significantly reduced active responses elicited by discrete cues. EA stimulation attenuated Fos expression in the core but not the shell of the NACc. Altogether, these results highlight the therapeutic benefit of EA in preventing relapse to drug addiction.

1. Introduction

Drug addiction is characterized by relapse to drug-seeking behavior during periods of abstinence [1]. Acupuncture and electroacupuncture (EA) have been applied with great success to attenuate various conditions related to drug addiction [2, 3]. In animal model, EA significantly attenuates morphine-induced conditioned place preference (CPP) and behavioral sensitization [4–6]. Recently, Yang et al. [7] and Yoon et al. [8] reported that acupuncture can suppress morphine and ethanol self-administration. Similarly, using the self-administration model of reinstatement, we found that EA attenuates the reinstatement of heroin-seeking behaviors induced by heroin priming [9]. These findings provided new evidence that EA or acupuncture might have therapeutic effect on drug-seeking behaviors.

One factor that contributes to drug-seeking behavior is the presence of cues and contexts that it previously has associated with past drug use. The motivational effect of cue presentation is illustrated most dramatically by the reports of drug craving and relapse to drug-seeking behavior by addicts while in the presence of drug-associated cues and contexts [10–13]. In laboratory animals, discrete conditioned stimuli (cues) (e.g., tone, light, and sound of infusion pump) or contextual conditioned stimuli (cues) (e.g., operant chamber fan and time of day) reinstate drug seeking after extinction of the drug-taking behavior in the absence of these cues [14, 15]. The experiments reported here designed to further examine the effects of EA on discrete or contextual cue-induced reinstatement of heroin-seeking behavior after heroin self-administration.

The neural circuitry of cue-elicited drug seeking involves the nucleus accumbens (NACc) [16, 17]. Exposure to drug-paired cue causes an elevation in NACc neuron firing rates [17] and extracellular dopamine (DA) levels [16], as well as increased levels of c-Fos, a marker for neuronal activation [18]. Interestingly, Yoon et al. [8] reported that acupuncture stimulation at bilateral Shenmen (HT7) attenuated c-fos expression in the NACc utilizing the immunocytochemical detection of Fos protein in nicotine-sensitized rats. Furthermore, acupuncture significantly decreased both dopamine release in the NACc and behavioral hyperactivity induced by a systemic morphine challenge [6]. These findings suggest that acupuncture produces a therapeutic effect on opioid or nicotine addiction, possibly by modulating postsynaptic neuronal activity in the NACc (for review, see [19]). Given that there are anatomical differences between NACc core and shell in both neuronal morphology and connectivity, and these subregions play different roles in drug seeking [20–22], we also applied Fos immunomapping to investigate different functional activation of the NACc core and shell after cue-induced reinstatement of heroin-seeking and EA stimulation.

2. Methods

2.1. Subjects. Male Sprague-Dawley rats (250–300 g) from the Zhejiang Center of Experimental Animals were used. They were randomly assigned and housed collectively (four per cage) under controlled environmental conditions (22°C, 12-h light/dark cycle) with free access to food and water. All animal treatments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were conducted during the light cycle.

2.2. Surgical Procedure. The animals were implanted with chronically indwelling intravenous catheters under sodium pentobarbital (50 mg/kg, ip) anesthesia. A silicon catheter (3.5 cm length, 0.5 mm inner diameter, 0.94 mm outer diameter) was inserted into the right external jugular vein and secured with thread so that the tip reached the right atrium. The other end of the catheter (10 cm length, PE20) exited from an incision on the back of the body. The catheters were flushed daily with 0.2 mL saline containing sterile benzylpenicillin sodium (60,000 units) and heparin (5 units), to prevent bacterial infection and maintain catheter patency and capped daily. All the animals were allowed to recover for at least 7 days. The rats were housed individually in stainless-steel mesh home cages (size 25 × 30 × 30 cm) after surgical procedure.

2.3. Self-Administration Apparatus. Training and testing were conducted in stainless-steel operant chambers (size 30 × 30 × 30 cm) placed in a sound-attenuated, temperature-controlled room; the light of the room was turned off during training. The apparatus consisted of 24 chambers equipped with two nose-pokes (ENV-114 M, Med Associates, Lafayette, IN) in the back wall. There were three LED lights (green, red, and yellow) inside each nose-poke hole. A cue-light (28 V, 0.1 mA, ENV-215 M, Med Associates) was situated on the wall above the nose-pokes. Drug solution was delivered through Tygon tubing, protected by a leash assembly (PHM-120, Med Associates), and suspended through the ceiling of the chamber from a plastic fluid swivel (PHM-115, Med Associates). The leash assembly was modified to fit a custom-made fluid connector fixed with an animal jacket. The Tygon tubing was attached to a syringe pump (PHM-100, Med Associates) that delivered fluid at a speed of 1.08 mL/min using a 10-mL syringe. The experimental events were controlled by an IBM-compatible PC using a MED Associates interface, running self-programmed software (OBSM v4.0, operant behavioral schedule manager) written in Borland Delphi 6.0. Heroin was obtained from the Institute of Forensic Science, Ministry of Public Security of the People's Republic of China and dissolved in physiological saline.

2.4. Self-Administration Procedure and Conditioning Protocols. All the rats underwent an identical sequence of behavioral training. Each rat was trained with one daily 4 h session for 14 consecutive days with either saline or heroin self-administration. The animals were transferred into the operant chambers before each training session and were put back in their individual home cages after the session where food was available. Water was always available both in the test cages and home cages. The rats were not placed in food restriction schedule. Enough food was provided to maintain natural weight gain.

The reinforcement schedule was a modified progressive ratio schedule that involved incrementing response requirements in a relatively gradual manner. The response requirement increased in a linear pattern as calculated according to the following equation: response requirement = truncate (0.2*(step-1) +1), where the results were truncated to integer value. The step number is the number of ratios completed. So in each daily session, the response requirements were 1 for the first five heroin infusions, 2 for the second five infusions, 3 for the third five infusions, 4 for the fourth five infusions, and 5 for the last five infusions. Based on our preliminary experiment, this schedule supported reliable heroin self-administration across a range of heroin doses, and the response training was as easy as a FR1 schedule but maintained a relatively high rate of responding, so the schedule could be kept constant throughout the sessions.

Each trial began with illumination of a green light inside the active nose-poke hole. Responding in the active hole resulted in an infusion of heroin (0.05 mg/kg) delivered by an infusion pump (PHM-100, Med Associates, Lafayette, IN). The green nose-poke light was turned off during heroin infusions. A 30 s intertrial interval (time out) followed and then another trial began. Responding in the inactive hole had no consequences. The response requirements started with one and increased one after each five heroin infusion. Each earned heroin infusion was also paired with a 5 s cue-light (situated on the wall above the nose-pokes) that served as the discrete cue stimulus. The session ended after 25 infusions were earned or 4 hours had passed, whichever came first.

2.5. Abstinence and Reinstatement. After 14 days of self-administration training, the rats were made to abstain from heroin for another 14 days during which they were confined to their individual home cages. The choice of abstinence duration was based on our previous work [23]. Using the same experimental procedure, we found that there were no significant differences of discrete CS-induced heroin seeking among the rats after 1, 2, and 4 weeks abstinence from self-administration.

The reinstatement testing lasted for 2 hours, and each animal was tested only once. During testing, the animals still wore their jackets, but the leash assemblies were not connected. The testing consisted of two consecutive 1-h phases. During the first phase, the rats were allowed to respond to the nose-pokes with all the conditioned stimulus lights kept off. The responses were recorded. This phase was used to measure contextual cue- (chamber environment) induced heroin seeking and was generally regarded as an extinction phase.

Immediately after the first phase, the second phase began. This was signaled by one 5 s presentation of the discrete cue stimulus (the nose-poke light, the cue light, and the pump noise), which was previously paired with each heroin infusion. During this phase, the green light inside the active nose-poke hole was turned on, and each active response resulted in another 5 s presentation of the discrete cue stimulus and the turning off of the green nose-poke light. After turning off the discrete cue stimulus, another trial began. This phase was used to measure discrete cue-induced heroin seeking.

2.6. EA Stimulation. Rats were kept in special holders with their hind legs and tails exposed. Two stainless steel needles of 0.3 mm diameter were inserted into each hind leg in the acupoints ST36 (5 mm lateral to the anterior tubercle of the tibia) and SP6 (3 mm proximal to the superior border of the medial malleolus, at the posterior border of the tibia). Constant current squarewave electric stimulation produced by an electroacupuncture apparatus (Model G-6805-2, Shanghai Medical Electronic Apparatus, China) was administered via the two needles. The frequency of stimulation used was 2 Hz. The intensity of the stimulation was increased stepwise from 0.5 to 1.0 mA, with each step lasting for 15 min.

2.7. Fos Immunohistochemistry. Four rats from each group were randomly selected for c-Fos immunohistochemistry. Immediately after behavioral testing, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, ip) and killed by transcardial perfusion of 200 mL ice saline followed by 200 mL 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB). Brains were dissected and postfixed in the same fixative and then stored in 30% sucrose at 4°C for 3–5 days. Coronal sections (30 μm in thickness; 1.6 mm from bregma according to the atlas of Paxinos and Watson [24]) were cut on the cryostat at −25°C. Sections were rinsed in 0.01 M phosphate-buffered saline (PBS) and incubated in PBS containing 5% normal goat serum and 0.3% Triton X-100 for 30 min and then in Fos antibody (rabbit polyclonal antibody. Santa Cruz, USA) diluted at 1 : 200 in PBS at 4°C for 48 h. After rinsing three times with PBS, sections were incubated in the biotinylated goat anti-rabbit secondary antibody (Sigma, USA, diluted 1 : 200 with PBS) for 2 h and washed again. Then all corresponding sections were placed in the avidin-biotin-peroxidase complex solution for 60 min. Finally, DAB was used for visualization of Fos immunoreactivity. The reaction was stopped by several PBS washes. Sections were then mounted on gelatin-coated slides, air-dried, dehydrated through graded alcohols, cleared in xylene, and coverslipped with Eukitt.

2.8. Quantification of Fos-Positive Nuclei in the NACc. The NACc is an integral part of the basal ganglia located within the ventral striatum. It is composed of two regions: core and shell (see Figure 4(f)). Sections were scanned using an Olympus BX51 microscope. Image analysis was carried out with the aid of an image analysis system (40x magnification). Three consecutive sections were taken from each animal, and the Fos-positive nuclei were counted bilaterally, based on a randomization procedure. A computer-generated rectangle (250 × 600 um) was placed in a fixed area of the NACc core and shell of each section, and the analysis software counted stained nuclei within the area.

2.9. Experimental Protocols. Forty rats were trained with heroin self-administration. Training sessions were conducted daily for 14 consecutive days. Then the rats were abstinent from heroin for two weeks, during which they lived in their individual home cages. The heroin-trained rats were divided randomly into four groups: the contextual-cue-induced reinstatement (No EA CONT, $n = 10$), contextual/discrete-cue-induced reinstatement (No EA CONT/DIS, $n = 10$), EA CONT/DIS ($n = 10$), and restraint CONT/DIS (kept in the special holders, $n = 10$). The same experimental procedures were used for the control (No EA SAL, $n = 6$) rats except the heroin was substituted with the same volume of saline. EA (or restraint) treatment was given once daily for 14 days during heroin abstinence. No EA CONT group was allowed to nose-poke for 2 h with all the light signals off. It was used to measure contextual cue- (chamber environment) induced heroin seeking. The No EA CONT/DIS, EA CONT/DIS, restraint CONT/DIS, and No EA SAL rats were allowed to nose-poke for two consecutive 1 h testing phases. All the light signals were turned off in the first phase, and discrete cue stimuli were presented in the second phase.

2.10. Statistical Analysis. Experimental data were expressed as mean ± SEM. The differences in total active responses and heroin infusions during heroin self-administration were analyzed using two-way analysis of variance (ANOVA) with session as a repeated within-subject factor and group as a between-subject factor. Cue-induced active responding during reinstatement testing was also analyzed using two-factor repeated ANOVA with time block (15 min) as a within subject factor and group as a between-subject factor. Significant effects were followed by post hoc Tukey tests. Fos protein expression was analyzed using one-way ANOVA. When significance was found using ANOVA procedures, post

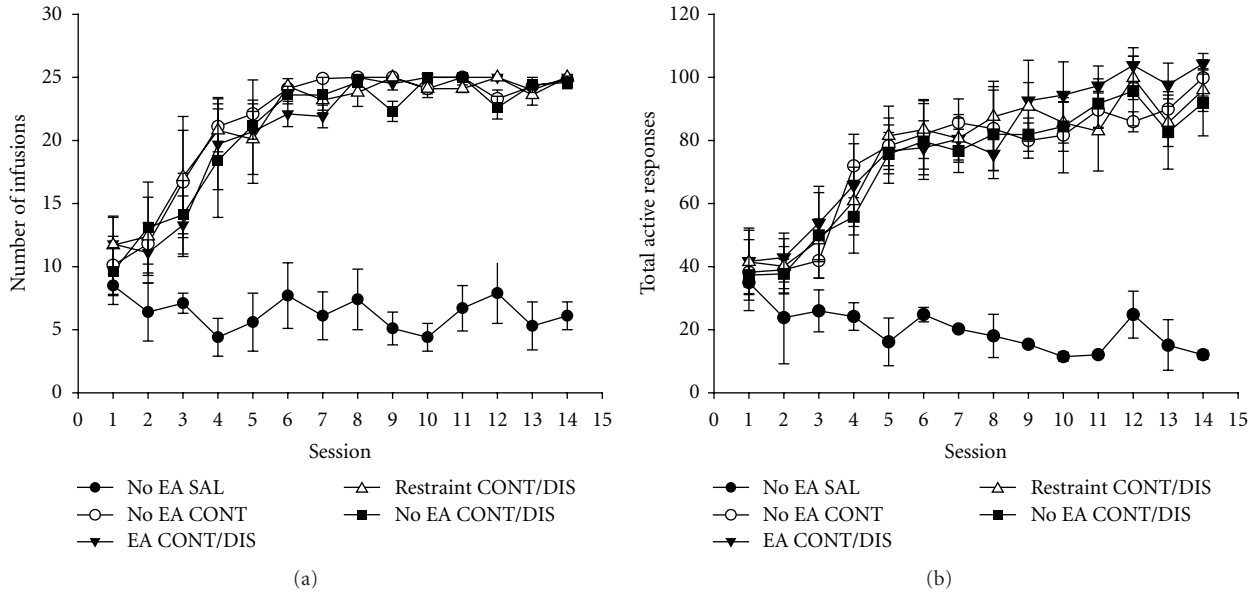


Figure 1: Acquisition of intravenous heroin self-administration. Data were expressed with mean ± S.E.M. of total infusions (a) and total active nose-poke responses (b) during each daily session.

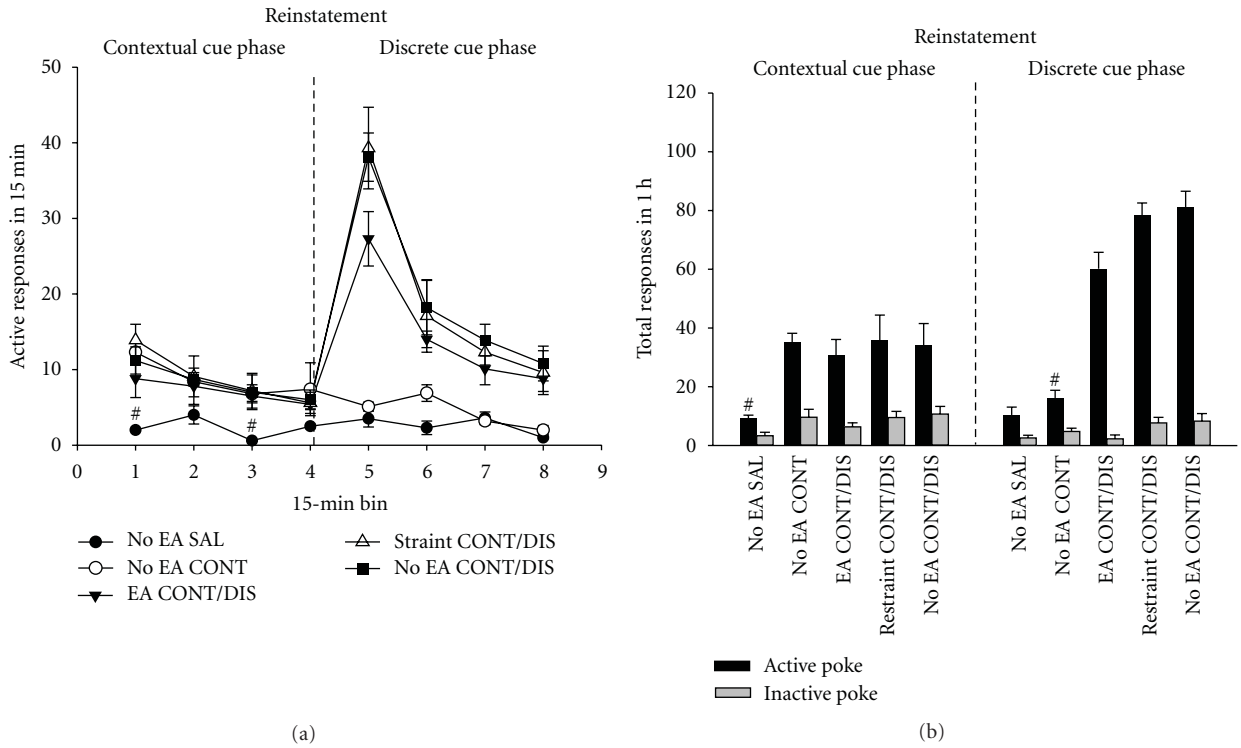
hoc analyses were conducted using Fisher LSD test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Heroin Self-Administration. As shown in Figure 1, all heroin-trained rats demonstrated reliable heroin self-administration, as indicated by the increase in infusions and active responses and reached stable levels of heroin infusions and active responses within 14 days of heroin self-administration training without there being any differences between groups in total active responses and infusions per session. This was reflected by the nonsignificant main effects of heroin groups ($F(3, 36) = 1.29$, $F(3, 36) = 0.83$, resp., for total responses and infusions per session, NS), interactions between heroin groups and training sessions ($F(39, 468) = 1.46$, $F(39, 468) = 0.65$, resp., for total responses and infusions per session, NS), and the significant main effect of training sessions ($F(13, 468) = 53.92$, $F(13, 468) = 42.74$, resp., for total responses and infusions per session; $P < 0.001$) (Figures 1(a) and 1(b)). Tukey post hoc test comparisons revealed that rats quickly learned to self-administer heroin such that a significantly higher number of the active responses and infusions were observed at the fourth training session ($P < 0.001$) with stable heroin self-administered behavior acquired after the sixth session. The rats that were trained with saline could not establish stable self-administration; just a few infusions were made within each session (Figure 1). Only minimal responding was observed at the inactive nose-poke for all the training groups and during all the training sessions (data not shown).

3.2. Effect of EA on Cue-Elicited Drug Seeking Behavior. After 14 days of abstinence from heroin self administration, the rats were returned to the operant chambers for testing cue-evoked heroin-seeking behavior. Two-factor ANOVA revealed significant main effects of block ($F(3, 123) = 14.31$, $F(3, 123) = 43.20$, resp., for contextual and discrete cues, $P < 0.001$), group ($F(4, 41) = 12.34$, $F(4, 41) = 35.13$, resp., for contextual and discrete cues, $P < 0.001$), and also a significant interaction between block and group ($F(12, 123) = 6.10$, $F(12, 123) = 11.90$, resp., for contextual and discrete cues, $P < 0.01$). As shown in Figure 2(a), heroin cue-induced reinstatement of active responding occurred mainly in the first 15 min blocks and decreased across blocks during both contextual and discrete cue-induced reinstatement testing phases. In contextual cue phase, Tukey post hoc test comparisons revealed active responses were higher in No EA CONT/DIS, EA CONT/DIS, restraint CONT/DIS, and No EA CONT group than in No EA SAL group in the first and the third block (all $P < 0.01$). There were no significant differences among No EA CONT/DIS, EA CONT/DIS, restraint CONT/DIS, and No EA CONT group, although there was a trend for EA to decrease active responses ($P > 0.05$). In discrete cue phase, EA produced significant reductions in active responses in the first 15 min block ($P < 0.05$), as compared with No EA CONT/DIS and Restraint CONT/DIS group.

When the total amount of active responses were analyzed, one-way ANOVA revealed significant effects of group for both contextual cues ($F(4, 41) = 11.76$, $P < 0.01$) and discrete cues ($F(4, 41) = 26.37$, $P < 0.001$) testing phases (Figure 2(b)). In contextual cue phase, total active responses were significantly higher in No EA CONT/DIS, EA CONT/DIS, restraint CONT/DIS, and No EA CONT group than in No EA SAL group (all $P < 0.01$). In discrete cue phase, the total amount of active responses was higher in No EA CONT/DIS and Restraint CONT/DIS group than in EA CONT/DIS and No EA SAL group (all $P < 0.05$).



(a) (b)

Figure 2: Effect of EA treatment on cue-induced heroin-seeking after two weeks of abstinence from heroin self-administration. Data were expressed with mean ± S.E.M. Number of active response rates in 15 min blocks (a) or total responses (b) in contextual cue phase and discrete cue phase. $^{*}P < 0.05$ indicates a difference from No EA CONT/DIS group and Restraint CONT/DIS group. $^{\#}P < 0.01$ indicates a difference from No EA CONT/DIS, restraint CONT/DIS, No EA CONT, and EA CONT/DIS group.

Active responses were also higher in EA CONT/DIS group than in No EA CONT group ($P < 0.05$). There were no significant differences between No EA CONT/DIS and restraint CONT/DIS group ($P > 0.05$). Responding at the inactive nose-poke was minimal during both testing phases (Figure 2(b)).

3.3. Effect of EA on Fos Protein Expression in the NACc. In the NACc core, One-way ANOVA revealed significant effects of group for Fos protein expression ($F(4, 55) = 78.21$, $P < 0.001$). As illustrated in Figures 3 and 4, only a few Fos positive neurons were detected in No EA SAL group (Figure 4(e)). Compared with No EA CONT group, No EA CONT/DIS and restraint CONT/DIS group exhibited an increase in Fos-positive nuclei (Fisher LSD test; $P < 0.01$). Of note, EA stimulation attenuated Fos expression relative to No EA CONT/DIS and restraint CONT/DIS group (Fisher LSD test; $P < 0.05$). No differences in Fos expression were noted between the No EA CONT/DIS group and Restraint CONT/DIS group ($P > 0.05$).

In the NACc shell, enhanced Fos protein expression was observed in No EA CONT/DIS, EA CONT/DIS, restraint CONT/DIS, and No EA CONT group relative to No EA SAL group. There were no differences in Fos expression among No EA CONT/DIS, EA CONT/DIS, restraint CONT/DIS, and No EA CONT rats, as determined by Fisher LSD post hoc test ($P > 0.05$).

4. Discussion

Exposure to environmental stimuli previously associated with drug intake can provoke drug relapse in humans. Environmental cues repeatedly associated with the subjective effects of heroin can elicit drug craving and, possibly, automatic behavioral responses that may lead to relapse in recovering heroin addicts [25–28]. In laboratory animals, discrete-conditioned stimuli that are explicitly paired with opiate injections and contextual-conditioned stimuli that are associated with a distinct opiate environment can reinstate opiate seeking after extinction of the drug-taking behavior in the absence of these cues [27]. Consistent with the mounting evidence suggesting the role of discrete and contextual cue in precipitating relapse [26, 29], our results further confirmed that self-administration environment (contextual cue) and discrete cue stimuli previously associated with heroin injections could elicit robust heroin-seeking behavior after 2-week heroin withdrawal.

In the present study, we found that 2 Hz EA attenuated discrete but not contextual cue-induced reinstatement of heroin seeking after heroin abstinence. These results demonstrate dissociable roles of EA in discrete versus contextual cue-induced reinstatement of heroin seeking. Discrete cues are different from contextual cues. In discrete cue appetitive Pavlovian conditioning, discrete cues with a defined onset and offset that typically activate one sensory modality are provided, accompanied by heroin delivery [30]. Discrete

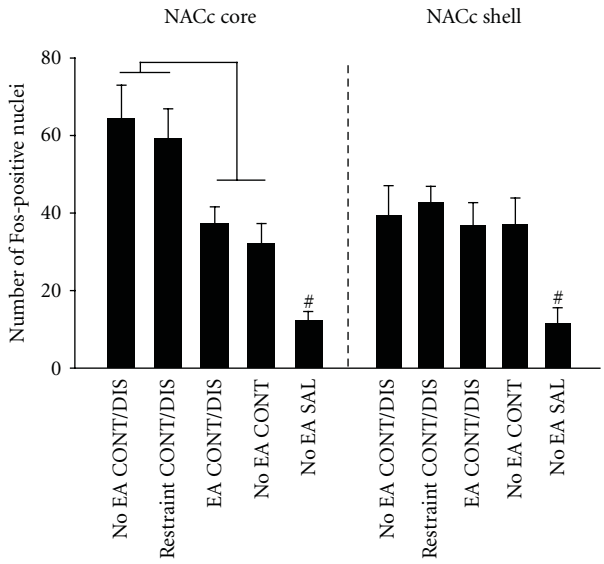


Figure 3: Quantitative analysis of Fos-positive nuclei in the NACc core and shell. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{\#}P < 0.01$ versus No EA CONT/DIS, Restraint CONT/DIS, No EA CONT and EA CONT/DIS group.

cue functions as a conditioned reinforcer during testing. Associative learning and memory processes may be involved in acquisition and maintenance of associations between drug and drug-paired discrete cues [18, 31]. In contrast, contextual cue is delivered as the animal explores the environment; thus, the temporal relationship between contextual stimuli and reinforcement is not an essential component of the learned associations [32, 33]. Some studies suggest that contextual cue induces heroin seeking by acquiring motivational properties via its direct association with heroin reward during training, independent of its learned associations with the discrete conditional stimuli [34, 35]. This motivational account of contextual cue-induced reinstatement of heroin seeking is supported by the finding that this effect involves the VTA, a brain area involved in the incentive motivational effects of drug and nondrug reinforcers [31]. Based on the above discussion, our current findings seem to suggest that the effects of EA on heroin seeking may be mediated partially by regulating associative learning and memory between drug and drug-paired cues but not motivational properties of drug seeking. Of course, further studies must be performed to clarify this issue.

The NACc core and shell subregions are differentially involved in the reinstatement of cocaine seeking, depending on the type of trigger that elicits this behavior. Our present results that discrete cue evoked an increase in Fos-positive nuclei in the NACc core are consistent with data from several studies using cocaine-trained rats [36]. Fuchs et al. [22] found that reversible inactivation (muscimol plus baclofen) of the NACc core attenuated cocaine seeking in cue-induced reinstatement, and NACc shell inactivation failed to alter cocaine seeking. Similarly, inactivation of the NACc core with muscimol-plus baclofen injection blocked cue-induced reinstatement of cocaine seeking, but inactivation of the NACc shell increaseds cue-induced reinstatement of the extinguished response that had previously delivered cocaine [37]. Moreover, inhibition of NACc core but not shell p70s6k and rps6 phosphorylation decreased discrete cue-induced reinstatement of cocaine seeking [38]. Di Ciano and Everitt reported that permanent lesions or antagonism of AMPA receptors in the core but not the shell decrease discrete cue-induced cocaine seeking, as assessed in a second-order reinforcement schedule [39]. Together, our results and those reviewed above suggest that activation of NACc core neurons mediates discrete cue-induced drug seeking. Our results that EA stimulation suppressed elevated Fos expression in the core but not the shell suggested that the effects of EA on discrete cue-evoked heroin seeking were mediated by suppressing neuronal hyperexcitability in the NACc core. Of note, we also found that contextual cue evoked an increase in Fos-positive nuclei in the NACc shell. It seems to indicate that activation of NACc shell neurons mediates context-induced drug seeking. Such a notion is consistent with the finding that injections of the dopamine D1 receptor antagonist SCH 23390 into the lateral or medial shell, but not core, decreased context-induced reinstatement of heroin seeking [40]. Another study showed that medial shell injections of the metabotropic glutamate 2/3 receptor agonist decreased context-induced reinstatement of heroin seeking [41]. Harris and Aston-Jones [42] reported that Fos induction after exposure to morphine-paired contexts is more pronounced in the shell than in the core. However, another study showed that GABA agonist-induced neural inhibition within the NACc core or shell disrupted context induced reinstatement of cocaine seeking [43]. Moreover, exposure to discriminative cues that predicted cocaine availability increased neuronal activity in the shell but not core [44]. Altogether, these results demonstrate that the NACc is a functionally heterogeneous structure with respect to its involvement in discrete cue- and context-induced cocaine seeking. In present study, we did not observe that EA stimulation reduced elevated Fos expression in the NACc shell. However, Kim et al. [6] demonstrated that acupuncture could decrease the DA release in NACc shell and core. Other studies indicated that the changes of endogenous opioids produced by EA were only observed in NACc shell [45, 46]. The discrepancy between these studies and the present one might be explained by differences in condition of animals and experimental protocols. Numerous examples reveal that the regulatory action of acupuncture is bidirectional. Its therapeutic actions are achieved by normalizing metabolism or pathogenic changes toward homeostasis. For example, differences in intensity and duration of acupuncture stimulation can lead to variation in the induced effects through different nervous pathways. Weak stimulation affects A-beta nerve fibers, whereas strong stimulation affects C fibers [47]. The specific direction of the acupuncture effect may depend on an appropriate selection of certain acupuncture points and variation in technique. For example, contrary to expectations and the results of prior research, Facchinetti et al. [48] demonstrated reduced beta endorphin levels with acupuncture treatment of primary headaches.

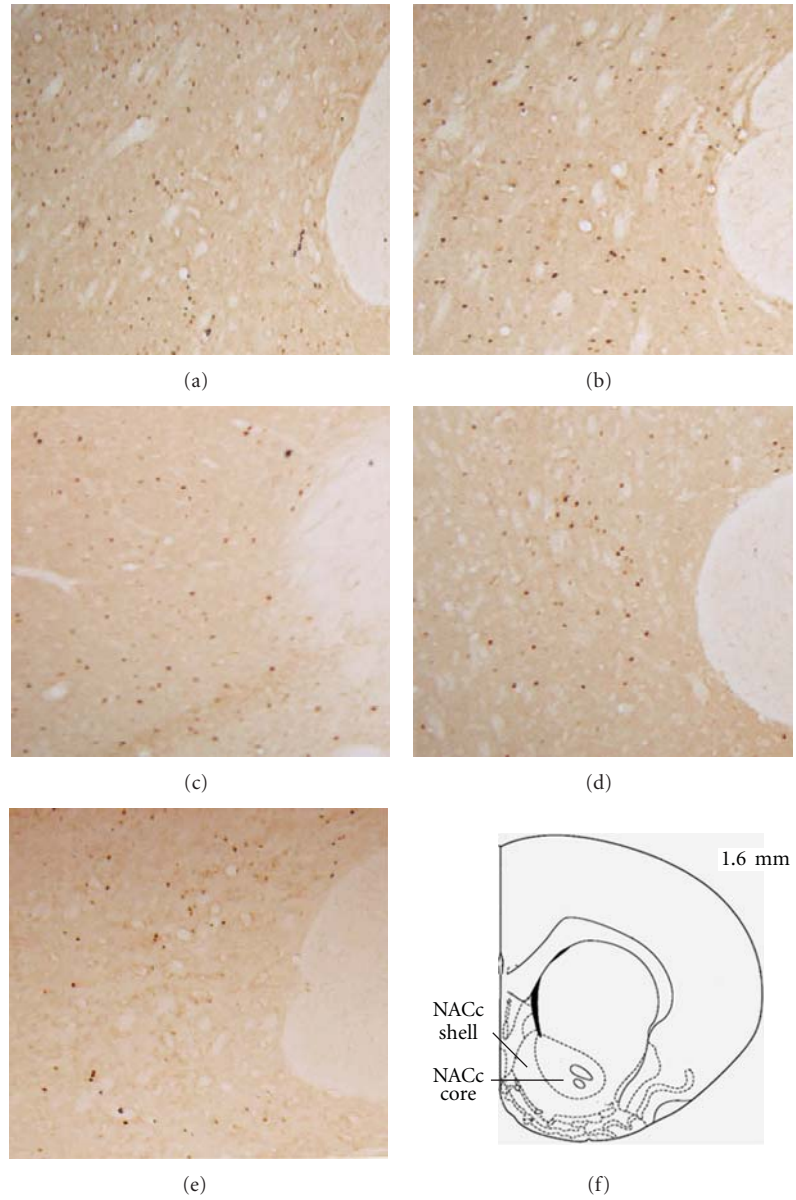


Figure 4: Representative coronal sections showing Fos immunoreactivity in the NACc core. No EA CONT/DIS group (a), restraint CONT/DIS group (b), EA CONT/DIS group (c), No EA CONT group (d), and No EA SAL group (e). Schematic coronal section analyzed. (f) Number at the top right of Figure 4(f) represents the distance (in millimeters) from bregma. The depicted coronal section was adapted from the atlas by Paxinos and Watson [24]. Scale bar, 200 μm.

Despite knowledge of neuronal activation in the NACc core underlying EA's effectiveness in discrete cue-elicited drug seeking, little is known about neurobiological mechanisms by which EA stimulation exerts a positive influence on drug-seeking behavior. It is possible that acupuncture reduces discrete cue-induced drug-seeking behavior by modulating dopamine release or activation of postsynaptic dopamine receptors in the NACc core. Kim et al. [6] evaluated the effect of acupuncture on repeated morphine-induced changes in extracellular dopamine levels using in vivo microdialysis and repeated morphine-induced behavioral changes. They found that acupuncture significantly decreased both dopamine release in the NACc and behavioral hyperactivity induced by a systemic morphine challenge. These results suggest that the therapeutic effect of acupuncture on morphine addiction occurs through inhibition of drug-induced elevation in dopamine levels in the NACc. On the other hand, 2 Hz EA stimulation was used in the present study. It has been demonstrated that the 2 Hz EA could stimulate the release of endogenous opioid peptide enkephalins and endomorphin in CNS, which interact with mu and delta opioid receptors [46, 49]. The mu and delta opioid receptors are involved in the inhibitory effects of 2 Hz EA on morphine-induced CPP [5]. Moreover, recent studies have shown that EA at 2 Hz increased preproenkephalin mRNA levels in the NACc of morphine CPP rats when the

morphine-induced CPP was attenuated by 2 Hz EA [45]. Hence, it is likely that the endogenous opioids and their interaction with mu and delta receptors in the NACc might be involved in the therapeutic benefit of 2 Hz EA in discrete cue-induced drug seeking-behavior.

With regard to Experimental protocols in the present study, there are some variable factors that need to be taken into account. Firstly, it is still controversial as to how to set suitable control group for acupuncture or EA. In order to exclude interference from restraint stress, we run restraint group as a control. However, the perfect control, of course, is one in which needles are inserted into acupuncture points but without electric stimulation. Secondly, in our experimental protocol, the animals tested contextual cue-induce reinstatement firstly, and then tested discrete cue-induced reinstatement. This raises the possibility that contextual cue itself might influence discrete cue-induced heroin seeking. In fact, during contextual cue phase, the rats were allowed to respond to the nose-pokes with all the conditioned stimulus lights kept off. The responses were recorded, but no consequences were produced. This phase was used to measure contextual cue- (chamber environment) induced heroin-seeking and was generally regarded as an extinction phase [50]. Furthermore, we observed that contextual cue-induced reinstatement of active responding occurred mainly in the first 15 min block and decreased across blocks. There were no significant differences in active responses between the groups in the last 15 min block.

5. Conclusion

EA treatment significantly reduced discrete cue-induced heroin seeking behavior in reinstatement of self-administration procedures. Fos protein expression consistent with conditioned enhancement by discrete cue stimuli was observed in the NACc core. 2 Hz EA stimulation attenuated Fos expression in the core but not the shell of the NACc. Altogether, these results support the hypothesis that EA can reduce drug-seeking and highlight the therapeutic benefit of EA in preventing relapse to drug addiction.

Conflict of Interests

The authors do not have any conflicts of interest or any circumstances that could be perceived as a potential conflict of interest.

Acknowledgment

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Research Article

Changes in Cytokine Expression after Electroacupuncture in Neuropathic Rats

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The production of proinflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) plays a key role in chronic pain such as neuropathic pain. We investigated changes in cytokine expression in injured peripheral nerves and dorsal root ganglia (DRG) following electroacupuncture (EA) treatment. Neuropathic pain was induced by peripheral nerve injury to the left hind limb of Sprague-Dawley rats under pentobarbital anesthesia. Two weeks later, the nerve-injured rats were treated by EA for 10 minutes. The expression levels of IL-1β, IL-6, and TNF-α in peripheral nerves and DRG of neuropathic rats were significantly increased in nerve-injured rats. However, after EA, the cytokine expression levels were noticeably decreased in peripheral nerves and DRG. These results suggest that EA stimulation can reduce the levels of proinflamtory cytokines elevated after nerve injury.

1. Introduction

Acupuncture has been widely used in traditional East Asian medicine for the clinical treatment of chronic pain and various diseases such as rheumatoid arthritis and inflammatory bowel syndrome [1], but the mechanism of acupuncture-induced analgesia remains unclear. Recent studies have documented the analgesic effects of acupuncture and electroacupuncture (EA) stimulation using behavioral and molecular biological methods in rats [2–6].

Holguin et al. [7] observed that the expression of proinflammatory cytokines was increased by the release of nitric oxide. In addition, spinal nitric oxide improves pain facilitation through glial activation [8] and the release of interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) [9]. Recently, interest in neuroinflammation and neuroimmune activation caused by neuropathic pain has grown rapidly. Inflammation and immune responses are caused by neurological disorders such as peripheral nerve injuries that are often associated with persistent pain [10]. Painful nerve injury results in rapid and sustained upregulation of IL-1β, IL-6, and TNF-α in the damaged nerve itself and in macrophages in the dorsal root ganglion (DRG) [11]. IL-1, IL-6, and TNF-α have been known to play an important role in the inflammatory response to pain [12–14]. In particular, Lee et al. [14] suggest that IL-1β and TNF-α function in the initiation of persistent neuropathic pain, while IL-6 is important for maintenance. However, the relationship between acupuncture analgesia and expression in inflammatory cytokines is unclear. Therefore, the present study was conducted to determine whether the expression levels of proinflammatory cytokines in peripheral nerves including the injured nerves and DRG are changed by EA that exerts analgesic effects.

2. Materials and Methods

2.1. Surgical Procedures. Adult male Spague-Dawley rats (220–250 g, $n = 32$) were used in this study. Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A segment of the sciatic nerve was exposed between the

mid-thigh and the popliteal fossa by skin incision and blunt dissection through the biceps femoris muscle. The three major divisions of the sciatic nerve (tibial, sural, and common peroneal nerves) were clearly separated based on individual perineuria. The tibial and sural nerves were tightly ligated and then transected, while the common peroneal nerve was left intact [15]. Complete hemostasis was confirmed and the wound was closed with muscle and skin sutures. There were four groups in this study: naïve (no surgery), sham operation (clearly separated sciatic nerve branches only), neuropathic surgery, and EA stimulation after neuropathic surgery. All animal experiments were performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of Laboratory animals and received approval from the Institutional Animal Care and Use Committee of Yonsei University Health System.

2.2. Behavioral Analysis. Behavioral tests to assess pain development were performed at postoperative days 1, 4, 7, and 14. To measure mechanical allodynia, rats were placed on a metal mesh floor under a custom-made transparent plastic dome (8 × 8 × 18 cm). Innocuous mechanical stimuli were applied to the sensitive area of each hind paw with a von Frey filament every 3-4 s (8 mN bending force, 10 repetitions). The frequency of foot withdrawal out of 10 trials with the von Frey filament application was expressed as a percentage (response rate (%) = number of foot withdrawals/number of trials × 100). To quantify cold sensitivity of the foot, brisk withdrawal in response to acetone applied to each paw every 5 min (5 repetitions) was documented. The frequency of foot withdrawal (expressed as a percentage) was used as a cold allodynia index.

2.3. EA Treatment. EA treatment was carried out after the behavioral test. The detailed methods for EA stimulation were described previously [2]. In short, rats were anesthetized with 2% enflurane in 95% O_2/5% CO_2. Stainless steel acupuncture needles (0.30 mm in diameter and 30 mm in length) were inserted percutaneously at a depth of 2-3 mm into the Zusanli (ST36) and Yinlingquan (SP9) acupuncture points. Electrical stimulation was produced by a stimulus isolation unit (A365, World Precision Instruments, Sarasota, FL, USA). Train pulses (0.6 mA, 1 Hz, 0.1 ms pulse width) were applied to the inserted needle for 10 min using the pulse master unit (A300, World Precision Instruments).

2.4. mRNA Quantitation Using Reverse Transcriptase. All rats were immediately sacrificed by decapitation after the final EA stimulation. The lumbar (L4-L5) DRG and the injured peripheral nerves were rapidly dissected and stored in 200 μL lysis buffer (easy-BLUE reagent, iNtRON Biotechnology, Seoul, Republic of Korea). Total RNA (2 μg) from each sample was reverse-transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was amplified by polymerase chain reaction (PCR) in a 20 μL reaction mixture containing 4 μL dNTP mix (2.5 mM each), 1 μL Oligo(dT) primer (500 ng/μL), 4 μL 5x first-strand buffer, and 2 μL 0.1 M DTT. Reverse-transcript PCR (Mastercycler Gradient, Eppendorf, Hamburg, Germany) was performed for 37 cycles in a thermal cycler using PCR PreMix (Bioneer Inc., Alameda, CA, USA) under the following conditions: denaturation, 30 seconds at 95°C; annealing, 1 minute at 62°C; and extension, 2 minutes at 72°C. At the 37th cycle, extension was continued for an additional 5 minutes at 72°C. Reverse transcript PCR was performed for the amplification of IL-1β, IL-6, TNF-α, and β-actin (Table 1).

Table 1: Primers used in reverse transcript PCR.

Gene name	Primer sequence
IL-1β	F: 5′-GGAAGGCAGTGTCACTCATTGTG-3′ R: 5′-GGTCCTCATCCTGGAAGCTCC-3′
IL-6	F: 5′-GGGACTGATGTTGTTGACAGCC-3′ R: 5′-CATATGTAATTAAGCCTCCGACTT-3′
TNF-α	F: 5′-CCCCGACTATGTGCTCCTCAC-3′ R: 5′-AGGGCTCTTGATGGCGGA-3′
β-actin	F: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ R: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

(F: Forward primer; R: Reverse primer).

Amplified products were electrophoresed on 1.5% agarose gels, visualized with ethidium bromide, and photographed. To quantify band intensities, negative controls were scanned and then analyzed using the NIH image program. Amplification of endogenous β-actin was used to normalize IL-1β, IL-6, and TNF-α mRNA levels.

2.5. Statistical Analysis. Values were expressed as the mean ± standard error of the mean (S.E.M) and compared using one-way or two-way ANOVA followed by Dunnett's *post hoc* pairwise comparison (SPSS Ver. 17.0, SPSS Inc., Chicago, IL, USA). A P value of less than 0.05 was considered significant.

3. Results

Behavioral signs of neuropathic pain were produced after nerve injury (Figure 1). Figure 1(a) shows the development of mechanical allodynia observed before EA stimulation. The brisk withdrawal responses were induced when von Frey filament was applied to the receptive fields on nerve-injured hind paw. Two-way ANOVA showed significant main effects in between groups ($F_{2,7} = 120.15$, $P < 0.01$), and different time points ($F_{4,7} = 12.82$, $P < 0.01$), and significant interaction ($F_{8,7} = 9.49$, $P < 0.01$). This implies that behavioral performances in three groups are different depending on time points after surgery. Subsequent Dennett's *post hoc* multiple comparisons showed that neuropathic group displayed significantly higher withdrawal responses to von Frey filament at all time points after nerve injury ($P < 0.05$). Figure 1(b) shows the development of cold allodynia observed before EA stimulation. The brisk withdrawal responses were induced when acetone was applied to the receptive fields on nerve-injured hind paw. Two-way ANOVA showed significant main effects in between groups ($F_{2,7} = 54.23$, $P < 0.05$), different time points

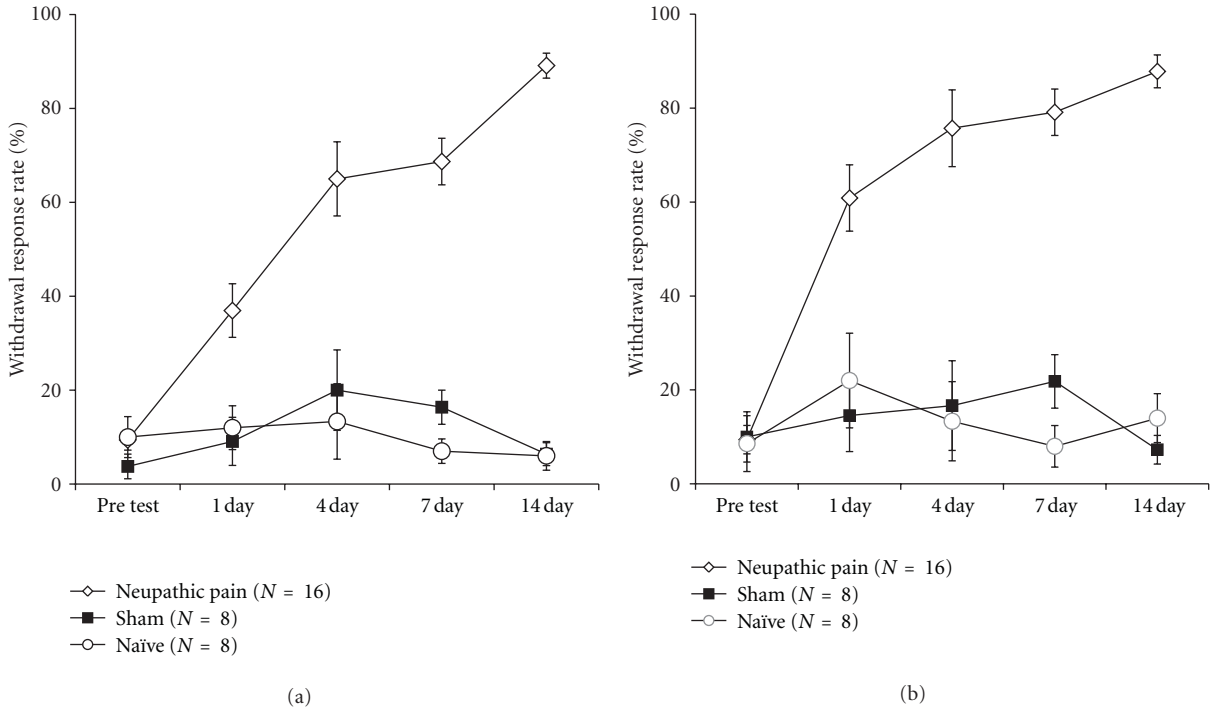

Figure 1: Development of mechanical (a) and cold allodynia (b) before EA stimulation. Naïve: the group without surgery; Sham: the sham-operated group; neuropathic: the neuropathic surgery group. Values are represented as mean ± SEM response rate as a percentage (number of foot withdrawals/number of trials × 100) ($^*P < 0.05$ versus Naïve).

($F_{4,7} = 7.59$, $P < 0.05$), and significant interaction ($F_{8,7} = 5.60$, $P < 0.05$). This implies that behavioral performances in three groups are different depending on time points after surgery. Subsequent Dennett's *post hoc* multiple comparisons showed that neuropathic group displayed significantly higher withdrawal responses to acetone at all time points after nerve injury ($P < 0.05$). These behavioral results indicated that nerve-injured rats were more sensitive to stimulations and the sensitivity was continued until postoperative day 14.

The expression of cytokines at the injured nerves ending following the development of pain was verified. Figure 2(a) shows the expression levels of cytokine mRNAs (IL-1β, IL-6, and TNF-α) in four different groups. The neuropathic group (NP) showed higher cytokine mRNAs expression than Sham and Naïve groups. However, when EA was applied (EA+NP), the expression level was decreased. Figure 2(b) shows the summarized results from 8 animals in each group. The neuropathic group only showed significant higher cytokine mRNAs expression levels for IL-1β, IL-6, and TNF-α. Furthermore, the EA application to neuropathic rats reduced the expression levels to the Sham and Naïve's level.

The expressions of cytokines in the DRG were also observed. The DRG neurons in the neuropathic group showed higher cytokine mRNAs expression as the injured nerves did. The EA application reduced cytokine mRNA levels in the neuropathic rats (Figure 3(a)). Figure 3(b) shows the summarized data in the DRG. The expression levels of IL-1β and IL-6 mRNAs were significantly higher in the neuropathic group than other groups. The expression of TNF-α tended to increase but the difference was not significant. EA application in neuropathic rats reduced the increased cytokine expressions.

4. Discussion

As shown in the present study, neuropathic pain was developed following peripheral nerve injury. Our previous studies demonstrated that the behavioral signs of neuropathic pain can be alleviated by manual acupuncture or EA [2, 3]. According to Cha et al. [2], in particular pain-relieving effects of EA on mechanical allodynia lasted for 180 min (3 hrs) in neuropathic pain rats. However, the precise efficacy and mechanisms of analgesic effects of acupuncture stimulation for the treatment of neuropathic pain syndromes remain unclear.

Several lines of evidence indicate that acupuncture stimulation mediates the release of neurochemical factors at some sites in the central nervous system (CNS) [16]. EA produces an antihyperalgesic effect in a rat model of inflammatory pain by activating the spinal endorphin/endomorphin system (for μ receptors) and the enkephalin system (for δ receptors) [17]. Serotoninergic and noradrenergic systems of pain inhibition may also mediate the analgesic effects of acupuncture [10, 13, 18–20]. Cha et al. [2] also reported that EA inhibits the expression level of nitric oxide synthase in the spinal cord of neuropathic rats. These studies suggest that EA stimulation may produce neurochemical changes in the nervous system.

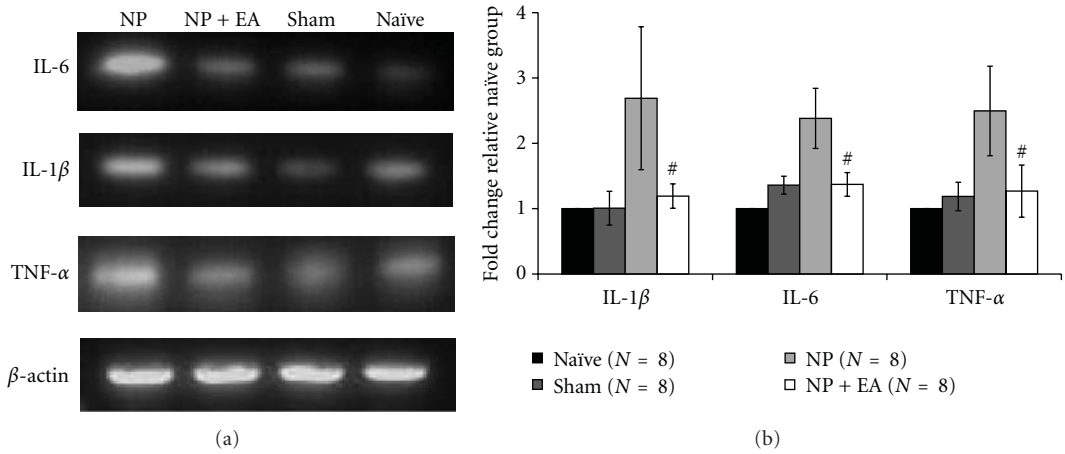

Figure 2: Effect of EA stimulation on cytokine mRNA expressions in injured nerves. (a) Representative photographs of cytokine expression in peripheral nerve tissue. (b) Expression levels of IL-1β, IL-6, and TNF-α in peripheral nerves of neuropathic rats were significantly higher than those in naïve rats. However, after EA stimulation, the cytokine expression levels were significantly decreased. NP: neuropathic group; NP+EA: neuropathic with electroacupuncture group; Sham: sham-operated group; Naïve: normal group ($^{*}P < 0.05$ compared to naïve, $^{\#}P < 0.05$ compared to neuropathic rats).

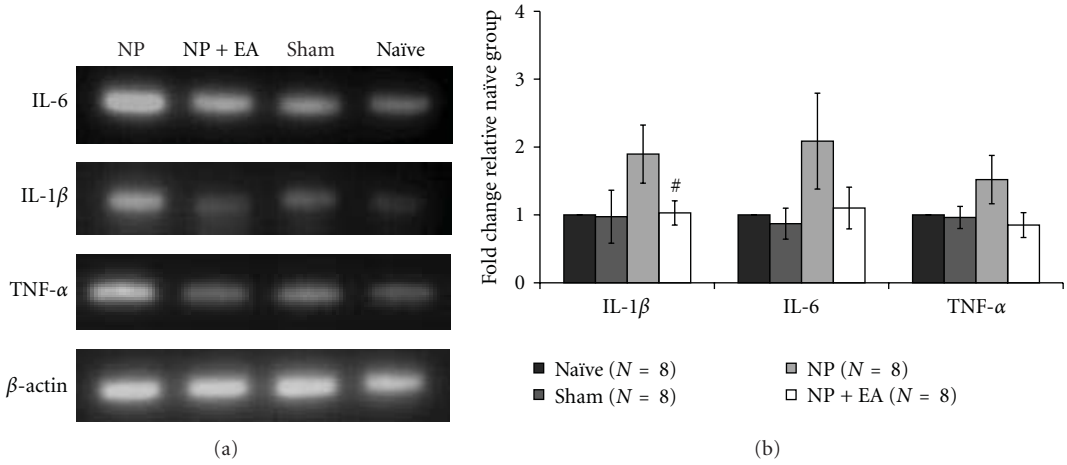

Figure 3: Effect of EA stimulation on cytokine mRNA expressions in the DRG. (a) Representative photographs of cytokine expressions in the DRG. (b) Expression levels of IL-1β and IL-6 in the DRG of neuropathic rats were significantly higher than those in naïve rats. However, the expression level of TNF-α was not significantly higher than in naïve rats. After EA stimulation, IL-1β cytokine expression level was significantly decreased. NP: neuropathic group; NP+EA: neuropathic with electroacupuncture group; Sham: sham-operated group; Naïve: normal group ($^{*}P < 0.05$ compared to naïve, $^{\#}P < 0.05$ compared to neuropathic rats).

Cytokines belong to glycoproteins which have low molecular weight. These are secreted by immune cells like T-cells, macrophages, and neutrophils. In the nervous system, Schwann cells and glial cells can synthesize and release cytokines [21, 22]. mRNA for proinflammatory cytokines including TNF-α, IL-1β, and IL-6 has been shown to increase in the DRG and spinal cord [14].

Recently, the relationship between cytokine expressions and neuropathic pain was reported. Evidence shows that cytokine signaling is related to pain development. For example, the time-dependent spinal cytokine expression levels showed a rapid and transient upregulation of TNF-α mRNA, a delayed upregulation of IL-1β mRNA, and a rapid and more sustained upregulation of IL-6 mRNA in the spinal nerve ligation model of neuropathic pain [23, 24]. Furthermore, it has been known that IL-1β and TNF-α are important for the initiation of persistent neuropathic pain, and IL-6 functions to maintain it [14]. Our results also showed significant increase in mRNA levels of proinflammatory cytokines in the injured nerves and DRG following the development of neuropathic pain.

However, the expression levels of TNF-α tended to increase but were not significantly high compared to the controls in DRGs while the expression levels significantly increased in the injured nerve. The TNF-α signaling pathway is activated early in abnormal pathological pain and plays a pivotal role in initiation of the proinflammatory cytokine cascades including IL-1β and IL-6 in the nervous system

including the DRG [25, 26]. It is not easy to explain the reason why there is no significant increase of TNF-α mRNA in the DRG. According to Lee et al. [14], TNF-α mRNA levels were immediately elevated in the DRG at 1 day after nerve injury and then gradually reduced. The expression levels of TNF-α mRNA were not different from the control group by 7 days after injury. Because the cytokine levels were investigated after behavioral test for 14 days, this may explain, at least in part, the lack of significant increase in TNF-α mRNA levels in nerve injured rats.

To date, however, the effects of acupuncture on proinflammatory cytokine expressions were not studied systematically yet. In the present study, we demonstrated that the expression levels of proinflammatory cytokines were dramatically decreased after EA stimulation in both the injured peripheral nerves and DRG of neuropathic rats. There were some studies which showed the effect of EA on pain attenuation and inhibition of cytokines expression in the central nervous system using an animal model of cancer pain [27] and on reduction of inflammation-induced cytokine expression by acupuncture using an animal model of carrageenan-induced inflammatory pain [28]. Similar to cancer pain and inflammatory pain, we demonstrated that EA inhibits the expressions of proinflammatory cytokines.

In summary, our results showed that the expression of cytokines in the DRG and injured peripheral nerves dramatically increased in neuropathic rats and significantly decreased after EA stimulation. This suggests that the increased levels of cytokines may be related to persistent pain which can be modulated by acupuncture stimulation including EA. However, the detailed mechanisms involved in cytokine expression related to acupuncture analgesia remain to be determined. A better understanding of the neurobiological mechanisms of EA stimulation-mediated analgesia may help to improve its effectiveness.

5. Conclusions

We generated neuropathic model and confirmed its sensitivity to pain behaviorally. The pain signaling significantly increased the cytokine levels in the injured nerves and DRG in neuropathic group than Sham and Naïve groups. But these increased cytokine levels were reduced by EA application after pain generation. The results indicate that EA stimulation can reduce the inflammatory cytokine expressions through pain signaling and modulation pathways. These suggest that EA stimulation is effective in the modulation of the inflammatory cytokine expression and it maybe an effective analgesic treatment on neuropathic pain symptoms.

Acknowledgment

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Research Article

Effects of Electroacupuncture on *N*-Methyl-*D*-aspartate Receptor-Related Signaling Pathway in the Spinal Cord of Normal Rats

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This study examined the influence of the N-methyl-D-aspartate receptor (NMDAR) on the modulation of related spinal signaling after electroacupuncture (EA) treatment in normal rats. Bilateral 2 Hz EA stimulations (1-2-3.0 mA) were delivered at acupoints corresponding to Zusanli (ST36) and Sanyinjiao (SP6) in men for 30 min. Thermal sensitization was strongly inhibited by EA, but this analgesia was reduced by preintrathecal injection of the NMDAR antagonist, MK801. Phosphorylation of the NMDAR NR2B subunit, cAMP response element-binding protein (CREB), and especially phosphatidylinositol 3-kinase (PI3K) were significantly induced by EA. However, these marked phosphorylations were not observed in MK801-pretreated rats. EA analgesia was reduced by preintrathecal injection with the calcium chelators Quin2 and TMB8, similar to the results evident using MK801. Phosphorylation of PI3K and CREB induced by EA was also inhibited by TMB8. Calcium influx by NMDAR activation may play an important role in EA analgesia of normal rats through the modulation of the phosphorylation of spinal PI3K and CREB.

1. Introduction

Electroacupuncture (EA), a new and modern type of traditional acupuncture, is widely used to treat various types of diseases in a clinical setting with the alterations of peripheral electrical stimulation rather than hand manipulation [1]. EA has an excellent pain relief efficacy profile, and it has been clinically used as a therapy in Korean medicine. Yet, the basis of the pain relief remains unclear.

Basic studies concerning the mechanisms of EA-mediated pain relief have been conducted using an animal model of pain [2]. The induction of EA analgesia involves the N-methyl-D-aspartate receptor (NMDAR), an ionotropic glutamate receptor [3]. The activation of NMDAR plays an important role in the induction and maintenance of hyperalgesia in the spinal dorsal horn [4–6]. Functional NMDAR are heteromeric complexes including the essential NR1 subunit and one or more of the four NR2 subunits (A–D). In particular, the NR2B subunit has an important function in spinal dorsal horn sensory pathways, and phosphorylation of this subunit plays a role in the induction of long-term potentiation (LTP), a phenomenon related to central sensitization [7, 8]. NMDAR containing the NR2B subunit localizes in the extrasynaptic membrane [9]. Their activations are involved in a variety of pain states including the development of central sensitization via the induction of LTP in the dorsal horn of the spinal cord [10, 11].

The induction of LTP requires an increase in the intracellular concentration of calcium in the postsynaptic neuron of the spinal cord [10, 12]. NMDAR-mediated influx of calcium into neurons may initiate the intracellular signaling pathways such as mitogen-activated protein kinase (MAPK) and other related proteins. Thus, NMDAR should be important for signaling cascades in the pain centralization in the spinal cord [13]. Noxious stimulation releases the neurotransmitter glutamate, and the activation of the corresponding glutamate receptors in postsynaptic dorsal horn neurons induces central sensitization [14, 15].

Activation of NMDAR has been implicated in noxious and inflammatory stimulation-evoked extracellular signal-regulated kinase (ERK) and cAMP response element-binding

protein (CREB) activation in dorsal horn neurons [15]. The ERK cascades are suggested to contribute to excitatory plasticity in the spinal cord [16]. Activation of intracellular signaling pathways involving p38 and ERK contribute importantly to synaptic plasticity underlying spinal neuronal sensitization. These activations in the spinal cord are reduced by antagonists of NMDAR [17]. The phosphatidylinositol 3-kinase (PI3K) inhibitor inhibits pain-related behavior in a dose-dependent manner and is a major factor in the expression of central sensitization after noxious stimuli [18].

Following concomitant use of EA with NMDAR antagonist, there was a difference in the experimental results between normal and pain animal models. The concomitant use of EA with NMDAR antagonist can synergistically alleviate pain in carrageenan-treated rats [19]. However, following the treatment with NMDAR antagonist, EA analgesia is impaired in normal rats [3]. The present study was performed under the hypothesis that EA analgesia has a different action between normal and pain animal models and produces a basic analgesic effect as a mild nociceptive stimulation.

The aim of the present study was to investigate the role of EA on the NR2B subunit of NMDAR and pain-related signaling proteins in a normal animal model. The link of phosphorylation between the NMDAR NR2B subunit and ERK, p38, PI3K, and CREB was assessed in the spinal cord of normal animal presenting EA analgesia.

2. Methods

2.1. Animals. Male Sprague-Dawley rats averaging 180 g in weight were obtained from Dooyeol Biotech (Seoul, Korea). The rats were housed at 22°C under alternating 12 hour cycles of dark and light and were fed a commercial diet and allowed tap water ad libitum starting 1 week before the study and continuing throughout the study. All experiments were approved by the Pusan National University Animal Care and Use Committee in accordance with the Council of the International Association for the Study of Pain of December 1982. Each group consisted of six rats for the behavioral test and tree rats for Western and immunohistochemical analysis. All treatments were administered under isoflurane (Choongwae, Seoul, Korea) anesthesia, which was provided using a model VIP 3000 calibrated vaporizer (Midmark, Orchard Park, OH, USA).

2.2. EA Stimulation. Under light gaseous anesthesia (1.0% isoflurane in air), two stainless-steel 0.2 mm-diameter needles were inserted to a depth of approximately 3 mm into each hind leg at the acupoints corresponding to Zusanli (ST36) and Sanyinjiao (SP6) in men and were connected to a Pulsemaster Multichannel Stimulator SYS-A300 electrical stimulator (Word Precision Instruments, Berlin, Germany). EA was accomplished with 2 Hz stimulation for 30 min and an intensity set at 1 mA and increasing stepwise to 2 mA and 3 mA, with each step lasting 10 min. For sham-EA control, acupuncture needles were inserted bilaterally at a point lateral to the aforementioned acupoints without any electrical stimulation.

2.3. Intrathecal Injection. Intrathecal catheterization was performed as previously described under 1% isoflurane anesthesia [20]. Briefly, a PE-10 intrathecal catheter was inserted through the slit in the L4-5 level of the vertebrate to reach the lumbar enlargement of the spinal cord. Two days after surgery, only those rats without overt signs of spinal cord or root damage, such as paralysis or lameness, were used for experimentation. The NMDAR antagonist MK801 and calcium chelator Quin2 or TMB8 were dissolved in sterile saline and injected intrathecally at a volume of 10 μL via a catheter within 1 min. The catheter was then filled with 8 μL of saline for flushing. Drugs were administered once into the subarachnoid space of the spinal cord 30 min prior to EA stimulation. The vehicle control group for drugs received injections of identical amounts of phosphate-buffered saline (PBS) via an identical method.

2.4. Measurements of Thermal Hyperalgesia. The heat paw withdrawal latency (PWL) of six rats each group was measured by the plantar test using a model 37370 apparatus (Ugo-Basile, Comerio, Italy). The rats were placed in six separate cages (17 × 11.5 × 14 cm) for 30 min after EA treatment, and thermal thresholds of the left hind paw were assessed three times with a 5 min interval between trials. The mean values were taken as the PWL. The intensity of the infrared generator was adjusted to produce withdrawal latencies of approximately 8–10 s (80 infrared intensity). A cut-off period of 15 s was used. The latency responses were monitored from 30 min after EA stimulation with or without injection of MK801 and the calcium chelators. Rats not treated with EA were also placed under gaseous anesthesia, and the PWL was then measured.

2.5. Western Blot. To examine changes in the NMDAR NR2B subunit, ERK, p38, PI3K, and CREB, the L4-5 segments of the spinal cords were removed 0, 10, 30, 60, 90 and 120 min after the beginning of EA stimulation. L4-5 segment of the spinal cord, 5 mm in length, involved partially lumbar enlargement of the spinal cord, in three rats of each group by laminectomy under anesthesia induced by intraperitoneal injection of 4% chloral hydrate (300 mg/kg). The spinal cords were washed in cold HEPES buffer and homogenized in nine volumes of lysis buffer. Equal amounts of proteins were then separated by 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), after which the resolved proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) that was subsequently blocked with 5% nonfat milk in Tris-buffered saline containing 0.4% Tween 20.

The membranes were incubated with anti-NR2B (Millipore, Billerica, MA, USA), anti-phospho-NR2B (pNR2B, ser1303; Upstate Biotechnology, Lake Placid, NY, USA), anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-ERK (pERK, thr202/tyr 204; Cell Signaling Technology, Danvers, MA, USA), anti-p38 (Santa Cruz Biotechnology), anti-phospho-p38 (pp38, thr180/tyr182; Cell Signaling Technology), anti-PI3K (Cell Signaling Technology), anti-phospho-PI3K(pPI3K, Tyr458; Cell Signaling Technology), anti-CREB (Cell Signaling Technology) or

antiphospho-CREB (pCREB, ser133; Cell Signaling Technology) for 1-2 h at room temperature, after which the blots were incubated with horseradish peroxidase-conjugated secondary antibody, and the antibody-specific proteins were visualized using an enhanced chemiluminescence detection system according to the recommended procedure (Pierce, Rockford, IL, USA). β-actin was used as a loading control for all experiments. Quantification of immunoreactivity corresponding to the total and phosphorylated bands was performed by densitometric analysis using a MultiGauge Version 3.0 (Fujifilm, Tokyo, Japan).

2.6. Immunohistochemistry. The L4-5 segments of the spinal cords were removed as described above for Western blot in three different rats in each group. Tissues were fixed in 4% paraformaldehyde and immersed in 30% sucrose for 48 h at 4°C for cryoprotection. Frozen 14 μm-thick sections were then prepared and preincubated in a blocking solution (CAS-block, Invitrogen-Molecular Probes, Camorillo, CA, USA) for 9 min at room temperature. The sections were incubated with the following primary antibodies overnight in PBS at 4°C: pPI3K (Tyr607, ABcam, Cambridge, UK), pCREB (Ser 133, Santa Cruz Biotechnology) and antineuronal nuclei (NeuN, Millipore-Chemicon, Billerica, MA, USA). After being washed with PBS-containing Tween-20 (PBST), the sections were incubated with the secondary antibody, goat anti-rabbit IgG-TR (Santa Cruz Biotechnology), and anti-mouse IgG-FITC (Vector Laboratories, Burlingame, CA) for 2 h at room temperature and then washed with PBST. Slides were mounted in the mounting medium for fluorescence (Vector Laboratories, Inc. Burlingame, CA), and images were captured using a LSM 510 laser scanning confocal microscope (Zeiss, Oberkohen, Germany).

2.7. Data Analyses. Data are expressed as the mean ± SEM. Data were analyzed by multifactorial analysis of variance (ANOVA) using the Sigmastat statistical program Version 11.0 (Systat Software, San Jose, CA, USA). Behavioral analysis was performed by a two-way ANOVA post hoc test via Tukey's test. Western blot analysis was performed using a one-way ANOVA post hoc test via Tukey's test. A $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Behavioral Analysis on EA with or without MK801 Pretreatment. Rats usually resumed full activity within 2–5 min of the cessation of isoflurane anesthesia, regardless of whether they received EA stimulation. Behavioral test measured the basal threshold of heat PWL for the left hindpaw 1 h before EA stimulations, and it was measured at 30 min intervals at 30, 60, and 90 min following EA stimulation. EA produced an analgesia characterized by a markedly higher PWL profile as compared with the normal control rat within 30 min after stimulation. But, findings were similar to control rats 60 min following EA. With EA stimulations following pretreatment with MK801, there was a lower degree of PWL as compared with EA-treated rats; the MK801 effect was dose dependent. Significant differences

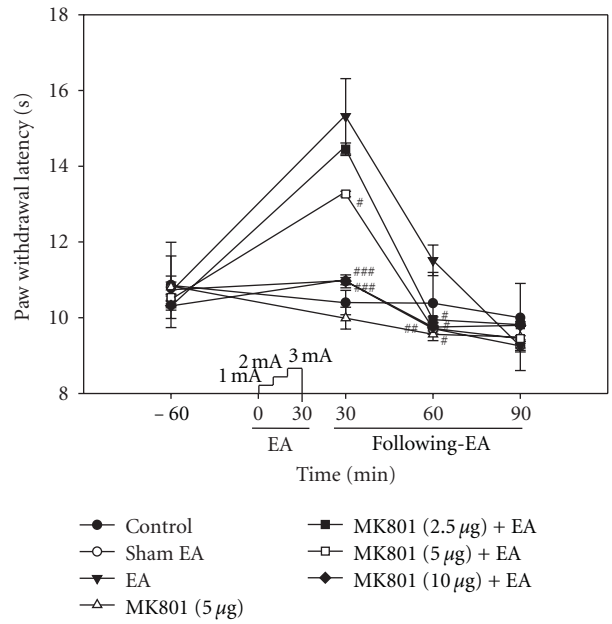

Figure 1: Effect of EA stimulation with or without MK801 pretreatment on the PWL to thermal stimuli. Each point indicates the mean ± SEM ($n = 6$). EA-stimulated rats showed a significant analgesic effect compared with normal control rats, and pretreatment with MK801 inhibited EA-induced analgesia. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with EA-treated rats.

in PWL were observed in the MK801-treated rats within 30 and 60 min after EA stimulation. These results suggested that the pretreatment with MK801 impaired EA analgesia, which implicated NMDAR in EA analgesia (Figure 1).

3.2. NMDAR NR2B Subunit and Related Proteins Analyses on EA with or without MK801 Pretreatment. The first experiment examined the induction of the total and phosphorylated NMDAR NR2B subunit, ERK, p38, PI3K, and CREB in normal rats. Next, the NMDAR antagonist MK801 was intrathecally preinjected to identify the proteins associated with NMDAR in the spinal cord and checked for time-dependent alterations. Following EA stimulations, there were no time-dependent alterations in the total protein, but there was a marked degree of changes in the phosphorylated form of NR2B, PI3K, and CREB. The phosphorylation of NMDAR NR2B was significantly increased at 30 min after the beginning of EA stimulation. Phosphorylation of PI3K was significantly increased at 30 min after the beginning of EA stimulation and between 30 and 90 min after EA. Phosphorylation of CREB was significantly increased 30 min after the beginning of EA stimulation. However, phosphorylation of ERK and p38 showed no significant changes (Figure 2).

Following treatment with the NMDAR antagonist MK801, the induction of the total and phosphorylated proteins was evaluated. Similar to the above results, there was no marked degree of change in the total protein. The significant alteration of NR2B phosphorylation was not observed at 30 min after the beginning of EA, and this expression

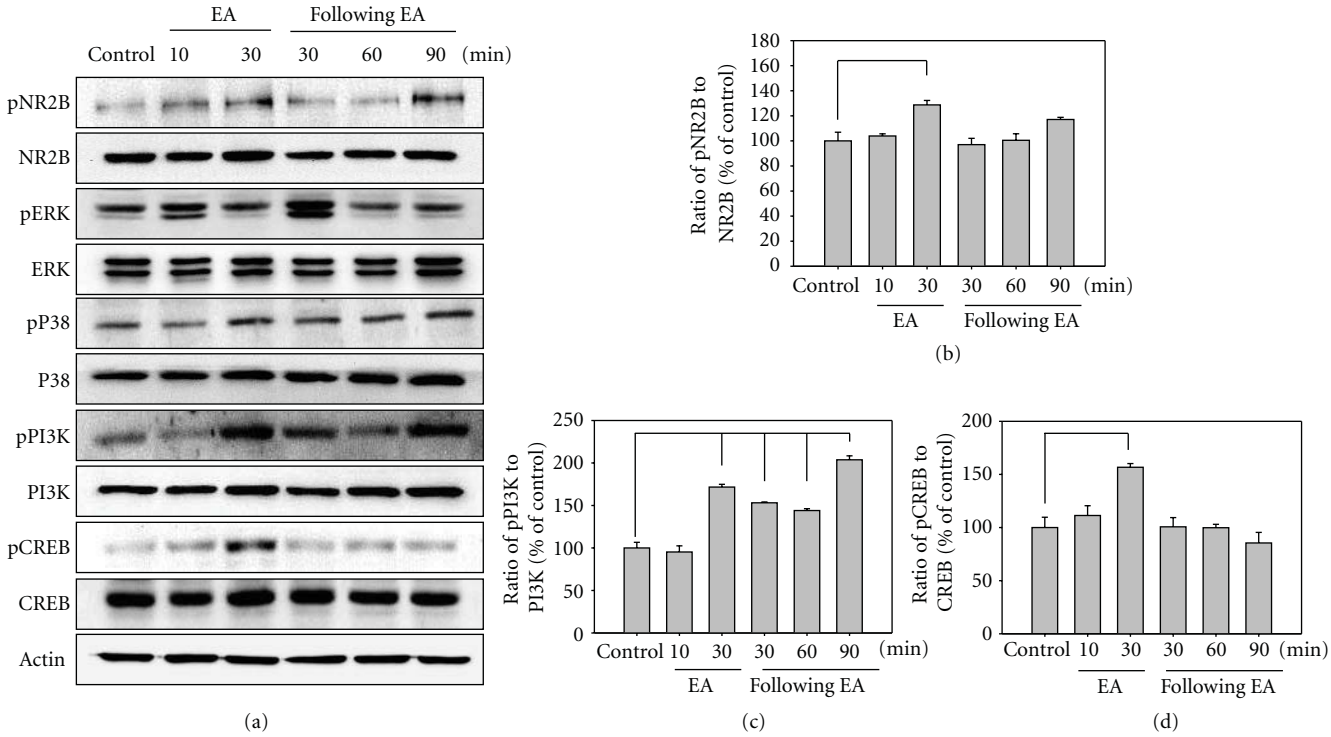

Figure 2: Western blot (a) and densitometric analysis for NMDAR NR2B subunit (b), PI3K (c), and CREB (d) in the L4-5 segment of the spinal cord in EA-treated rats. The level of each protein is expressed as a percentage of the control. Each single experiment was conducted on three pooled animals. The panel represents a typical result from three independent experiments. The phosphorylation of NR2B subunit increased significantly at 30 min from the beginning of EA stimulation. The phosphorylation of PI3K was significantly increased at 30 min from the beginning of EA stimulation and between 30 and 90 min after EA. The phosphorylation of CREB was significantly increased at 30 min from the beginning of EA stimulation. $^{**}P < 0.01$, $^{***}P < 0.001$.

was somewhat decreased after EA stimulation. Although there were no significant changes, ERK phosphorylation was increased at 10 min from the beginning of EA. Additionally, the marked increases of PI3K and CREB phosphorylation, which were formed following EA stimulation, were not observed following MK801 pretreatment. Especially, CREB showed a significantly lower phosphorylation from 60 min on following EA stimulations with MK801 pretreatment (Figure 3).

3.3. Effects of Calcium Chelator on EA with or without MK801 Pretreatment. NMDAR is a receptor involved in calcium influx into neurons. Accordingly, with the assumption that the above results originated from the intracellular calcium influx via NMDAR, pretreatment with the calcium chelators Quin2 and TMB8 was carried out. Both calcium chelators impaired EA analgesia in a similar manner to the pretreatment with NMDAR antagonist MK801 in the behavioral test (Figure 4). Following the pretreatment with TMB8, changes in the phosphorylation of CREB and PI3K were evaluated at 30 min from the beginning of EA stimulation. Following the pretreatment with calcium chelator, the phosphorylation of CREB and PI3K due to EA stimulations was significantly decreased (Figure 5). To localize pPI3K and pCREB expression and distribution in the spinal cord, we employed immunofluorescence staining with neuron markers NeuN in normal and EA-treated rats. Double-labeling staining showed a large proportion of pPI3K or pCREB and NeuN colocalization in the laminae IV-VI of the dorsal horn (Figure 6). While a similar distribution of pPI3K and pCREB was observed in neuronal cells, strong expression of PI3K was evident in EA-treated rats compared with normal rats.

4. Discussion

EA stimulation markedly reduces inflammatory hyperalgesia by inhibiting the release of glutamate in the spinal dorsal horn, and NMDAR antagonists display an antinociceptive action in an inflammatory pain model [19]. Induction of EA analgesia involves NMDAR and is inhibited by a NMDAR antagonist in a normal rat [3]. However, NMDAR-mediated EA-induced analgesic effects, especially the underlying mechanism(s) of EA in normal rats, have received relatively little attention.

To clarify the mechanisms by which EA alleviates pain, studies conducted on normal rats as well as investigations on pain alleviation in a pain model would also be of significance. We performed 2 Hz EA stimulation at ST36 and SP6 acupoints, which previously showed significant analgesic effects and phosphorylation of the NMDAR subunit [21]. The goal of the present study was to observe the time-dependent alteration in the spinal NMDA NR2B subunit, ERK, p38,

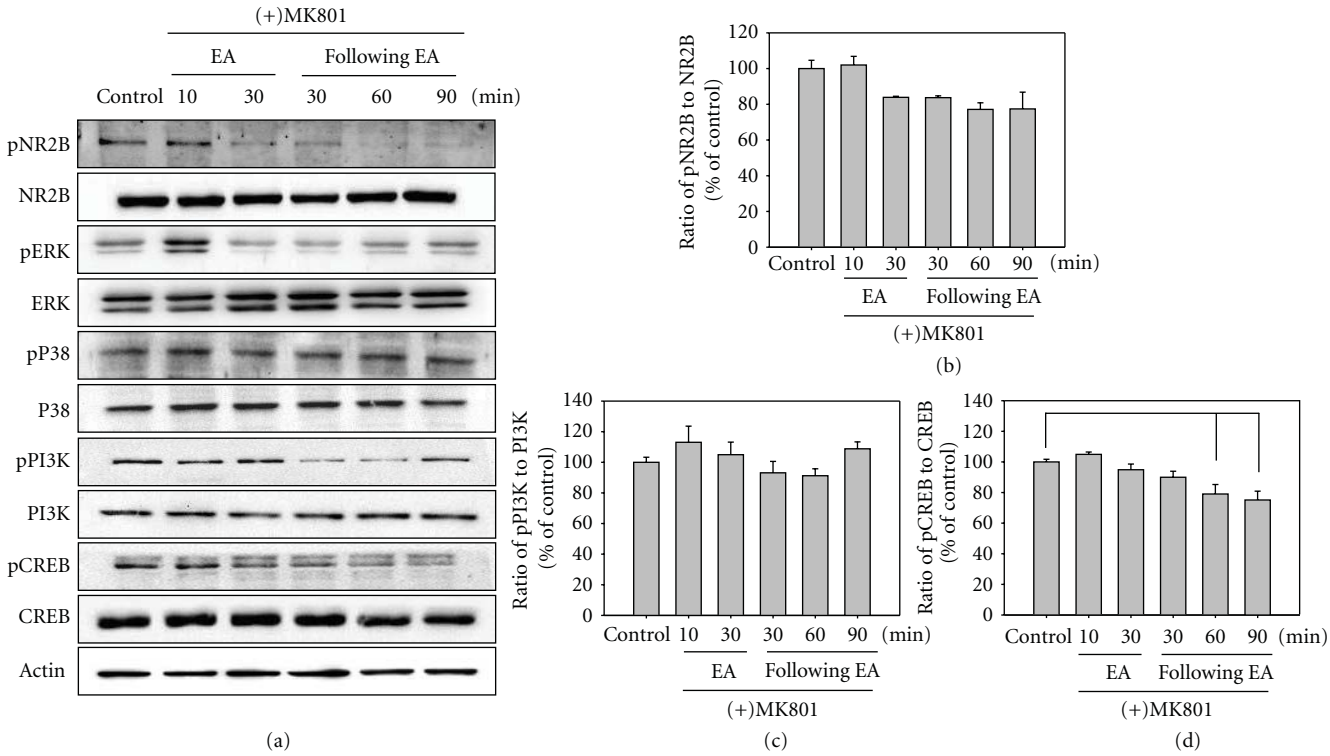

Figure 3: Western blot (a) and densitometric analysis for NMDAR NR2B subunit (b), PI3K (c), and CREB (d) in the L4-5 segment of the spinal cord in MK801- and EA-treated rats. The level of each protein is expressed as a percentage of the control. Each single experiment was conducted on three pooled animals. The panel represents a typical result from three independent experiments. EA-induced phosphorylation of NR2B subunit and PI3K was not observed by MK801 pretreatment, and a significant lower phosphorylation of CREB was detected from at 60 min after EA stimulations; $^{*}P < 0.05$.

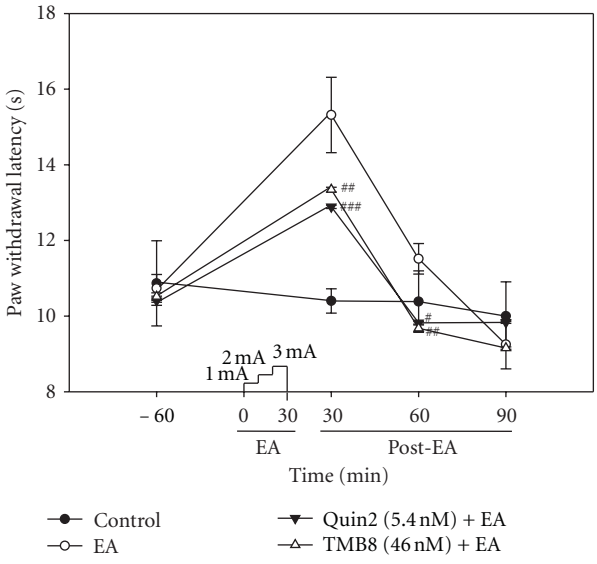

Figure 4: Effect of EA stimulation with calcium chelator pretreatment on the PWL to thermal stimuli. Each point indicates the mean ± SEM ($n = 6$). EA-stimulated rats showed a significant analgesic effect compared with normal arts, but Quin2 and TMB8 pretreatment inhibited EA-induced analgesia. $^{***}P < 0.001$ compared with control rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with EA-treated rats.

PI3K and CREB phosphorylation in EA-stimulated rats that had or had not been pretreated with the NMDAR antagonist MK801.

Analgesia induced by EA was observed, and the effects of MK801 pretreatment on heat PWL were assessed. EA stimulation resulted in persistent analgesia within 60 min after EA treatment. However, EA-induced analgesia was significantly abolished by pretreatment with MK801 (Figure 1). Behavioral studies demonstrated that MK801 profoundly inhibited the PWL of EA-induced analgesia, similar to previous observations [3]. Our behavioral results may indicate the involvement of NMDAR in induction or maintenance of EA analgesia.

NMDARs are important in the plasticity of the synaptic processes of the nervous system, such as sensitization of the nociceptive pathways [22]. The inhibition of NMDAR containing the NR2B subunit in the superficial dorsal horn of the spinal cord suppresses nociceptive transmission, and these receptors seem to have a higher conductance than other NMDARs [10, 23]. The NR2B subunit may be important in pain states where a possible build-up of glutamate activates extrasynaptic NMDAR in the spinal cord [9, 10].

NMDAR containing the NR2B subunit plays a role in the development of central sensitization via the induction of LTP in dorsal horn nociceptive synaptic transmission [11]. LTP requires an increase in the intracellular concentration of

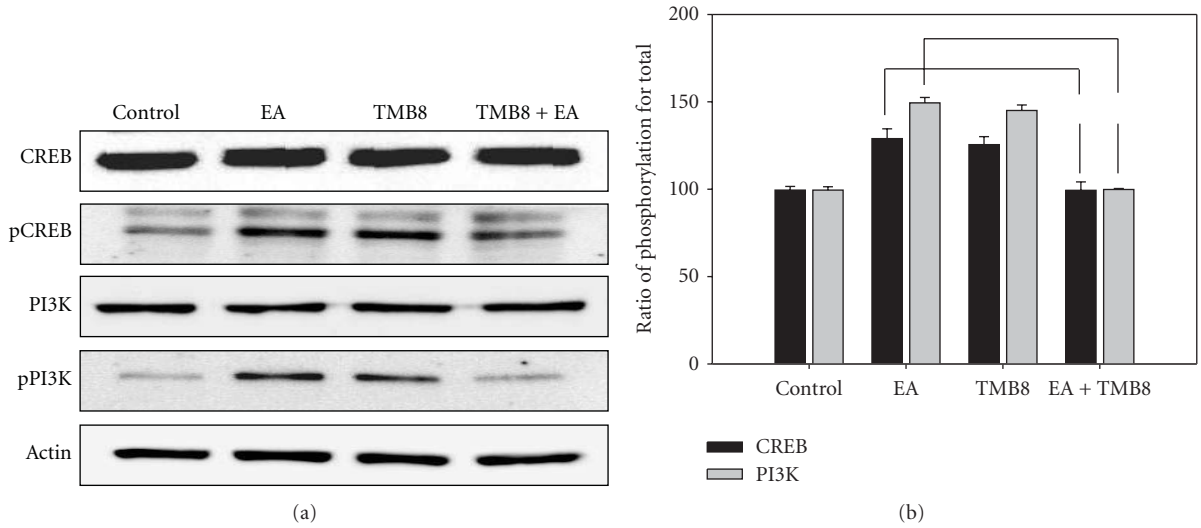

Figure 5: Western blot (a) and densitometric analysis (b) for induction of total and phosphorylation of PI3K and CREB in the L4-5 segment of the spinal cord in TMB8- and EA-treated rats. The level of each protein is expressed as a percentage of the control. Each single experiment was conducted on three pooled animals. The panel represents a typical result from three independent experiments. EA-induced phosphorylation of PI3K and CREB was arrested by TMB8 pretreatment. $^{**}P < 0.01$, $^{***}P < 0.001$.

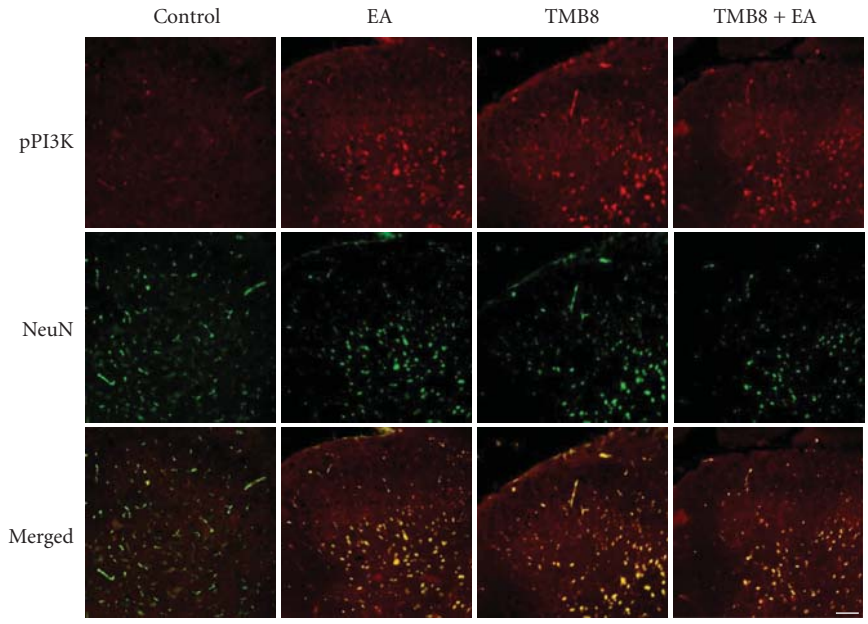

Figure 6: Immunohistochemical localization of pPI3K in the dorsal horn L4-5 segment in normal and EA-treated rats. The expression of pPI3K (red) was usually detected in the laminae IV-VI regions of dorsal horn of the spinal cord. NeuN (green) was also detected in the similar region, and merged image showed that pPI3K and NeuN were colocalized. Scale bar = 200 μm.

calcium in the postsynaptic neurons of the spinal cord [12]. NMDARs function as a calcium channel. LTP in dorsal horn neurons are dependent on NMDAR containing the NR2B subunit, and this receptor is involved in use-dependent sensitization at the spinal level [10].

The elevation of intracellular calcium activates a cascade of biochemical events and ultimately leads to altered gene expression [24, 25]. Calcium entry into neurons via NMDAR may initiate MAPKs and the PI3K signaling cascade. Thus, it was appropriate to examine the induction of total and phosphorylated ERK, p38, PI3K, and CREB, as well as the NMDA NR2B subunit during and following EA stimulation.

Phosphorylation of the NMDAR NR2B subunit was significantly induced by EA treatment. In addition, phosphorylation of PI3K, CREB, and especially PI3K was strongly induced by EA stimulation, but that of ERK and p38 was not induced (Figure 2). To demonstrate the possible involvement of NMDAR, the NMDAR antagonist MK-801 was administrated intrathecally before the EA stimulation. EA-induced phosphorylation of the NMDAR NR2B subunit,

PI3K, and CREB was strongly inhibited by MK801 pretreatment (Figure 3). These results indicate that EA analgesia may be produced by phosphorylation of PI3K and CREB via NMDAR NR2B subunit activation in the spinal dorsal horn. CREB and PI3K may be important intracellular controllers of EA analgesia in the spinal cord with the NMDAR NR2B subunit.

PI3K is a lipid kinase that generates membrane-associated second messengers, which are able to activate several signaling cascades and cellular processes [18, 26]. PI3K is involved in a transcription-independent and short-term form of spinal plasticity, termed wind-up, which may underlie central sensitization in C-fiber-mediated evoked responses, and PI3K inhibition reduces the phosphorylation of the NR2B subunit of NMDAR [18].

CREB signaling plays a role in the long-term facilitation after noxious stimuli in the spinal cord neurons. CREB phosphorylation represents a better marker than *c-fos* expression for neuronal activity after noxious stimulation because its induction is more rapid and more sensitive [27]. The NMDAR antagonist MK801 markedly suppressed EA-induced CREB phosphorylation in the present study, corroborating the previous demonstrating that MK801-mediated suppression of spinal cord associated pain in a formalin-induced pain model [27].

Influx of calcium via NMDARs leads to the phosphorylation and activation of CaMKII, and CAMKII activation may also affect phosphorylation of the NMDAR NR2B subunit [18]. Therefore, we hypothesized that calcium might be involved in EA analgesia in the spinal cord. To assess this hypothesis, we investigated the effect of calcium chelators on EA analgesia, animal behavior (PWL) and subsequently examined the phosphorylation of PI3K and CREB by Western blotting. A diminished PWL was apparent in rats pretreated with calcium chelator as compared with EA-treated rats; similar results were obtained upon pretreatment with NMDAR antagonist (Figure 4). The phosphorylation of PI3K and CREB due to EA stimulation was decreased by pretreatment with calcium chelator (Figure 5). These results implicate PI3K and CREB as key players in EA analgesia, as in the central sensitization of noxious stimulation. Phosphorylation of the NMDAR NR2B subunit provokes increased calcium influx upon EA stimulation, which may induce PI3K and CREB phosphorylation as sensitization-like mechanisms in the dorsal horn of the spinal cord.

Low-frequency EA activates betaendorphin and enkephalin systems through their receptors, which are expressed in the spinal cord and which contribute to the modulation of nociceptive transmission [1]. The relationship between PI3K activation and EA analgesia in normal rats remains unclear in the nervous system. But PI3K activation contributes to calcium-regulated opioid release from polymorphonuclear cells, the major source of opioids, and thereby inhibits inflammatory pain [28]. Further studies are need concerning the interaction between opioid system and PI3K signaling in spinal nociception during EA stimulation.

Concerning the localization of pPI3K and pCREB induced by EA stimulation, these reactions were colocalized with neuronal marker and were found mainly in laminae IV-VI in the dorsal horn (Figure 6). Primary afferents of low-threshold Aα/Aβ mechanoreceptors terminate mainly in laminae III–V [29]. These results suggest that EA stimulation induces expression of PI3K and CREB in neuronal cells distributed in laminae IV-VI.

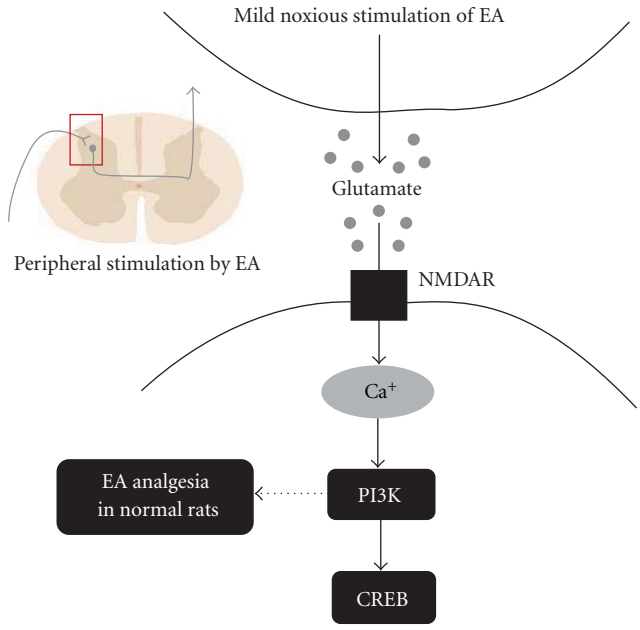

Figure 7: Proposed schematic diagram for of EA-induced analgesia in normal rats. Peripheral stimulation by EA induces glutamate release in the spinal cord dorsal horn this neurotransmitter activates NMDAR. Appropriate influx of calcium via activated NMDAR leads to the phosphorylation of the PI3K and CREB cascade, especially PI3K, manifesting as analgesia. The dotted line denotes where further study is needed to clarify an interaction between PI3K and EA analgesia in normal rats.

In a pain animal model, calcium influx via NMDAR is involved in the spinal centralization and induces persistent pain. However, depending on the intracellular concentration of calcium, the results might vary. An appropriate level of calcium produces analgesia with the activation of signaling protein. But, an excessively higher level of intracellular calcium contradictorily induces and maintains pain.

In normal rats, the intracellular calcium influx was induced through the activation of NMDAR. Thus, the related proteins were activated, and this led to the EA analgesia. Put another way, as an appropriate mild noxious stimulation, EA stimulation may induce an appropriate degree of intracellular calcium influx by NMDAR and phosphorylate PI3K and CREB, producing EA analgesia.

In the present study, normal rats were sequentially administered EA stimulations at magnitudes of 1, 2, and 3 mA in 10 min intervals and induced EA analgesia over a total period of 30 min. The degree of pain control reached a maximum level when the stimulations were given for approximately 20 min with lower 1 mA in an inflammatory pain model in our lab. In a prior study, only a high frequency of electrical stimulation for C-fibers (3 mA) was capable of activating NMDAR and inducing the intracellular signal

pathway in the spinal cord [13]. The frequency and intensity of electrical stimulation may be an important factor to activate the intracellular signal pathway.

Accordingly, if EA stimulations evoke EA analgesia as a mild noxious stimulation, this would produce an analgesic effect with the application of EA whose magnitude was of an appropriate degree in a dependent manner to the severity of pain. Based on cases not appropriate for a pain model, following the treatment with extremely high degree of stimulations or a long-term treatment with EA, pain might be aggravated.

Consequently, we suppose that EA analgesia in a normal rat has a different effect on modulating spinal NMDAR-related signaling in rats with inflammatory or neuropathic pain and propose possible schematic diagram (Figure 7). The present results suggest that appropriate influx of calcium via NMDAR in normal rats induces related PI3K and CREB phosphorylation, especially PI3K, manifesting as analgesia. EA analgesia in normal rats may depend on the intensity of the applied EA stimulation. In the application of EA to a pain model, basic studies should also be conducted to examine such parameters as the frequency and intensity of EA depending on the diseases.

Acknowledgment

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Research Article

The Cortical and Striatal Gene Expression Profile of 100 Hz Electroacupuncture Treatment in 6-Hydroxydopamine-Induced Parkinson's Disease Model

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Electroacupuncture (EA), especially high-frequency EA, has frequently been used as an alternative therapy for Parkinson disease (PD) and is reportedly effective for alleviating motor symptoms in patients and PD models. However, the molecular mechanism underlying its effectiveness is not completely understood. To implement a full-scale search for the targets of 100 Hz EA, we selected rat models treated with 6-hydroxydopamine into the unilateral MFB, which mimic end-stage PD. High-throughput microarray analysis was then used to uncover the regulated targets in the cortex and striatum after 4-week EA treatment. In the differentially regulated transcripts, the proportion of recovered expression profiles in the genes, the functional categories of targets in different profiles, and the affected pathways were analyzed. Our results suggested that the recovery of homeostasis in the transcript network and many regulated functional clusters in the cortex and striatum after EA treatment may contribute to the behavioral improvement of PD rats.

1. Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disease that affects about 1–3% of the population over 65 years of age and is characterized by the progressive loss of DAergic neurons in the substantia nigra pars compacta and an associated decline in striatal dopamine [1]. It is known that "idiopathic" PD (>85% of all cases) does not appear to exhibit heritability [2]. In addition, there is no cure for PD, and the underlying pathogenesis of the disease is still unknown [2]. In general, we understand that the pathology of PD is complex and is most likely a "consequence of an unspecified combination of genetic and environmental factors, which induce a common pathogenic cascade of molecular events" [3]. Pharmacological and surgical therapies are available that can alleviate some of the symptoms, but these interventions are associated with serious side effects and generally lose their efficacy over time [4]. Acupuncture, however, has long been known to have therapeutic effects on chronic and acute pain. Today, electro-acupuncture (EA) is frequently used as an alternative therapy for PD and reportedly leads to subjective improvements in PD patients [5, 6]. An increasing number of clinical studies and experimental data support EA, especially high-frequency EA, as an effective therapy for alleviating motor symptoms in patients and PD models [7–9]. However, the underlying neuroprotective mechanisms of acupuncture treatment in PD are not yet understood. Some experimental results reveal that EA could increase the number of tyrosine hydroxylase- (TH-) positive neurons in the substantia nigra and striatum (STR) of an MPTP- (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-) treated mouse model [10, 11]. Using the hemiparkinsonian rat model induced by unilateral transection of the medial forebrain bundle (MFB), our previous studies showed that 100 Hz EA

could significantly improve the apomorphine-induced motor disorder symptoms, but the dopamine (DA) content did not increase significantly [12, 13]. Moreover, the effects of high-frequency EA on the motor symptoms of Parkinsonian rats involved the upregulation of endogenous neurotrophins [8, 9] and BDNF and trkB receptors in a 6-hydroxydopamine- (6-OHDA-) induced rat PD model [14], as well as the restoration of the homeostasis of DAergic transmission in the basal ganglia circuit and the suppression of inflammatory responses in the ventral midbrain [9, 12, 15]. These results suggest that the mechanisms underlying the benefits of acupuncture are systemic and unknown. Thus, it is especially important to find EA-targeted transcripts. Gene expression profiling using microarray technology could re veal the complex nature of disease genesis and progression [16] and the many targets of certain therapeutic measures at the genomic level [17]. To search for EA-affected transcripts after 6-OHDA treatment and obtain insight into the potential mechanism of EA's effect, we applied the microar ray method to explore gene expression profiles in a control group, a 6-OHDA-unilateral lesioned rat model, and an EA-treated group for 4 weeks after 6-OHDA lesion. We ob served the differential genes at the transcription level and the changed gene profiles in the cortex and STR.

2. Materials and Methods

2.1. Animal Care and Unilateral MFB Injection with 6-OHDA. Adult female Sprague Dawley rats weighing 200–220 g were obtained from the laboratory animal center, Capital University of Medical Sciences, and housed in a standard 12-h on/off light cycle with ad libitum access to food and water. The experimental procedures were approved by the Committee on Animal Care and Usage, Capital Medical University, and all efforts were made to minimize animal suffering.

The artificial lesion with 6-OHDA into the MFB is considered an appropriate model to study late-stage PD [18]. After adapting to their surroundings for 3 days, SD rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and then fixed on a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with the mouth bar set at −3.3 mm. The skull was exposed, and a burr hole was drilled to introduce a syringe for a single injection of the 6-OHDA solution (containing 2 μg 6-OHDA per μL in 0.01% ascorbicacid, pH = 5). To minimize variability due to degradation of the toxin, the 6-OHDA solutions were freshly made, kept on ice, and protected from exposure to light. The solution was injected into the left MFB according to the atlas of König and Klippel (1963); the coordinates relative to bregma were ML: −2.5 mm, AP: −3.8 mm, and DV: −8.1 mm. A total volume of 4 μL 6-OHDA was injected at a flow rate of 1 μL/min, and the syringe was left in place for 2 min and then slowly removed over a 1-2 min time period. The skin was sutured, and then the animals received 4 mg/kg ketofen i.p., as an analgesic and were allowed to recover before returning to the animal housing facilities. The lesion was allowed to progress for 5 weeks, after which the animals were sacrificed for postmortem analyses.

2.2. Behavioral Testing. Rotational testing was performed blindly and in automatic rotameter bowls (Columbus Instruments, USA) prior to MFB lesion and 7 (one week), 14 (two weeks), and 35 days (5 weeks) following the lesion (Figure 1(b)), a method previously reported by Liang et al. [9]. Changes in rotational behavior were assessed by monitoring body rotations induced by apomorphine (0.5 mg/kg, s.c.). The net number of rotations (contralateral-ipsilateral) was recorded over a time span of 45 min, and the number of turns per minute was calculated [13, 14, 19]. This behavioral test was performed blindly. Data were reported as means ± SEM. Statistical significance was assessed using one-way ANOVA followed by the Newman-Keuls post hoc test of differences between groups. A $P < 0.01$ was considered statistically significant in this portion of the experiment.

2.3. High-Frequency EA Stimulation. EA stimulation at 100 Hz was performed following our previously described method [8, 9, 12]. Rats were randomly divided into three groups: the sham group, the 6-OHDA-injected group, and the 6-OHDA-injected group with EA stimulation at 100 Hz. Animals in the sham group underwent the same operation without injection of 6-OHDA. EA stimulation was administered from day 8 following the 6-OHDA injection after behavioral testing at one week. Two stainless steel needles 0.25 mm in diameter and 5 mm in length were inserted obliquely at the acupuncture point DAZHUI (GV 14, directly below the spinous process of the vertebra prominens) and horizontally at BAIHUI (GV 21, at the midpoint of the line connecting the two ears). Bidirectional square-wave electrical pulses (0.2 ms duration), designated EA, were administered for a total of 30 min each day, 6 days per week. The duration of EA treatment was limited to 4 weeks. The intensity of the stimulation was increased stepwise from 1 to 2 mA and then to 3 mA, with each step lasting for 10 min. The animals remained in the cage during EA administration in an awake, unrestrained condition.

2.4. Immunohistochemical Analysis. Rats were deeply anesthetized with 450 mg/kg chloral hydrate and then transcardially perfused with 100 mL saline followed by 200 mL 4% paraformaldehyde in phosphate buffer. Brains were dissected and postfixed in the same fixative and cryoprotected in 30% sucrose solution for 3–5 days. Frozen sections were cut into 30 μm thick sections on a cryostat and processed for TH-immunohistochemistry. Every sixth section of the SN or STR was selected from each brain. After several washes, brain slices were incubated with TH antibody (diluted 1 : 2000; Chemicon, USA) for 24 h at 4°C. The antibody was detected using an ABC Elite kit (Vector laboratories, USA) with 3, 3′-diaminobenzidine (DAB) and nickel enhancement [13].

2.5. Protein Extraction and Western Blot. To obtain protein extracts, rats were sacrificed by cervical dislocation 2 days after the last electroacupuncture treatment, and each encephalic region was dissected rapidly and frozen using liquid nitrogen and kept at −80°C until use. The frozen substantia nigras were homogenized 4 times with sonication at 70% intensity in 10 s bursts with 500 μL of routine SDS-protein

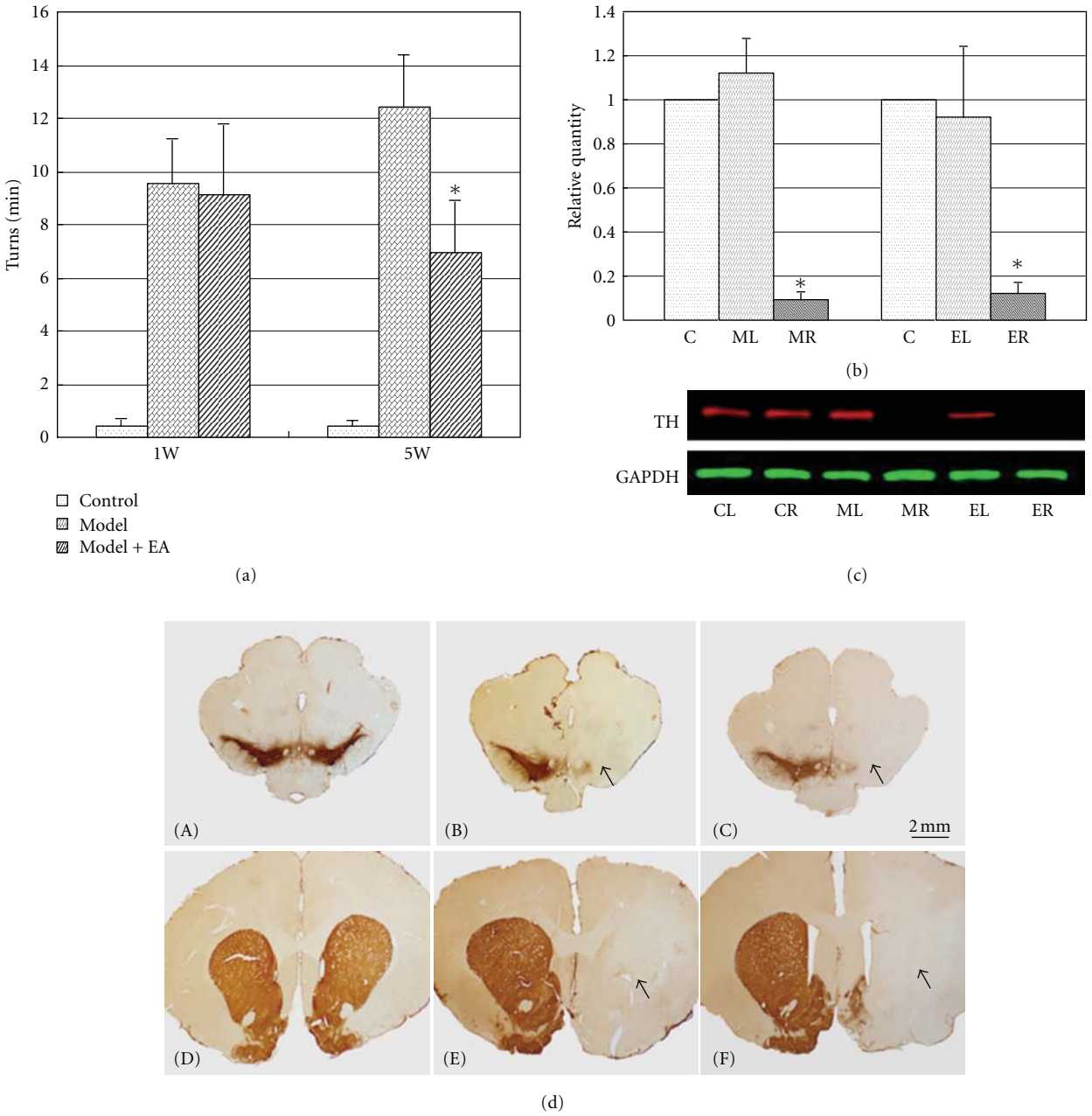


Figure 1: PD rat models were prepared using 6-OHDA and were treated with 100 Hz EA. EA at 100 Hz was applied at the acupuncture points DAZHUI (GV14) and BAIHUI (GV21) for 28 days. Rotational behavior induced by apomorphine was measured 1, 2, and 5 weeks after 6-OHDA treatment of the MFB. (a) The Y-axis represents the net number of turns of rats per minute. The X-axis represents the detection time. The net number of turns per minute of the 5-week EA-treated group decreased significantly compared to the corresponding 5-week model group ($P < 0.001$). ((b) and (c)) Western blotting results revealed that the expression of TH was significantly downregulated in the right STR of the 6-OHDA-treated rats (MR) and the EA-treated model rats (ER) compared to the nonlesioned side (c). CL and CR represent the left and right STR of the control rats, respectively. The relative grey level is presented in (b) ($^*P < 0.01$). The Y-axis represents the relative grey value versus the control. The X-axis represents different groups. In addition, the extensive loss of DA neurons in the rat SN and STR was detected using Tyrosine hydroxylase- (TH-) specific immunostaining (scale bar 2 mm, (d)-(A)–(d)-(F)). As indicated by the arrows in (d), representative microphotographs of the right STR ((d)-(E) and (d)-(F)) and SN ((d)-(B) and (d)-(C)) after TH immunostaining showed an extensive reduction in the density of TH-immunoreactive axons in the STR and SN of 6-OHDA-treated rats, regardless of EA treatment (right STR of (d)-(E) and (d)-(F) and right SN of (d)-(B) and (d)-(C)), compared to the control ((d)-(A) and (d)-(D) or the left of model STR or SN).

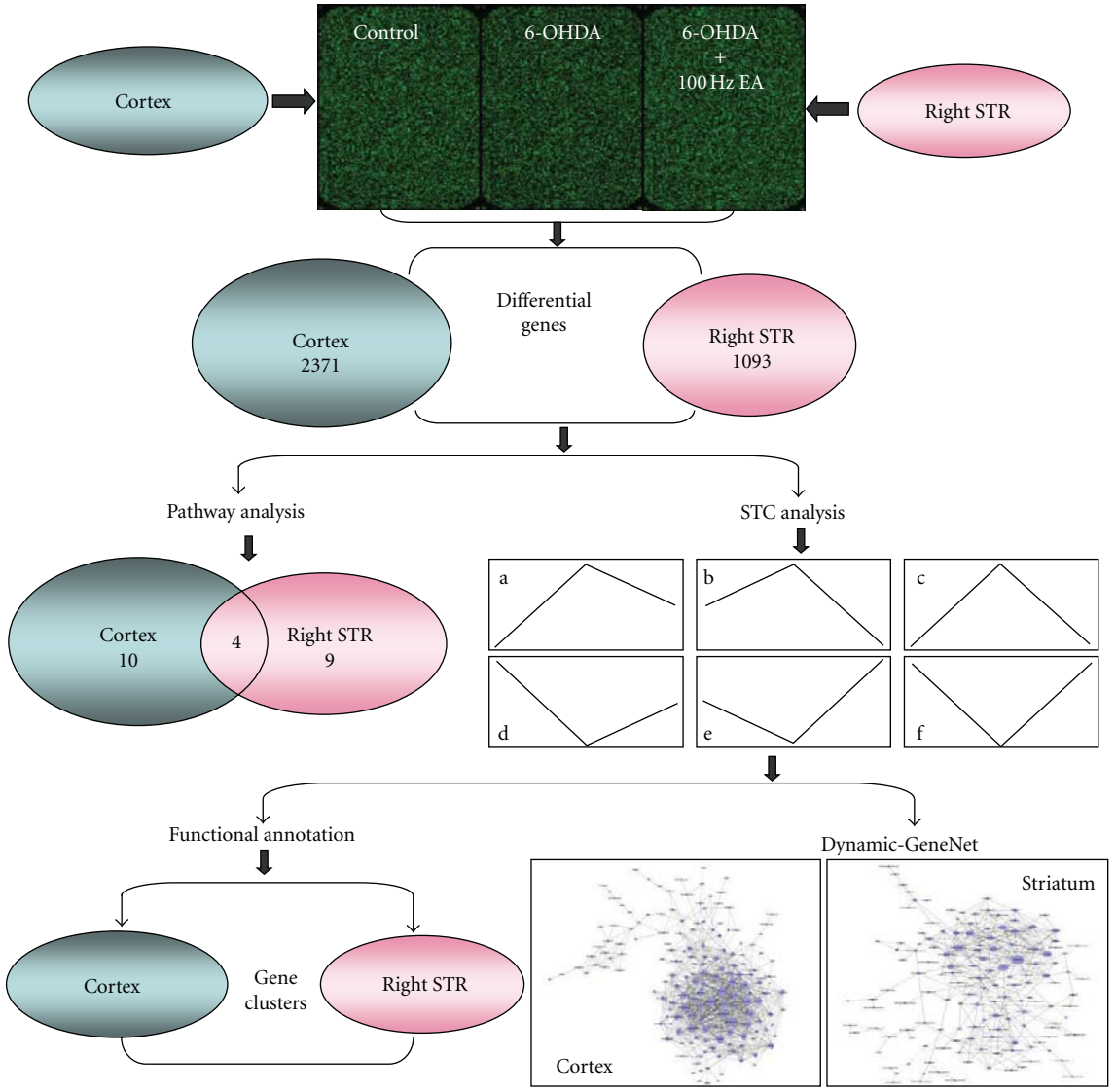


Figure 2: The flow-sheet was used for the data analysis of genomic profile. Three biology duplicate samples in each group were used for microarray analyzing. The cortex and right STR were examined, and the differential genes in this figure came from comparison of three groups ($P < 0.05$). To observe these genes in depth, STC (Figure 3) and pathway analysis were performed. Dividing differential genes into groups according to 16 profiles by STC, we then completed a functional annotation (Tables 1–3, S1–4). Then, genes from the 6 types of recovery expression profiles (A)–(F) after EA-treatment were subjected to Dynamic-GeneNet analysis. The genes of three profiles (STC (a), (b), and (c)) were the principal part in the spatial network.

lysing buffer (including protease inhibitor cocktail, P2714, Sigma). The protein concentration of the final extract solution was determined using the BCA method. The western blotting procedures followed the standard protocol. Protein lysates were separated by electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.2% Tween 20 for 1 h and then incubated overnight at 4°C with the indicated primary antibody. Membranes were then treated with IRDye 800 (green) or IRDye 700 (red) conjugated affinity purified anti-mouse, anti-rabbit secondary IgG for 1 h, followed by three washes with PBS containing 0.1% Tween 20 and two washes with PBS alone. The fluorescent bands were visualized using an LI-COR Odyssey infrared double-fluorescence imaging system (American Company LI-COR).

2.6. Sample Preparation and Microarray Image Analysis. Total RNA was separately extracted from the nine individual samples using the RNeasy Mini Kit (QIAGEN, 74106). Microarray analysis was performed and repeated 3 times using a biological sample in each group with the Whole Rat Genome

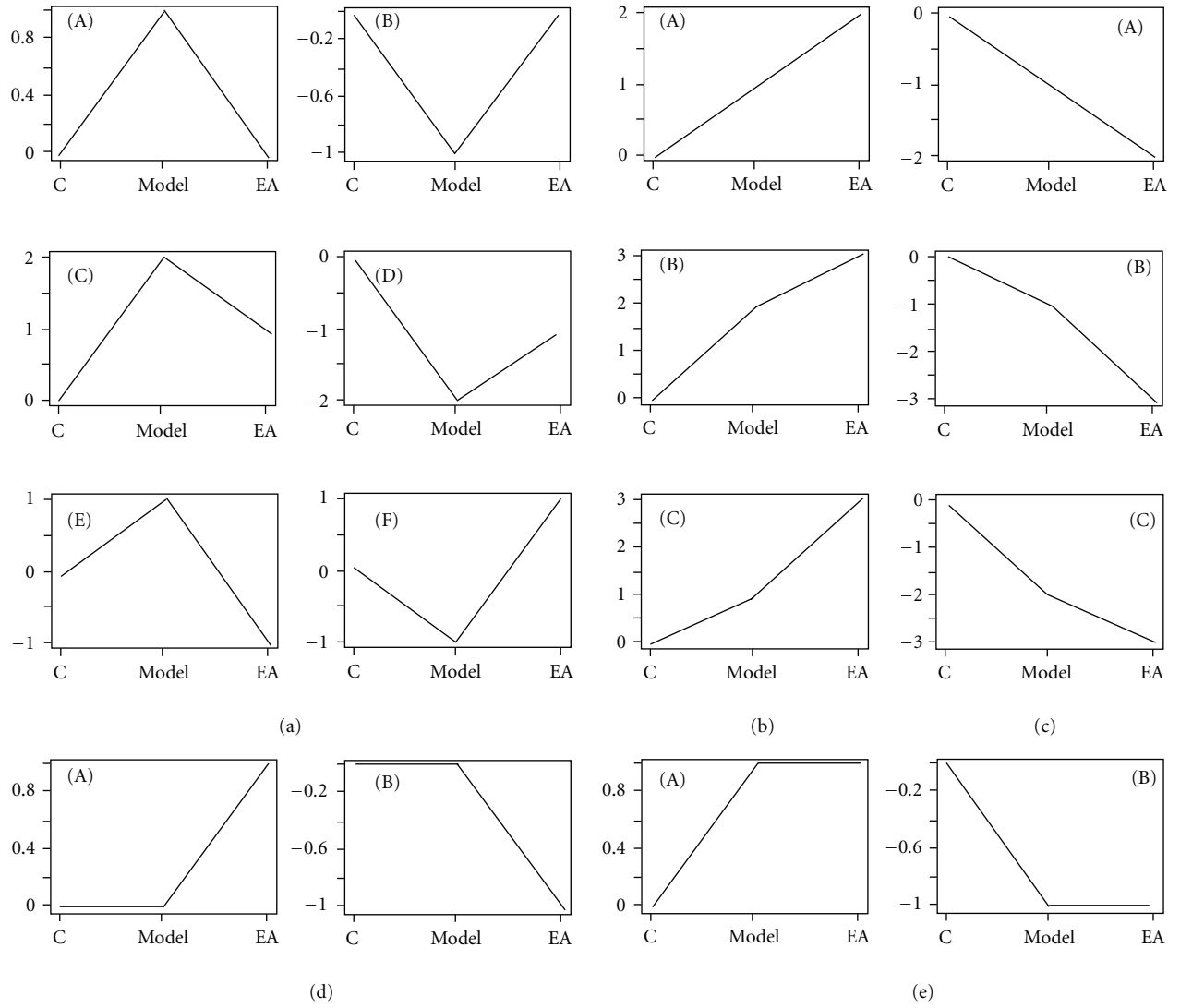


Figure 3: STC analyzed for differential genes in the cortex and STR after 6-OHDA or 6-OHDA/EA treatment. There were 16 types of profiles for differential genes in three experimental groups of the cortex or STR (control, model, and EA group). The number on the *Y*-axis is the degree of change and not a concrete value. These profiles can be divided into 5 subgroups. (a) Recovery expression profiles group ((a), (A)–(F)). After EA treatment, these genes tended to recover the normal level. Genes of (a)-(A) and (B) were completely recovered. Genes of (a)-(C) and (D), however, were only partially recovered. (a)-(E) and (F) represent the overcorrect genes. (b) Upregulated group ((b), (A)–(C)). These genes continued to be upregulated after EA treatment. (c) Downregulated group ((c), (A)–(C)). These genes continued to be downregulated after EA treatment. These two part of genes ((b) and (c)) need to be considered individually and they belong to the portion of genes regulated by EA. (d) This group was only regulated by EA ((d)-(A) and (d)-(B)). These genes were up- or downregulated only after 100 Hz EA treatment. (e) The group continued to maintain the model group level. These genes displayed no change after EA treatment but were upregulated or downregulated compared to control. A detailed functional annotation is displayed (see Tables S2–8 in supplementary material available on line at doi:10.1155/2012/908439) and it shows that the target genes of 100 Hz EA are extensive and involved in multifunctional regulation.

4×44 K microarrays (Gene expression hybridization kit, 5188-5242, Agilent). Following the protocols of the Low RNA Input Linear Amplification and Labeling Kit Plus (Agilent, #5184-3523), we synthesized double-stranded cDNA and applied it as a template to label cRNA. For each hybridization, 2 μg of total RNA from each sample (control or model) was used. Each sample cRNA (labeled with Cy3 (Cy3 NHS ester, PA13105 GE healthcare)) was individually hybridized on microarrays. The labeling and hybridization steps were carried out according to the Agilent protocol, and the images were scanned using a microarray scanner (Agilent, G2565BA).

2.7. Mul-Class Dif Analysis. We applied the RVM *F*-test to filter the differentially expressed genes in the control and experiment group because the RVM *F*-test can effectively

increase the degrees of freedom in cases of small samples. After the significance analysis and FDR analysis, we selected the differentially expressed genes according to the P value threshold [20, 21].

2.8. STC Analysis. The series test of cluster (STC) algorithm of gene expression dynamics was used to profile the gene expression time series and to identify the most probable set of clusters generating the observed time series. This method explicitly took into account the dynamic nature of the temporal gene expression profiles during clustering and identified the number of distinct clusters [22, 23].

We selected differential expression genes in a logical sequence according to RVM (Random variance model) corrective ANOVA. In accordance with the different signal density change profiles of genes under different conditions, we identify a set of unique model expression profiles. The raw expression values were converted into $\log_2$ ratios. Using a strategy for clustering short time-series gene expression data, we defined a set of unique profiles. The expression model profiles are related to the actual or expected number of genes assigned to each model profile. Significant profiles have higher probability than expected by Fisher's exact test and multiple comparison test [24].

2.9. STC-Gene Functional Annotation. For transcripts in each profile of STC analysis, functional annotation and classification were completed using DAVID 6.7 b (current release, Jan. 2010) of DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/) [25, 26]. Functional annotation is applied to the genes belonging to certain specific profiles. It is used to find the main function of the genes with the same expression trend. Gene ontology (GO) is a key functional classification of DAVID.

2.10. Dynamic-GeneNet (Coexpression Network). We also construct gene coexpression networks to find the interactions among genes in selected STC profiles (Figure 3(a), (A)–(F)) [27]. Gene coexpression networks were built according to the normalized signal intensity of specific expression genes. For each pair of genes, we calculated the Pearson correlation and chose the significant correlation pairs to construct the network (Figure 1S) [28].

In the network analysis, the degree centrality is one of the simplest and most important measures of the centrality of a gene and its relative importance within a network. Degree centrality is defined as the number of links that one node has to all of the other nodes [29]. Moreover, in studying some properties of the networks, k-cores in graph theory were introduced as a method of simplifying the analysis of graph topologies. A k-core of a network is a subnetwork in which all nodes are connected to at least k other genes in the subnetwork. A k-core of a protein-protein interaction network usually contains cohesive groups of proteins [29].

The purpose of Network Structure Analysis is to locate core regulatory factors (genes), in one network. Core regulatory factors connect the most adjacent genes and have the largest degrees. When considering different networks, the core regulatory factors were determined by the degree of difference between two class samples [30]. The core regulatory factors always have the largest degree differences. The distinct figures of our coexpression network comprising the selected genes are shown in Figures S1A and B.

2.11. Pathway Analysis. Similarly, pathway analysis was used to determine the significant pathway of the differential genes according to KEGG, Biocarta, and Reatome. We used Fisher's exact test and the chi-square test to select the significant pathway, and the threshold of significance was defined by the P value and FDR. The enrichment was calculated as previously described [31–33].

2.12. Clustering and TreeView for Identified Transcripts. Hierarchical clustering was performed in Cluster 3.0 soft. For nine samples (Cortex or STR), a list was prepared of the selected genes to be clustered. The normal signal data for selected genes were adjusted to become log transform data. Then the data were arranged according the requirements of Cluster 3.0 and "median" was selected for center genes and arrays. After that, the results of clustering of selected genes were presented by Java TreeView and exported to the images.

2.13. Selected Gene Expression Changes Were Validated Using qRT-PCR. Total RNA was extracted from the cortex or STR using a standard trizol (Invitrogen, 15596-018) procedure from each tissue sample. RNA was treated with RNase-free DNase I, and 3 μg of total RNA was subjected to cDNA synthesis with M-MLV reverse transcriptase (Promega, M170) in a 30 μL liquid phase reaction for RT-PCR, from which 1 μL of cDNA was used for PCR amplification. The primers used as an internal control for RT-PCR to amplify human 18 S were 5′-GGAAGGGCACCACCAGGAGT and 5′-TGC AGCCCCGGACATCTAAG. The primers used for amplifying 6-OHDA-targeted RNAs in real-time PCR (Stratagene MX3000P Sequence Detection system, USA) are listed in Table S1 (Supplementary Materials). The trials were completed by SYBR Premix Ex Taq kit (Takara, DRR041B). The PCR protocol consisted of 1 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C (Figure 4(b)). The levels of target gene expression were quantified relative to the level of 18 S using the standard curve method. The specificities of RT-PCR products were confirmed by the presence of both a single dissociation curve for the product and a single band, with corresponding molecular weight revealed by agarose gel electrophoresis. The statistical significance of the cluster gene rate and the discrete rate was assessed using t-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. EA at 100 Hz Attenuated the Rotational Behavior in a 6-OHDA PD Model. As seen in Figure 1, a nearly complete denervation of the SN in a rat model was observed in our experiments (Figures 1(d)-(B) and 1(d)-(E)). As hypothesized, the high-frequency EA stimulation did not to prevent the reduction in TH-positive dendritic fibers in the lesion-lateral

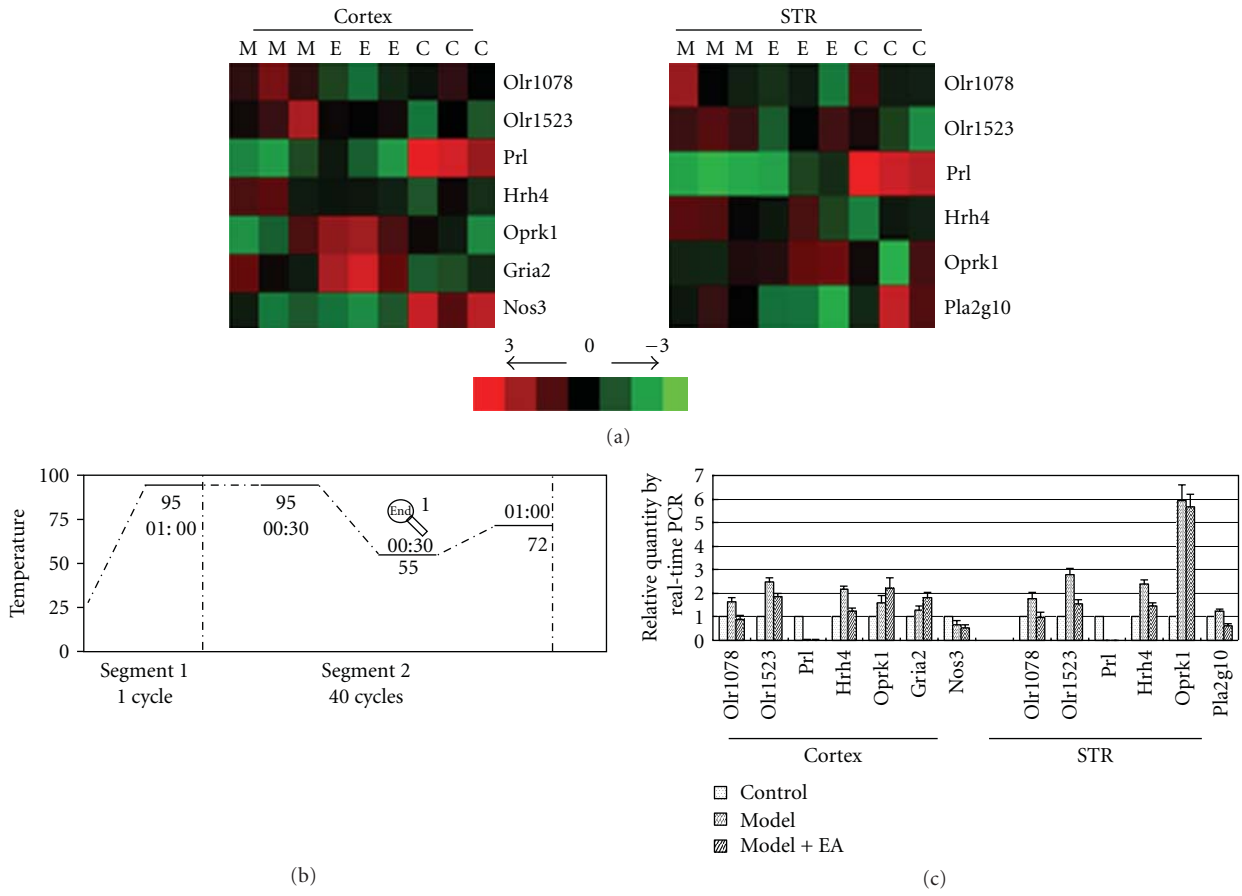


Figure 4: Selected transcripts clustered and validated with real-time PCR. After pathway analysis, we selected genes involved in major pathways (the common pathways in the cortex and STR, Table 7). First, selected genes were clustered using Cluster 3.0 according to the LOG value in the STR groups (a) Red is relatively upregulated and green is relatively downregulated in different samples. The three "Cs" are the three control samples. The three "Ms" are the model samples. The three "Es" are the EA-treated samples. Then, to verify the reliability of the microarray analysis, we verified these selected genes from the clustering diagram using real-time RT-PCR. The real-time PCR primers are listed on Table S1 (devised and synthesized by Takara). The reaction procedure was present on (b). If the level of control was regarded as 1, the corresponding gene expression in model and EA-treated groups were present on (c). These transcripts analyzed here showed coherent profiles with cluster (a).

SN and STR (Figures 1(d)-(C) and 1(d)-(F)). However, EA stimulation at 100 Hz reduced the number of rota tions exhibited by the unilaterally 6-OHDA lesioned PD models (Figure 1(a)). The rotational behavior induced by apomorphine (0.5 mg/kg) was measured 1 (7 days), 2 (14 days), and 5 weeks (35 days) after the 6-OHDA-treatment of the MFB. The net number of turns was calculated as the number of contralateral turns minus the number of ipsilateral turns. The values are expressed as means ± SEM (n = 10–14 per group, $P < 0.01$ between model/EA group and control). The turns/min of the control group was 0.42 ± 0.28 turns/min for 1 week, 0.33 ± 0.12 turns/min for 2 weeks and 0.43 ± 0.19 turns/min for 5 weeks. The rats in the 6-OHDA-treated group exhibited greater rotational asymmetry in the direction contralateral to the lesion (9.52 ± 1.69 turns/min at 1 weeks; 8.24 ± 2.10 turns/min at 2 weeks; and 12.44 ± 1.94 turns/min at 5 weeks) compared to the rats in the EA-treated group (9.13 ± 2.65 turns/min for 1 week; 7.88 ± 2.30 turns/min for 2 weeks; 6.92 ± 1.98 turns/min for 5 weeks). The number of turns of the 5-week EA-treated group decreased significantly compared to the corresponding 5-week model group ($P < 0.001$, the results from week 1 and week 5 are presented in Figure 1(a)). These results revealed that the number of apomorphine-induced rotations exhibited by 6-OHDA PD rats is reduced after EA treatment, although a histological recovery to normal levels could not be identified.

3.2. EA at 100 Hz Caused a Multimolecular Change In Vivo. An ANOVA for the three groups (Control, Model and EA group) in our studies revealed that there were 2371 differential genes in the cortex and 1093 differential genes in the STR ($P < 0.05$, Figure 2). After the STC analysis, these differential genes were divided into 16 types of expression profiles according to the alignment of the control, model, and EA-treated groups (Figure 3). The transcripts in profile (a) showed that the effect of EA in 6-OHDA lesioned animals return to the proximalis levels of gene expression that were

observed in the control group. The functional annotations for these transcripts in the 16 profiles refer to Tables S2–8 (Supplementary Material). Among the results of the functional annotations, the majority of the regulated genes belonged to profile (a) (recovered), (b) (upregulated), (c) (downregulated), or (e) (sustained) after EA treatment. The functions of these transcripts were found to be involved in many aspects, which were presented in supplementary Tables S2–5 and Tables S7–8, after DAVID 6.7 analysis. Therefore, 100 Hz EA may cause a multimolecular change in vivo in a PD model.

In addition, our results reveal that there were 255 mutual genes, with 16 types of profiles between the cortex and STR, and that 87.06 percent of these genes (222/255) were in the noncontradicted profiles (profiles in addition to the opposite regulated genes by EA). After the DAVID 6.7 analysis, 74.36 percent of the genes (29/39) listed in Table S9, which had names and functional annotations, belonged to the genes in profile (a) of Figure 3. After the functional annotation for the genes in Table S9, the categories of "cognition and sensory perception," "olfactory transduction and receptor activity," "G-protein coupled receptor protein signaling pathway," "integral to membrane," and "neurological system process" were found to be regulated by high-frequency EA ($P \leq 0.01$, Table S10). Multiple transcripts of these functional categories trended toward recovery to normal levels (Table S9).

The identification of the significantly regulated genes (the $\log_2$ difference absolute ≥ 1 compared to model, $P < 0.05$) after EA treatment was important to explain the internal changes to disorder and turnover of the genes. According to the standard in the previous bracket, 71 genes in the cortex were selected, and 50 genes, or 70.42% (50/71), were in profile (a) of Figure 3 (Table S11). In addition, 38 genes in the STR were selected, of which 78.95%, or 30 genes, were in profile (a) of Figure 3. The notations of these genes are listed in Table S11. This table shows which transcripts belong to each profile.

3.3. Multipathways Targeted by 100 Hz EA. The following ten pathways in the cortex and nine pathways in the STR were predominantly affected by 100 Hz EA ($P < 0.05$, Table S12): the olfactory transduction pathway ($P < 0.001$), Alzheimer's disease pathway ($P < 0.001$), Huntington's disease pathway ($P < 0.001$), neuroactive ligand-receptor interaction pathway ($P < 0.001$), inositol phosphate metabolism pathway ($P < 0.01$), calcium signaling pathway ($P < 0.05$), systemic lupus erythematosus pathway ($P < 0.05$), amyotrophic lateral sclerosis (ALS) pathway ($P < 0.05$), p53 signaling pathway ($P < 0.05$), and long-term depression pathway ($P < 0.05$) in the cortex; the neuroactive ligand-receptor interaction pathway ($P < 0.01$), olfactory transduction pathway ($P < 0.01$), arachidonic acid metabolism pathway ($P < 0.01$), amyotrophic lateral sclerosis (ALS) pathway ($P < 0.05$), long-term depression pathway ($P < 0.05$), ECM-receptor interaction pathway ($P < 0.05$), taurine and hypotaurine metabolism pathway ($P < 0.05$), cytokine-cytokine receptor interaction pathway ($P < 0.05$), and MAPK signaling pathway ($P < 0.05$) in the lesion-lateral STR (Table S12).

To validate the microarray results, we selected several transcripts to cluster (Figure 4(a)) that were involved in major pathways (Table S12) and validated the clustering result using real-time PCR (Figure 4(c)). The functions of the protein products for these transcripts are listed in Table S13. To some extent, the identified results confirmed the reliability of the array analysis.

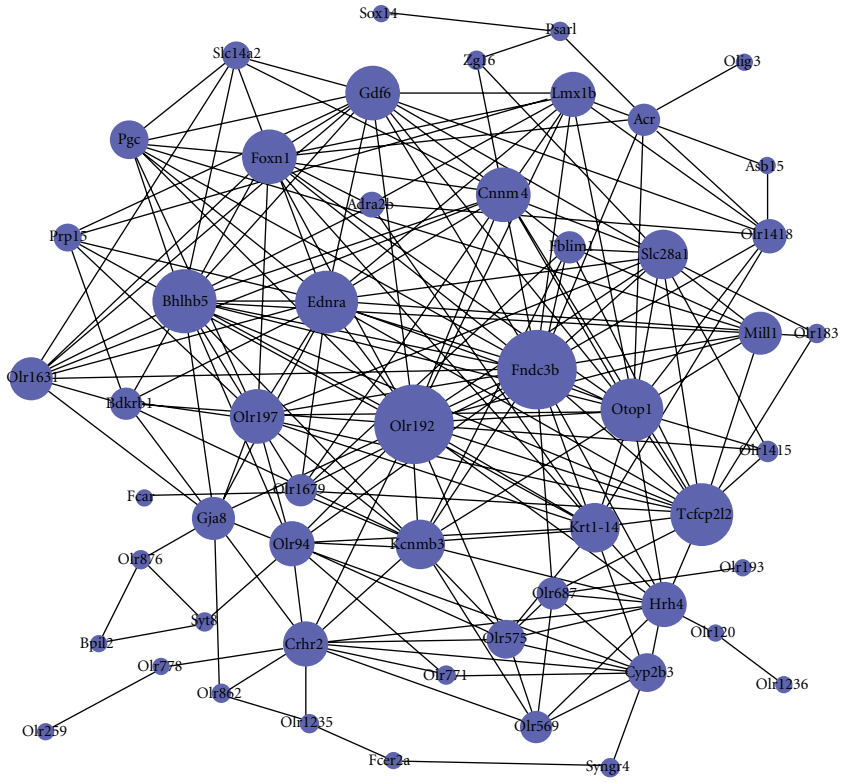
3.4. Internal Molecule Network Targeted by 100 Hz EA. The integrity coexpression network for the six recovered profiles of the cortex and STR is presented in Figure S1. The simplified network is shown in Figure 5 (genes without names or with poor interactions were deleted). Combining these figures, the network density and nodes' degrees suggested that the cortex assumes a more important role in the regulation of internal balance after EA treatment. As seen in Figure S1 and Figure 5, FAD104 (FNDC3B, XM_226988, degree = 42), olfactory receptor 192 (OLR192, NM_001000549, degree = 40), basic helix-loop-helix domain containing class B5 (BHLHB5, XM_345190, degree = 32), otopetrin 1 (OTOP1, NM_181433, degree = 32), and endothelin receptor type A (ENDRA, NM_012550, degree = 31) are the core nodes in the cortex network. Mitochondrial protease presenilins-associated rhomboid-like protein (PARL/PSARL, ENSRNOT00000030837, degree = 20), olfactory receptor 522 (OLR522, NM_001000562, degree = 18), and member 4a1 of the solute carrier organic anion transporter family (SLCO4A1, NM_133608, degree = 15) are the most important nodes in the impaired STR network.

4. Discussion

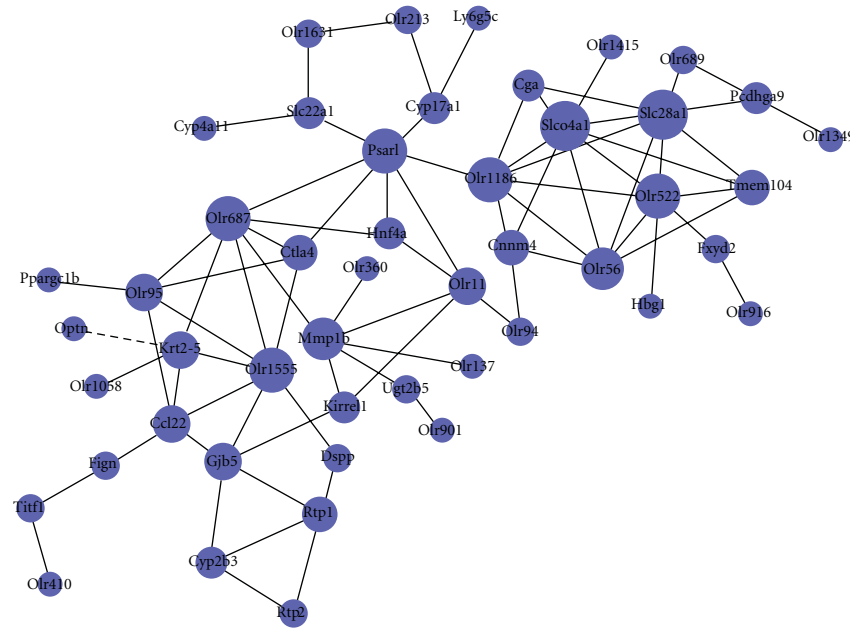
Acupuncture is a popular alternative therapy in patients with Parkinson's disease (PD) and may provide benefit in the clinical setting [34]. Although acupuncture has not been used to cure PD, a survey of PD patients demonstrated that patients who received acupuncture reported an improvement in their symptoms [7]. In addition, acupuncture is very safe and well tolerated in clinical practice. However, the mechanisms underlying the neuroprotective effects of this technique are not yet clear.

The best course of PD therapy may be to increase the DA content of the striatum. However, increasing evidence suggests that a poor relationship exists between the dopamine content of the striatum and the improvement of motor symptoms during PD treatment [35, 36]. Recently, Park et al. reported that the mechanism of acupuncture involves multiple factors [10, 11, 17]. In addition, it has been argued that DA neurons and other cells within the SN and adjacent brain regions are involved in PD pathology [37, 38]. Our results and those of work aiming to quantify the effects of EA demonstrate that the effects may be produced by the coordination of several internal brain regions including cortex and STR.

To treat the neurochemical phenomenon of dopamine depletion, we aimed to identify the molecular effects of EA. A rodent model with 6-OHDA injected into the MFB mimics end-stage PD [18]. The number of rotations exhibited by



(a)



(b)

FIGURE 5: Simplified dynamic gene network for genes in profile of Figure 3(a). The global dynamic gene networks of the cortex and STR are shown in Figures S1A and S1B. A simplified network is presented here with the genes without names or some minor genes in the network deleted. The blue dots indicate the differentially regulated genes. The lines show the relationship between genes. The solid line denotes positive regulation, and the dashed line denotes negative regulation. The size of the dots indicates the capability of the gene to interact with others, quantified by "degree" (refer to Section 2). The larger the degree, the more the genes interacted with the corresponding genes and the more important this gene was in the network.

an animal after amphetamine administration can be used to distinguish between partial and near complete (>90%) denervation in the SN [18]. Thus, a nearly complete denervation in the SN of a rat model was observed in our experiments (Figure 1(d)). Previously, we demonstrated that long-term, high-frequency EA stimulation on the points of DAZHUI (GV 14) and BAIHUI (GV 21) attenuated the rotational behavior of PD rats with partial lesions and improved motor activity [8, 9, 12]. In addition, previous results suggest that the effects of EA may be multitargeted and may be effective at PD without increasing dopamine (DA) [12, 13]. Thus, the 6-OHDA-injected rat model may provide an opportunity to test how the cortex and lesion-lateral STR develop a concerted reaction and change after EA treatment. High-frequency EA may also attenuate the rotational behavior of the end-stage PD models without significantly increasing the number of TH-positive dendritic fibers in the lesion-lateral SN and STR (Figures 1(a) and 1(d)). The network regulation of the central nervous system (CNS) and endocrine system may also be involved in the mechanism of EA at PD.

At present, PD is no longer believed to be a disorder that only affects the DAergic system [35, 36]. The elucidation of important gene expression profiles will enable the identification of genetic susceptibility markers, biomarkers of disease progression, and new therapeutic targets [3]. According to the standard of FCA $\geq$ 3 (EA versus model; $\log_2$ difference absolute $\geq$ 1), $\geq$70% of the genes in the cortex and STR were in profile (a) of Figure 3 (Table S11). Lesions induced by 6-OHDA may activate a compensatory cascade of neurotrophic activity within the nigrostriatal system as a physiological response to the loss of DAergic neurons in rats [39, 40]. These percentages also suggest that the effect of EA in 6-OHDA lesioned animals may result in a return of the partial gene expression levels to those of control values. FCA $\geq$ 3 transcripts that can be searched for in the literature were listed in Table S13. References to the functions of these transcripts in the supplementary literature 1–4 indicate that the stable expression of these transcripts is important for homeostasis.

In addition, several of the transcripts that were significantly regulated only by EA were particularly noteworthy (Table S6, profile (d) in Figure 3). EA at 100 Hz upregulated the functions of "epidermal growth factor receptor binding" in the cortex and of "angiogenesis or vasculature development" in the STR ($P \leq 0.01$). AREG and EPGN participated in the function of epidermal growth factor receptor binding. TBX4, ANGPT2, and VEGFA (Table 3) participated in angiogenesis or vasculature development. The concrete functions of these transcripts are shown in Table S13 referred to the supplementary literature 5–9. These genes that were upregulated by EA (Table S6) were extremely important for angiogenesis or vasculature development, which contributes to the delivery of important signal molecules from the cortex to the STR or other regions.

After the relative expression level, which is either up- or downregulated, was quantified, the altered transcripts were applied to a bioinformatics analysis using the program "pathway" (Section 2). Ten pathways in the cortex and nine pathways in the STR were predominantly affected by 100 Hz EA (Section 3). The most commonly regulated pathways between the two encephalic regions included the olfactory transduction pathway, neuroactive ligand-receptor interaction pathway, amyotrophic lateral sclerosis (ALS) pathway, and long-term depression pathway. PD is a neurodegenerative disease characterized by the selective degeneration of DAergic cells in the SN and the nigrostriatal pathway. In the 6-OHDA-induced PD rat model, cotransplantation of fetal ventral mesencephalic cells with olfactory ensheathing cells (OECs) has been reported to enhance DA neuron survival, striatal reinnervation, and functional recovery [41]. OECs constitute a unique population of glial cells that accompany and ensheath the primary olfactory axons. These cells are believed to be critical for the spontaneous growth of olfactory axons within the developing and adult olfactory nervous system and have recently emerged as potential candidates for the cell-mediated repair of neural injuries [42]. However, the relationship between the olfactory transduction pathway and OECs has not yet been identified. Another important regulated pathway by EA was the neuroactive ligand-receptor interaction pathway. The signal connection was significantly enhanced in the cortex and in the lesion-lateral STR. The pathway analysis for the differential regulated genes and the functional annotation for the differential transcripts in the regulated profiles by 100 Hz EA suggest that EA functions through network regulation. In addition, we selected several transcripts in the commonly regulated pathways to identify their expressions using QRT-PCR (Figure 4(c)). Several concrete functions of these transcripts are shown on Table S13 referred to the literature 10–13. From the corresponding literature, pathways related to hormone levels and inflammatory stimuli may be targets of EA at 100 Hz.

Multiple microarray studies have compared the gene expression profiles of cells within the midbrain of normal controls with those from Parkinson's diseased brains [37, 38, 43–45]. As Section 3 presents, the differential genes were divided into 16 types of expression profiles after the STC analysis (Figure 3). Transcripts in profile (a) revealed that the effect of EA in the 6-OHDA lesioned animals is to return the levels of gene expression to control values. By summarizing the functional annotations for these transcripts in the 16 profiles (Supplementary Tables S2–8), we were able to draw a schematic to present the probable relationship between the cortex and lesion-lateral STR (Figure 6). Several important functional categories were identified in this schematic. In the mutual functional clusters in the cortex and lesion-lateral STR of profile (a), the categories of "olfactory transduction and receptor activity," "G-protein coupled receptor protein signaling," "cognition and sensory perception," "neurological system process," and "integral to membrane" were the functional categories of the mutual regulated transcripts between the cortex and STR (Table S10). The identical results of the two differential analytic process revealed that there were several functional clusters in the cortex and lesion-lateral STR that are targeted by EA at 100 Hz. In addition, the transcripts in these functional clusters returned the levels of gene expression to control values. These clusters were connected vinculums between the cortex and lesion-lateral STR.

Although we observed a therapeutic effect of high-frequency EA, it was essential to explain the possible molecular

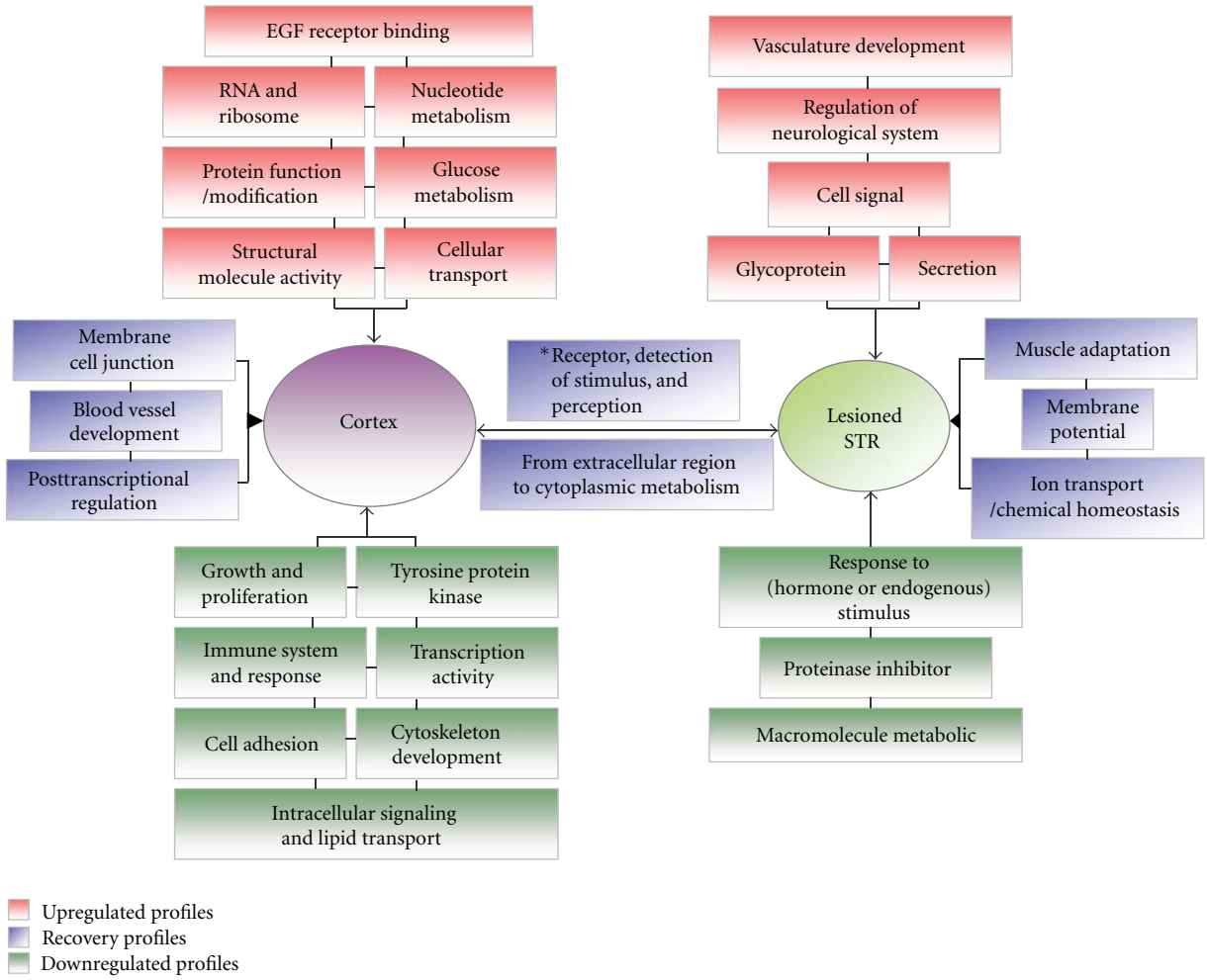


Figure 6: A scheme of functional annotation for transcripts in the 16 types of profiles (for discussion). There were 16 types of profiles for the differential transcripts in the three experimental groups of the cortex or lesion-lateral STR (control, model, and EA group). These profiles can be mainly divided into 5 subgroups after 100 Hz EA treatment (Figure 3). Here, the schematic summarizes the related major functional categories of the transcripts regulated in 16 types profiles (Tables S2–8). Red represents the functional annotation for the upregulated transcripts. Green represents the functional annotation for the downregulated transcripts. Blue represents the functional annotation for the transcripts that returned the levels of gene expression to control values. *This box includes olfactory receptor activity, detection of chemical stimulus involved in sensory perception of smell, G-protein coupled receptor protein signaling pathway, cell surface receptor linked signal transduction, cognition or neurological system process, and sensory perception (Table S2).

mechanism. Previously, no detailed and complete information was available about how genes in the cortex and STR were regulated by EA to maintain homeostasis. In the present work, we observed that the transcriptional levels of many functional clusters were regulated in the cortex or lesion-lateral STR when DAergic neurons were nearly inactivated. In addition, the functions of these transcripts were found to be involved in many aspects (shown in supplementary Tables S2–8). Our results also suggest that lesions induced by 6-OHDA may activate a compensatory cascade, as described above. Previously, microglial activation was observed in the lesion-lateral SN of PD rat models, and 100 Hz EA was observed to significantly inhibit the activation of microglia in the SN [15]. In the present study, many transcripts, which can positively regulate functions related to immune system processes and immune responses, were downregulated in the model cortex and were further downregulated after EA treatment (Table S5, Figures 3(c) and 6). These findings suggest that the immune suppression signal from the cortex was present after the 6-OHDA lesion, possibly because of a compensatory cascade. However, EA was able to further enrich the suppressive signal. The relationship between the suppressive signal and the two encephalic regions needs to be investigated further.

High-frequency EA resulted in a reduction in the number of apomorphine-induced rotations. We also found that EA resulted in the up- or downregulation of several other functions in the two encephalic regions (Figure 6). Combining

the functional categories for the recovery profiles, these clusters form a functional network for the cortex and lesion-lateral STR, which is regulated by 100 Hz EA. In addition, the functional category of "response to hormone or endogenous stimulus" in the lesion-lateral STR is interesting. The transcripts in this functional cluster were downregulated. The level of one transcript, prolactin (PRL), is known to be below the detection limit in PD patients [46] and was also extremely low in our results (Figure 4(c)). EA at 100 Hz was unable to upregulated the level of PRL (Table S9). However, several transcripts that respond to hormones or endogenous stimuli were downregulated. The effect mechanisms underlying the phenomenon about the downregulated transcripts need to be investigated further. Moreover, the coexpression network of the transcripts in the six recovered profiles (Figure 3(a)) of the cortex and STR that were targeted by 100 Hz EA is presented in Figure S1. and Figure 5. By carefully observing the transcripts in the networks, we identified that FAD104, OLR192, BHLHB5, OTOP1, and ENDRA are the core nodes in the targeted cortex network (degree $\geq$ 30). PSARL, OLR522, and SLCO4A1 are the most important nodes in the lesion-lateral STR network targeted by 100 Hz EA. The major functions of the eight transcripts are shown in Table S13 referred to the supplementary literature 14–26. Combining these figures, the network density and nodes' degrees suggested that the cortex assumes the most important role in the regulation of internal balance after EA treatment (Figure 5). In addition, the cortex may be the center that gives the regulation signal. To some extent, the most commonly presented transcripts between the cortex and STR and the profiles of these transcripts in three groups may reflect the main trends of molecular regulation in vivo after EA treatment. Furthermore, we analyzed the recovered gene rate of the mutually presented genes between the cortex and STR and the rate of the regulated transcripts ($\log_2 > 1$ or $\log_2 < -1$). Both of these rates were greater than 70% (see Section 3). A dynamic gene network including several of these genes, especially genes in profiles (a)-(A), (C), and (E), may be one part of the basic framework for high-frequency EA effects (Table S2, Figures 3, 5, and 6).

Although information at the transcriptional level does not directly correlate with the levels and functions of the corresponding proteins, transcription is the first response and defense when an organism is subjected to a stimulus. As a result, changes in global transcripts may reveal the molecular mechanism of complicated disorders after high-frequency EA treatment.

5. Conclusions

High-frequency EA may attenuate the rotational behavior of a PD model when there was a near complete (>90%) denervation in the lesion-lateral SN. A microarray analysis of the cortex and STR revealed that 100 Hz EA is a multitarget treatment for PD and that the curative effect may be generated through regulating targeted transcripts, pathways, and functional clusters and through the spatial network interaction of targeted transcripts in different encephalic regions. In conclusion, recovering homeostasis may be an important part of high-frequency EA mechanisms.

Acknowledgments

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Review Article

Neuroendocrine Mechanisms of Acupuncture in the Treatment of Hypertension

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Hypertension affects approximately 1 billion individuals worldwide. Pharmacological therapy has not been perfected and often is associated with adverse side effects. Acupuncture is used as an adjunctive treatment for a number of cardiovascular diseases like hypertension. It has long been established that the two major contributors to systemic hypertension are the intrarenal renin-angiotensin system and chronic activation of the sympathetic nervous system. Recent evidence indicates that in some models of cardiovascular disease, blockade of AT1 receptors in the rostral ventrolateral medulla (rVLM) reduces sympathetic nerve activity and blood pressure, suggesting that overactivity of the angiotensin system in this nucleus may play a role in the maintenance of hypertension. Our experimental studies have shown that electroacupuncture stimulation activates neurons in the arcuate nucleus, ventrolateral gray, and nucleus raphe to inhibit the neural activity in the rVLM in a model of visceral reflex stimulation-induced hypertension. This paper will discuss current knowledge of the effects of acupuncture on central nervous system and how they contribute to regulation of acupuncture on the endocrine system to provide a perspective on the future of treatment of hypertension with this ancient technique.

1. Introduction

Hypertension affects approximately 1 billion individuals worldwide [1]. Hypertension is the most common chronic disorder in the United States, affecting 29% of the adult population [1]. The prevalence of this disorder increases with age; for normotensive middle-aged adults in the US, the lifetime risk of developing hypertension approaches 90% [2]. Although a number of treatment strategies have been developed for this disease, treatment has not been perfected and often is associated with adverse side effects.

Hypertension is the final outcome of a complex interaction between genetic and environment factors that act on physiological systems involved in blood pressure (BP) regulation (i.e., those that influence intravascular fluid volume, myocardial contractility and vascular tone) [3]. Evidence suggests that increased sympathetic neural activity plays a role in causing hypertension in some subjects who have a genetic tendency toward increased sympathetic activity as a result of repetitive psychogenic stress, obesity, or high sodium intake [3]. An important hypothesis in the pathogenesis of essential hypertension involves an interaction between high dietary sodium intake and defects in renal sodium excretion, which can be influenced by sympathetic neural activity and the renin-angiotensin-aldosterone system [3]. Enhanced sympathetic activity increases the secretion of renin and angiotensin. Angiotensin II enhances renal tubular sodium reabsorption directly and indirectly through increased release of aldosterone.

Acupuncture increasingly is being accepted as an alternative medical therapy in the United States. Manual acupuncture and its potent alternative, electroacupuncture (EA), have been used in Asia to treat a number of cardiovascular diseases including hypertension. Many Western physicians, however, are reluctant to recommend acupuncture, because its action in the treatment of hypertension remains controversial and because the physiological mechanisms determining its actions are largely unknown. This paper will discuss

current knowledge of the effects of acupuncture on central nervous system and how they contribute to regulation by acupuncture of the endocrine system to provide a perspective on the future of treatment of hypertension with this ancient technique.

2. Clinical Study of Acupuncture in Treatment of Hypertension

In the past three decades, there have been a number of clinical studies focused on the effectiveness of acupuncture at specific acupoints to reduce BP in essential hypertension. As early as the 1950s, publications in China reported that acupuncture effectively reduced BP in hypertensive patients [4, 5]. In 1975, Tam found that acupuncture produced a significant reduction in systolic and diastolic BP in 24 out of 28 patients with essential hypertension [6]. Figure 1 shows a number of acupoints found to be effective in reducing BP, including pericardium 5, 6 (P 5, 6), stomach 36 (ST 36), large intestine 4, 11 (LI 4, 11), bladder 18, 20 (BL 18, 20), and gallbladder 34 (GB 34) [7, 8].

3. Acupoints Selection

We have evaluated the point specificity in EA treatment of reflex-induced hypertension caused by the gallbladder or splanchnic nerve (SN) stimulation in cats. This visceral reflex leads to stimulation of the sympathetic nervous system through the activation of cardiovascular premotor sympathetic neurons in the rostral ventrolateral medulla (rVLM). We observed that EA at P 5-6 (pericardial meridian, overlying the median nerve) and LI 10-11 (large intestine meridian, overlying the deep radial nerve) are most effective in reducing reflex-induced hypertension. EA at LI 4–L7 (large intestine and lung meridians, overlying branches of median and the superficial radial nerve) and ST 36-37 (stomach meridian overlying the deep peroneal nerve) are less effective, while EA at LI 6-7 and K1-B67 does not influence BP. Furthermore, direct stimulation of the deep or superficial nerves underneath the acupoints produces similar results [9, 10]. Similar observations have been made in a rat model employing gastric distension to elevate BP [11, 12].

4. Stimulation Parameters

EA rather than manual acupuncture has been used in many studies on cardiovascular related diseases, because the parameters of EA can be precisely controlled so the results are reproducible, whereas the outcome from manual acupuncture is operator dependent and therefore, is not as reproducible. A low frequency of 2 Hz is used more frequently to treat hypertension, because EA induces frequency-dependent release of neuropeptides. EA at 2 Hz produces a significant increase in enkephalin-like immunoreactivity but not in dynorphin immunoreactivity, whereas 100 Hz increases dynorphin immunoreactivity but not enkephalin immunoreactivity [13]. The similar results were confirmed in humans [14]. In the brain, enkephalins and endorphins as well as their associated δ- and μ-opioid receptors have been shown to be more important in modulating the cardiovascular actions of EA than dynorphin (κ-opioid) [15].

In our rat model of reflex hypertension, sham acupuncture involving needle insertion without manipulation at P 5-6 or LI 6-7 acupoints did not attenuate the gastric distention-induced hypertension, thus demonstrating that this procedure can serve as a control for EA. However, EA at P 5-6, H 6-7 (overlying the ulnar nerve) or ST 36-37 with low current (2 mA) and low frequency (2 Hz) for 30 min inhibited the reflex-induced hypertension. Increasing the stimulation frequency to 40 or 100 Hz did not inhibit the elevated BP. In this regard, we observed a reciprocal relationship between the frequency of stimulation and the afferent response. Thus, it appears that low-frequency, low-current EA in a point-specific manner optimally influences reflex-induced hypertension [11, 12].

5. Central Regulation of Blood Pressure

An increasing number of studies have demonstrated a critical role for the central nervous system in the development and maintenance of hypertension. In particular, increases in sympathetic nerve activity and alterations in arterial baroreflex function appear to contribute to the pathogenesis of this disease [16]. The development of hypertension in various animal models of hypertension, such as the spontaneously hypertensive rat (SHR), the renin transgenic (TGR mRen2) rat, the Dahl salt-sensitive rat, and the deoxycorticosterone acetate- (DOCA-) salt rat, is associated with increases in sympathetic activity. Increased sympathetic nerve activity elevates BP through arteriolar constriction and by increasing the force and rate of contraction of the heart to increase cardiac output. Renal sympathetic nerve activity also stimulates renin secretion that activates the systemic renin-angiotensin system leading to angiotensin (Ang) II-induced vasoconstriction and sodium retention [17]. Alteration of arterial baroreflex function has also been implicated in the development of hypertension [18]. Carotid sinus and aortic arch baroreceptors respond to changes in BP by modulating parasympathetic and sympathetic outflow and, hence, heart rate, cardiac output, and vascular tone. In response to a static increase in BP, the baroreflex resets towards a higher pressure [19]. In hypertensive conditions, resetting of the operational point of the arterial baroreflex, therefore, contributes to maintaining increased BP rather than opposing it. Similar to animal models of hypertension, hypertension in human subjects is associated with increases in sympathetic activity and blunted arterial baroreflexes [3, 18, 20, 21].

In hypertensive animals, functional changes within the central nervous system have been detected largely in hypothalamic and medullary areas that modulate sympathetic outflow [22]. Ang II contributes to cardiovascular regulation via its action at various hypothalamic and medullary areas to enhance sympathetic outflow, blunt the sensitivity of the baroreflex, and stimulate secretion of vasopressin [23, 24].

Over the past decade, we have examined the central neural regulation of visceral reflex-induced hypertension by acupuncture in different regions of brain, including the

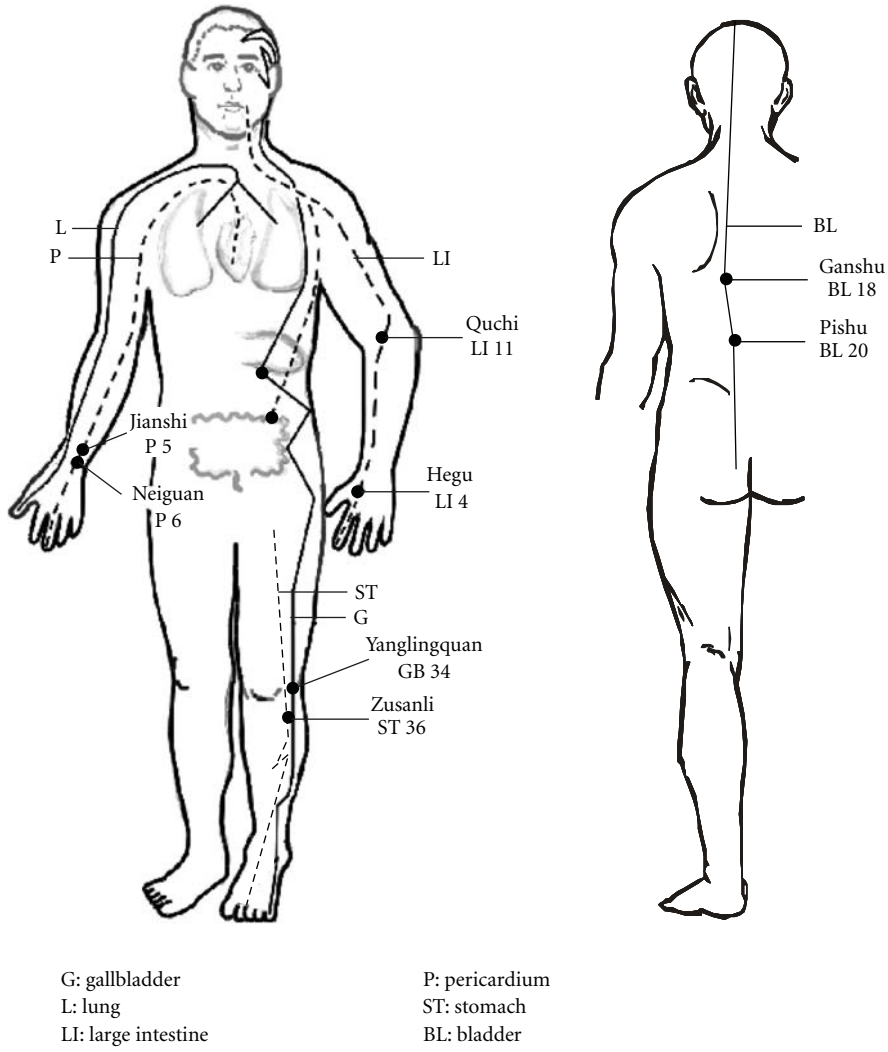

Figure 1: Location of acupoints along meridians. Note that although all meridians are bilateral, they are only drawn on one side for simplicity. Abbreviations of meridians: G: gallbladder; L: lung; LI: large intestine; P: pericardium; ST: stomach; BL: bladder.

rVLM, hypothalamic arcuate nucleus, midbrain ventrolateral periaqueductal gray (vlPAG) nuclei, medullary nucleus raphé pallidus (NRP), and dorsal horn and intermediolateral column of the spinal cord.

6. EA Inhibition of Neural Activity in the rVLM

The rVLM plays a critical role in the regulation of BP [25]. Inhibition of neuronal function in this nucleus results in large decreases in BP [26]. We have demonstrated previously that both low-frequency electro- and manual acupuncture inhibit elevated BP as well as premotor sympathetic neural firing in the rVLM [12]. Administration of naloxone (nonspecific opioid receptor antagonist) or gabazine (γ-aminobutyric acid or GABA type A receptor blocker) in the rVLM abolishes the EA modulation [27]. The rVLM is an important brain stem region that processes somatic inputs during acupuncture stimulation. Electrophysiological studies of neuronal activity in the rVLM have shown that as compared to cardiovascular inactive points (LI 6-7, G 37–39), P 5-6 and certain acupoints along the large intestine meridian (LI 4–11), located over deep somatic neural pathways (median and deep radial nerves), provide more afferent input to cardiovascular premotor sympathetic neurons in the rVLM [10]. This observation likely explains why acupuncture over these deep nerves most effectively lower elevated sympathetic outflow and BP.

7. Neurotransmitters in rVLM, Arcuate, and vlPAG

Early studies in several models of hypertension suggested that EA lowers the elevated BP through the release of opioids, GABA, nociceptin, and serotonin (or 5-hydroxytryptamine, 5-HT) in the rVLM [28–32]. More recently, we have demonstrated that the EA inhibition of visceral reflex-induced hypertension in cats is related to the activation of μ- and δ-, but not κ-opioid receptors in the rVLM, suggesting that

endorphins, enkephalins, and perhaps endomorphin, but not dynorphin, are mainly responsible for EA modulation of cardiovascular responses.

Immunohistochemical staining studies have demonstrated the presence of enkephalinergic neurons in the rVLM and endorphinergic neurons in the arcuate nucleus that project directly to the rVLM and that both neurotransmitter systems are activated by EA [33]. EA inhibits the reflex hypertension through opioid-mediated inhibition of glutamate in the rVLM [34]. Electrophysiological studies [24] have shown that reciprocal excitatory glutamatergic (NMDA and non-NMDA) projections exist between the arcuate nucleus and vlPAG that may participate in the EA inhibition of cardiovascular function. This reciprocal projection may include a cholinergic component in the arcuate nucleus but not in the vlPAG [35].

Furthermore, EA, through presynaptic endocannabinoid CB1 receptor stimulation, reduces the vlPAG release of GABA but not glutamate during EA [36]. Reduced GABA disinhibits vlPAG neurons, thus increasing their activity, which, in turn, through an action in the NRP inhibits rVLM cardiovascular sympathetic neurons and related sympathoexcitatory reflex responses [37]. It is clear, therefore, that a variety of neurotransmitter systems underlie the cardiovascular modulation of EA. This includes both excitatory and inhibitory neurotransmitters, with their importance varying between the different nuclei.

8. Long-Loop Pathway for EA Cardiovascular Modulation

The role of the hypothalamic arcuate nucleus and its interaction with the vlPAG and rVLM in the EA-cardiovascular sympathoexcitatory responses has been extensively studied [10, 31, 38, 39]. Microinjection of the excitatory amino acid DLH, into the arcuate nucleus augments the responses of vlPAG neurons, while microinjection of small volumes (50 nL) of kainic acid (KA) causes reversible depolarization blockade that transiently deactivates arcuate neurons and decreases the vlPAG responses to SN stimulation [31]. Additionally, EA increases SN-evoked discharge of vlPAG neurons, a response that can be blocked by microinjection of KA into the arcuate nucleus. Microinjection of DLH into the arcuate nucleus, like EA, inhibits the reflex increase in BP induced by application of bradykinin to gallbladder for approximately 30 min. Finally, microinjection of KA into the arcuate blocks the inhibitory influence of EA on the reflex hypertension. As such, these results suggest that excitatory projections from the arcuate nucleus to the vlPAG appear to be essential to the inhibitory influence of EA on the reflex increase in BP induced by SN and gallbladder afferent stimulation.

9. vlPAG-rVLM Projections

The vlPAG provides inhibitory input to premotor sympathetic neurons in the rVLM to ultimately reduce sympathetic outflow and reflex elevations in BP [39]. Direct axonal projections from the vlPAG to the medulla have been documented in tract tracing studies [40]. However, a vlPAG projection to the raphé, in particular the nucleus raphé obscurus (NRO) also exists and has been thought to form an indirect pathway from the vlPAG to the rVLM that is involved in the EA-cardiovascular response [41]. Recent studies have suggested, however, that the NRP, located more ventrally than the NRO or the nucleus raphé magnus, contains more cells activated during median nerve stimulation with EA at the P 5-6 acupoints, as judged by the concentration of c-Fos labeling [42]. Chemical blockade of the NRP with KA or kenurenic acid transiently reverses activation of neurons in the rVLM during stimulation of the vlPAG as well as EA modulation of visceral excitatory reflexes [43]. Furthermore, the NRP inhibits rVLM activity, including activity of bulbospinal premotor sympathetic neurons. Serotonin projections from the raphé acting on 5-HT_{1A} receptors in the rVLM complete the vlPAG-NRP-rVLM circuit to modulate cardiovascular activity [43]. Thus, an indirect connection from the vlPAG to the rVLM involving a serotonergic connection between the NRP and the rVLM plays an important role in the long-loop modulation of cardiovascular sympathetic outflow during reflex visceral stimulation. These studies do not eliminate the possibility that direct projections between the vlPAG and the rVLM also might serve a functional role in EA-cardiovascular modulation. The direct or indirect projections from the vlPAG to the rVLM complete the long-loop pathway and provide an important source for the inhibitory influence of EA on rVLM premotor neurons and ultimately sympathoexcitatory cardiovascular responses [41].

10. Arcuate rVLM Projections

As noted previously, neurons in the vlPAG receive convergent input from a number of somatic nerves stimulated during EA as well as from the arcuate nucleus. Bilateral microinjection of KA into the rostral vlPAG partially reverses rVLM neuronal responses and cardiovascular inhibition during DLH stimulation of the arcuate. Conversely, depolarization blockade of the caudal vlPAG completely reverses arcuate evoked rVLM responses [41]. In parallel studies, we have observed that arcuate neurons can be antidromically activated from the rVLM and that arcuate perikarya are labeled with a retrograde tracer microinjected into the rVLM [41]. Many neurons from the arcuate that project to the rVLM are activated by EA stimulation (c-Fos positive) and they frequently contain opioid peptides, particularly β-endorphin [44]. As such, the vlPAG, particularly the caudal vlPAG, appears to be required for inhibition of rVLM neuronal activation by the ARC and subsequent EA-related cardiovascular activation. However, direct projections from the arcuate nucleus to the rVLM, likely serve as an important source of β-endorphin since this projection contains this opioid peptide [41]. This latter observation is consistent with our earlier anatomical study showing that cells in the rVLM contain enkephalin but not β-endorphin [44]. Hence, EA-cardiovascular responses that result from the action of β-endorphin on μ-opioid receptors located on

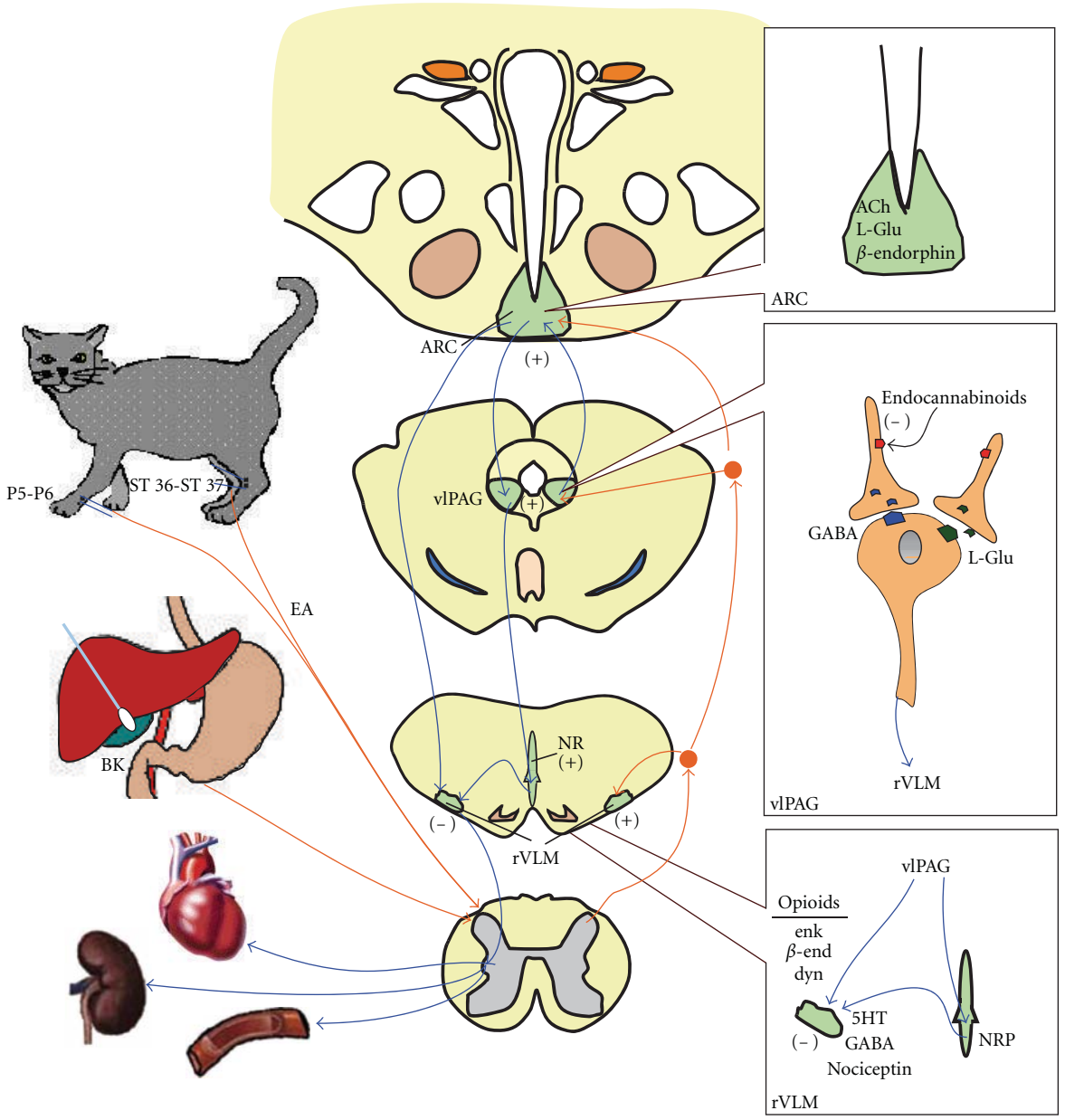

Figure 2: Neural circuits of acupuncture's action on cardiovascular sympathoexcitatory visceral reflex elevation of blood pressure. Abbreviations: ARC: arcuate nucleus; vlPAG: ventrolateral periaqueductal gray; NR: nuclei raphe; rVLM: rostral ventrolateral medulla. From[38].

rVLM sympathoexcitatory premotor neurons depend on this hypothalamic-medullary projection [45].

11. Role of Spinal Cord in Acupuncture-Cardiovascular Response

The spinal cord processes somatic and visceral reflexes as well as outputs from the central nervous system to effector organs involved in cardiovascular reflex regulation [46]. Anatomical and physiological studies indicate that the dorsal horn of the spinal cord serves as a major center for EA-induced analgesia [47, 48]. Both low- and high-frequency EA at *Zusanli* (*ST 36*) acupoint increase Fos immunoreactive neurons in the superficial laminae (I and II) in the dorsal horn of the spinal cord [48]. Since opioid or nociceptin-like immunoreactivity is present in the spinal sympathetic nuclei (i.e., intermediolateral column, IML) [49, 50], we have considered the possibility that EA also influences the neurotransmission between the brain stem and the IML [51]. In this regard, our studies have found that both opioid and nociceptin reduce the response to rVLM-induced sympathoexcitation, indicating that the two peptides can regulate sympathetic outflow [52, 53]. In addition, there has been a suggestion that descending pathways from the

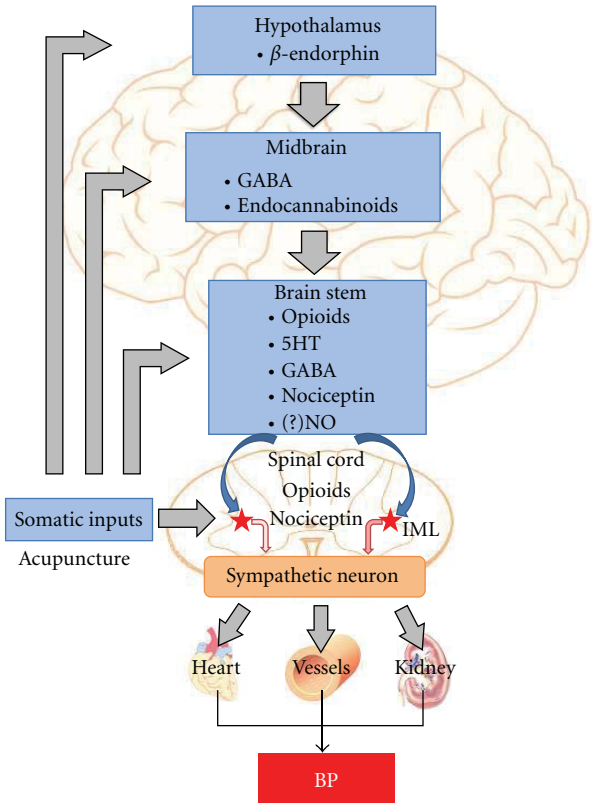

Figure 3: Neuroendocrine modulation of blood pressure by acupuncture. Abbreviations: GABA, γ-aminobutyric acid; 5HT, 5-hydroxytryptamine or serotonin; NO: nitric oxide; IML: intermediolateral column of the spinal cord.

brain stem (presumably to the dorsal horn of the spinal cord) may influence the segmental processing of somatic inputs during EA [54, 55]. Afferent stimulation can modulate sympathetic activity through the inhibition of excitatory interneurons [56]. Furthermore, somatic stimulation can elicit excitatory and inhibitory responses in both IML and dorsal horn interneurons, depending on the dermatome stimulated [57]. These interneurons appear to form important links in the spinal cord circuitry involved in autonomic control [58]. Taken together, these results indicate that opioid and nociceptin play a role in the processing of spinal cord interneuron activity in the EA response. However, spinal circuits controlling the cardiovascular visceral reflex responses during EA require further elucidation.

12. Endocrine and Vascular Actions of Acupuncture

Acupuncture reduces BP through modulation of the endocrine system, including decreases in plasma renin, aldosterone, and angiotensin II activity [59–61], and increased excretion of sodium [62]. Also, plasma norepinephrine, serotonin, and endorphin levels are reduced by acupuncture, reflecting its ability to modulate the neurohumoral system [63]. A laboratory-based study has demonstrated that long-term treatment with EA delayed hypertension development and restored nitric oxide in the plasma of SHRs [64]. Endothelial neuronal nitric oxide synthase (NOS) expression was significantly increased by EA in the mesenteric artery of SHRs, whereas neuronal (nNOS) expression was significantly attenuated. Additionally, EA at ST 36 induced nNOS expression in the gracile nucleus and medial nucleus tractus solitaries, and increased nNOS in the nuclei may modify central cardiovascular regulation, which contributes to hypotensive effects of acupuncture [65].

13. Short-Term and Long-Lasting Effect of Acupuncture

Williams and colleagues found that EA induced a significant and immediate poststimulation short-term reduction of diastolic blood pressure [66]. In 1997, a small study of 50 patients with essential hypertension found that shortly after 30 minutes of acupuncture both systolic and diastolic BP were lowered by 10–20 mmHg [61]. These data suggest that there is an immediate postacupuncture phenomenon. Our experimental studies in anesthetized animals have shown that the inhibitory effect of acupuncture on BP reflex responses occurs after 10–20 min of the start of EA stimulation and can last for as much as 60–90 min after termination of EA. In addition, in a preliminary study utilizing 24 hr ambulatory blood pressure monitoring [67], we have observed that 8 week of acupuncture lowers BP of hypertensive patients with mild-to-moderate hypertension (BP 140–180/90–110 mmHg) by 12–18 mmHg. This effect lasts for 4 weeks after termination of EA. These data suggest that EA at select acupoints (P5-P6 and ST 36-ST 37) known to have strong cardiovascular actions, performed once weekly for 8 weeks, significantly reduces BP. Importantly, this beneficial effect appears to persist for a prolonged period of time.

Several mechanisms might be involved in the long-lasting inhibitory action of acupuncture in hypertension. For example, the modulation by EA of rVLM sympathetic premotor neuronal responses to reflex-induced hypertension lasts for 30–40 min after the cessation of EA as a result of opioid and GABA modulation in this medullary region [68]. A recent study from our laboratory shows that reciprocal excitatory projections between the arcuate nucleus and the vlPAG may form a reinforcing circuit that can be activated for prolonged periods by EA, lasting as long as 30–60 min [41]. In addition, preliminary data from our laboratory using real-time PCR demonstrate that preproenkephalin in the rVLM is increased after completion of a single 30 min application of EA P 5-6 acupoints of rats [38]. The possibility that EA induces the production of opioid mRNA in the brain stem suggests that over time, EA may exert a long-lasting effect by stimulating increased production of opioid precursors.

14. Summary

Acupuncture has been shown to decrease BP in hypertensive patients and in animal models of hypertension. The mechanisms underlying the beneficial effects of acupuncture

are associated with modulation of sympathetic outflow and possibly the endocrine system. Experimental studies have shown that EA inhibits the reflex-induced hypertension by modulating the activity of cardiovascular presympathetic neurons in the rVLM. Activation of neurons in the arcuate nucleus of the hypothalamus, vlPAG in the midbrain, and NRP in the medulla by EA can inhibit the activity of premotor sympathetic neurons in the rVLM. Glutamate, acetylcholine, opioids, GABA, nociceptin, serotonin, NO, and endocannabinoids in the brain all appear to participate in the EA antihypertensive response (Figure 2). The central action of EA may also affect the endocrine system and lead to a decrease in plasma renin, aldosterone, angiotensin II, norepinephrine, and serotonin. The neuroendocrine mechanisms of acupuncture in the treatment of hypertension are not yet fully understood, and thus are worthy of further investigation (Figure 3).

Abbreviations

EA: Electroacupuncture
BP: Blood pressure
rVLM: Rostral ventrolateral medulla
ARC: Arcuate nucleus
vlPAG: Ventrolateral periaqueductal gray
NRP: Medullary nucleus raphé pallidus
NRO: Nucleus raphé obscurus
IML: Intermediolateral column
GABA: γ-aminobutyric acid
nNOS: Neuronal nitric oxide synthase
KA: Kainic acid.

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Research Article

Acupuncture Stimulation Alleviates Corticosterone-Induced Impairments of Spatial Memory and Cholinergic Neurons in Rats

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The purpose of this study was to examine whether acupuncture improves spatial cognitive impairment induced by repeated corticosterone (CORT) administration in rats. The effect of acupuncture on the acetylcholinergic system was also investigated in the hippocampus. Male rats were subcutaneously injected with CORT (5 mg/kg) once daily for 21 days. Acupuncture stimulation was performed at the HT7 (Sinmun) acupoint for 5 min before CORT injection. HT7 acupoint is located at the end of transverse crease of ulnar wrist of forepaw. In CORT-treated rats, reduced spatial cognitive function was associated with significant increases in plasma CORT level (+36%) and hippocampal CORT level (+204%) compared with saline-treated rats. Acupuncture stimulation improved the escape latency for finding the platform in the Morris water maze. Consistently, the acupuncture significantly alleviated memory-associated decreases in cholinergic immunoreactivity and mRNA expression of BDNF and CREB in the hippocampus. These findings demonstrate that stimulation of HT7 acupoint produced significant neuroprotective activity against the neuronal impairment and memory dysfunction.

1. Introduction

Acupuncture has long been known to modulate the biochemical balances in the central nervous system (CNS) and to maintain homeostasis [1]. Recently, much attention has been paid to acupuncture as an alternative therapy to improve memory-deficit symptoms through modulating the hypothalamic-pituitary-adrenal (HPA) axis and chronic stress-induced neurobiological responses [2–4]. In behavioral studies using a rat stress model induced by immobilization or chronic injection of corticosterone (CORT), we have previously demonstrated that hand acupuncture and low-frequency electroacupuncture stimulation significantly improved depression-like and anxiety-like behavior and restored the expression levels of neuropeptide Y and c-Fos in the brain [3, 5]. However, the underlying mechanism of acupuncture stimulation in modulating spatial cognitive function with respect to the cholinergic system in the CNS remains poorly understood.

Chronic stress causes dysregulation of the HPA axis in the neuroendocrine system, as evidenced by observations that the elevation of circulating CORT levels disrupts circadian regulation of CORT secretion as well as the glucocorticoid (GC) receptor-negative feedback circuit [6, 7]. Whereas acute stress increases GC levels and stimulates cognitive function, thus facilitating memory consolidation [8], prolonged and repeated exposure to high GC levels evokes adverse effects on cognition and memory function in rodents and humans [9, 10]. Accordingly, adrenalectomy or the restoration of CORT to normal blood levels prevents stress-induced cognitive deficits [11]. Many studies have shown that stimulation and sustained action of the HPA axis is attenuated via negative feedback actions of circulating GC by exogenous CORT administration, and this is closely associated with the development of psychosomatic disorders and produces serious changes in affective behavior indicative of memory deficit symptoms [12, 13].

The mammalian hippocampus has the highest density of GC receptors and participates in GC-mediated negative feedback of the HPA axis [14]. Therefore, hippocampal neurons, which are known to play an important role in learning and memory function, are particularly vulnerable to neuronal injury induced by chronic CORT injection [15], resulting in deficits in spatial memory and synaptic plasticity [16, 17].

The aim of the present study was to explore the efficacy of acupuncture therapy for healing chronic CORT-induced spatial memory impairment in an animal model using behavioral and neurobiological methodologies. To this end, acupuncture stimulation of HT7 (Sinmun) was evaluated for its efficacy in alleviating spatial learning and memory deficits in rats repeatedly exposed to exogenous CORT using the Morris water maze (MWM) test, and its underlying mechanism was elucidated by analyzing cholinergic markers in the hippocampus.

2. Materials and Methods

2.1. Animals. Adult male Sprague-Dawley (SD) rats weighing 260–280 g were obtained from Sam taco Animal Co. (Seoul, Korea). The rats were housed in a limited-access rodent facility with up to five rats per polycarbonate cage. The room controls were set to maintain the temperature at 22°C ± 2°C and the relative humidity at 55% ± 15%. Cages were lit by artificial light for 12 h each day. Sterilized drinking water and standard chow diet were supplied ad libitum to each cage during the experiments. The animal experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications no. 80-23), revised in 1996 and were approved by the Kyung Hee University Institutional Animal Care and Use Committee. All animal experiments began at least 7 days after the animals arrived.

2.2. Experimental Groups

2.2.1. Experiment 1. This study was designed to compare the CORT concentration in rat blood and brain tissue, immediately after CORT injection or immobilization stress for 21 days. The rats were randomly divided into three groups of four individuals each as follows: nontreated normal group (NOR group, $n = 4$), restraint-stressed group (STR group, $n = 4$), and CORT-injected and nontreated group (5 mg/kg, s.c., CORT group, $n = 4$). The immobilization stress procedure was performed by daily placing animals in 20 × 7-cm plastic tubes for 2 h for 21 days [18]. There were several 3-mm holes at one end of a tube for breathing. The animals had ample air but were unable to move.

Also, the other experiment was designed to compare the effects of acupuncture stimulation to enhanced CORT concentration in the blood of chronic CORT-injected rats for 21 days. The rats were randomly divided into five groups of four individuals each as follows: vehicle saline-injected group, instead of CORT (0.9% NaCl, s.c., CON group, $n = 4$), CORT-injected and nontreated group (5 mg/kg, s.c., CORT group; $n = 4$), CORT-injected and Sinmun (HT7) acupoint-stimulated group (CORT-HT group; $n = 4$), CORT-injected and Waiguan (TE5) acupoint-stimulated group (CORT-TE group; $n = 4$), CORT-injected and nonacupoint- (on the tail) stimulated group (CORT-TA group; $n = 4$). Corticosterone (Sigma-Aldrich Chemical Co., St Louis, mo, USA), dissolved in absolute ethanol, and subsequently diluted in water to the final concentration of 10% ethanol was injected at a daily dose of 5 mg/kg [19]. This CORT dose was selected because it induces plasma levels of the steroid comparable to those elicited by substantial stress. The CORT and vehicle injections were given in the morning between 9 and 10 am once daily for 21 consecutive days. As a vehicle control, animals in the CON group were subcutaneously given the equivalent volumes of saline to the final concentration of 10% ethanol. The daily doses and duration were determined based on the previous studies by Trofimiuk et al. [19].

2.2.2. Experiment 2. This study was designed to explore the efficacy of acupuncture therapy for healing chronic CORT-induced spatial memory impairment in an animal model using behavioral and neurobiological methodologies. The rats were randomly assigned to divided into five groups of seven individuals each as follows; vehicle saline-injected group (CON group, $n = 7$), CORT-injected and nontreated group (CORT group; $n = 7$), CORT-injected and Sinmun (HT7) acupoint-stimulated group (CORT-HT group; $n = 7$), CORT-injected and Waiguan (TE5) acupoint-stimulated group (CORT-TE group; $n = 7$), CORT-injected and nonacupoint- (on the tail) stimulated group (CORT-TA group; $n = 7$).

The experimental schedule of CORT injection, acupuncture treatment, behavioral test, and tissue and blood sampling are shown in Figure 1.

2.3. Acupuncture Stimulation. Acupuncture stimulation was bilaterally performed every second day for 5 min before the CORT injection during the CORT-injection period. The acupoint on tail and the Waiguan acupoint were selected as a nonacupoint and a comparison acupoint, respectively. The acupuncture stimulation was performed as previously described [5]. Briefly, small holders with five holes for four limbs and a tail were manufactured to restrain the rat bodies for acupuncturing. During the acupuncture treatment, animals were maintained within the cage with right and left forepaw taken out and fastened to the wall of the cage with tape. Sterilized disposable stainless steel acupuncture needles (0.30 × 25 mm, Suzhou Kangnian Medical Devices Co., Ltd., Shzhou, China) were inserted perpendicularly as deep as 2-3 mm at HT7 or TE5 acupoint. The depth of needle insertion at each acupoint was arbitrarily determined on the basis of several previously studies [20] and in the animal acupuncture atlas [21]. The HT7 point is anatomically located on transverse crease of wrist of forepaw, radial to the tendon of the m. flexor carpi ulnaris. The TE5 point is located 2 inches up to wrist bracelet, 0.5~1.0 inch deep. This acupoint is located between two bones. In addition, the nonacupoint needling was performed at one-fifth point of

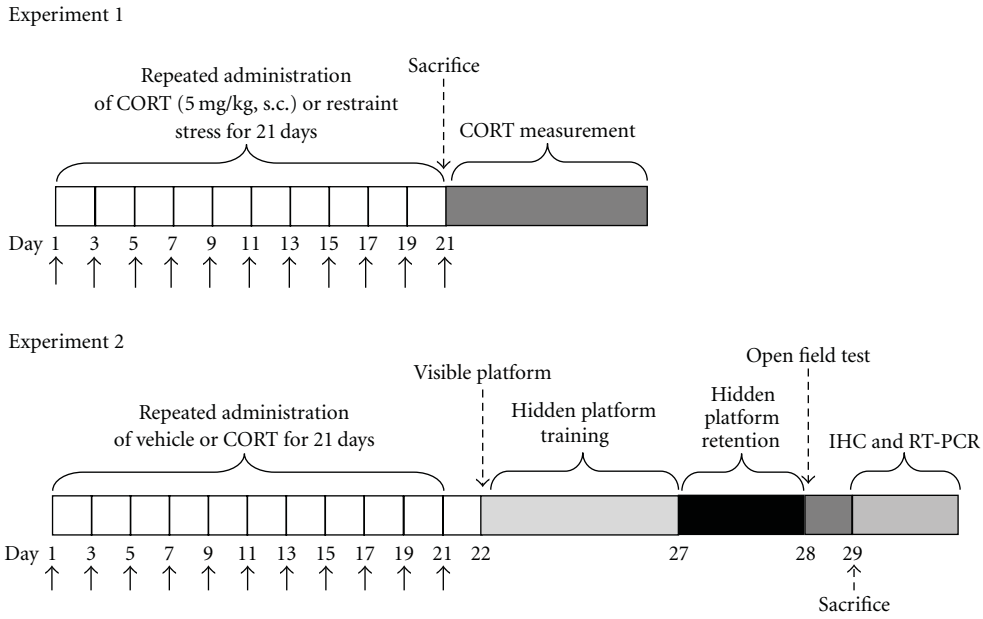

Figure 1: Experimental schedules of corticosterone-induced impairment of spatial memory in the rat. Black arrows indicate acupuncture treatments. Experiment 1 was designed to compare the CORT concentration in rat blood and brain tissue, immediately after CORT injection or immobilization stress for 21 days. It also was designed to compare the effects of acupuncture stimulation to enhanced CORT concentration in the blood of chronic CORT-injected rats for 21 days. Experiment 2 was designed to explore the efficacy of acupuncture therapy for healing chronic CORT-induced spatial memory impairment in an animal model using behavioral and neurobiological methodologies. IHC; Immunohistochemistry.

tail length from the proximal end of the tail to avoid the tail acupoints located at proximal or distal region of the tail. The CON group and CORT group were handled for 5 min before the CORT injection during the CORT-injection period to reduce stress and facilitate handling, instead of acupuncture stimulation, as described previously [5].

2.4. Corticosterone Measurement. After immobilization stress or CORT injection for 21 days, CORT concentration in blood and brain tissues was determined. For this, the unanesthetized rats were rapidly decapitated, and blood was quickly collected via the abdominal aorta. The hippocampus was rapidly removed from the rat brains in randomized order. Special care was taken to avoid predecapitation stress—while rats were rapidly decapitated, the other animals were left outside the room and handled for a few minutes prior to sampling. The blood and tissue samples were stored at −80°C until use. Hippocampus were homogenized in a lysis buffer containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, 10 mg/mL aprotinin, 1 mg/mL leupeptin and 0.5 mM sodium vanadate. Homogenization was carried out on ice using a tissue homogenizer and incubated for 1 min at 4°C with shaking. Homogenates were centrifuged and supernatants were collected. Protein concentrations were estimated by the procedure of Lowry et al. [22] with BSA as the standard. The CORT concentration was measured by a competitive enzyme-linked immunoassay (ELISA) using a rabbit polyclonal CORT antibody (OCTESIA Corticosterone kit; Alpco Diagnostics Co., Windham, NH, USA) according to the manufacturer's protocol. Samples (or standard) and conjugate were added to each well, and the plate was incubated for 1 h at room temperature without blocking. After wells were washed several times with buffers and proper color developed, the optical density was measured at 450 nm using an ELISA reader (MultiRead 400; Authos Co., Vienna, Austria).

2.5. Morris Water Maze Test

2.5.1. Morris Water Maze Apparatus. The MWM test was performed using a polypropylene circular pool (painted white internally, 2.0 m in diameter and 0.35 m high). The pool contained water maintained at a temperature of 22 ± 2°C. The water was made opaque by adding 1 kg of skim milk powder. During the MWM, a platform 15 cm in diameter was located 1.5 cm below the water in one of four sections of the pool, approximately 50 cm from the sidewalls. The pool was surrounded by many cues external to the maze. The pool was divided into four quadrants of equal area. A digital camera was mounted to the ceiling above the pool and was connected to a computerized recording system equipped with a tracking program (S-MART: PanLab Co., Barcelona, Spain), which permitted on- and offline automated tracking of the paths taken by the rat.

2.5.2. Hidden Platform Trial for Acquisition Test. The MWM task was performed on 22st day after the CORT injection and acupuncture treatment were commenced. The animals received three trials per day. The rats were trained to find

the hidden platform, which remained in a fixed location throughout the test. The trials lasted for a maximum of 180 s, and the time it took to find the submerged platform was recorded each time. The animals were tested in this way three per day for 6 consecutive days, and they received a 60-s probe trial on the seventh day. Finding the platform was defined as staying on it for at least 4 s before the acquisition time of 180 s ended. If the rat failed to find the platform in the allotted time, it was placed onto the platform for 20 s and assigned a latency of 180 s. Between one trail and the next, water was stirred to erase olfactory traces of previous swim patterns. The entire procedure took seven consecutive days, and each animal had three training trials per day, with a 30- to 40-min intertrial interval.

2.5.3. Visible Platform (Cued Trial) Test. The cued trial (three trials per rat) was performed on the first day (on 22st day) to assess the rats' motivation to escape from the water and to evaluate their sensor-motor integrity. The platform was placed in the north quadrant and had a visible black cue. The animal was placed in the pool and given 90 s to reach the platform, which was identified by a visible black platform above the surface of the water. Latency to reach the visible platform was automatically calculated.

2.5.4. Probe Trial for Retention Test. For the probe trial, each rat was placed into the water diagonally from the target quadrant (north), and for 60 s, was allowed to search the water, from which the platform had been removed. The time (% for total time) spent searching for the platform in the former platform quadrant (north) and in the other three quadrants was measured for each rat.

2.6. Open Field Test. Prior to water maze testing, the rats were individually housed in a rectangular container made of dark polyethylene ($45 \times 45 \times 35$ cm) in a dimly lit room equipped with a video camera above the center of the floor, and locomotor activity was measured. The locomotor activity was monitored by a computerized video-tracking system using S-MART program (PanLab Co.). The distance they traveled in the container was recorded during the 5-min test. The locomotor activity was measured in meters. The floor surface of each chamber was thoroughly cleaned with 70% ethanol between tests. The Plexiglas square arena was divided into nine equal-sized squares on the floor. The number of lines crossed (with all four paws) between the squares area was recorded for 5 min.

2.7. Choline Acetyltransferase (ChAT) Immunohistochemistry. For immunohistochemical studies, four rats in each groups were deeply anesthetized with sodium pentobarbital (80 mg/kg, by intraperitoneal injection) and perfused through the ascending aorta with normal saline (0.9%) followed by 300 mL (per rat) of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, postfixed overnight, and cryoprotected with 20% sucrose in 0.1 M PBS at 4°C. Coronal sections 30 μm thick were cut through the septal region or hippocampus using a cryostat (Leica CM1850; Leica Microsystems Ltd., Nussloch, Germany). The sections were obtained according to the rat atlas of Paxinos and Watson (hippocampus; between bregma −2.6 and −3.6) [23]. The sections were immunostained for ChAT expression using the avidin-biotin-peroxidase complex (ABC) method. Briefly, the sections were rinsed three times for 5 min each in PBS and then incubated with primary rabbit anti-ChAT antibody (1 : 2000 dilution; Cambridge Research Biochemicals Co., Bellingham, UK) in PBST (PBS plus 0.3% Triton X-100) for 72 h at 4°C. The sections were washed for 5 min in PBS and then incubated for 120 min at room temperature with biotinylated antirabbit goat IgG (for the anti-ChAT antibody). The secondary antibodies were obtained from Vector Laboratories Co. (Burlingame, Calif, USA) and diluted 1 : 200 in PBST containing 2% normal goat serum. To visualize immunoreactivity, the sections were incubated for 90 min in ABC reagent (Vectastain Elite ABC kit; Vector Labs. Co.), washed three times for 5 min in PBS, and incubated in a solution containing 3,3′-diaminobenzidine (DAB; Sigma) and 0.01% H_2O_2 for 1 min. Finally, the tissues were washed in PBS, followed by a brief rinse in distilled water, and mounted individually onto slides. Slides were allowed to air dry and were then cover-slipped. Images were captured using the AxioVision 3.0 imaging system (Carl Zeiss, Inc., Oberkochen, Germany) and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, Calif, USA). The sections were viewed at 100x magnification, and the numbers of cells within $100 \times 100\text{-mm}^2$ grids were counted by observers blinded to the experimental groups. Medial septum or hippocampal area cells were obtained according to the stereotactic atlas of Paxinos and Watson [23]. The cells were counted in three sections per rat within the hippocampal area.

2.8. Acethylcholinesterase (AchE) Immunohistochemistry. For AchE histochemistry, the sections were washed in PBS and incubated in a solution with 25 mg of acetylthiocholine iodine for 1 h. The solution was composed of 32.5 mL of 0.1 M sodium hydrogen phosphate buffer ($NaH_2PO_4 \cdot H_2O$, pH 6.0), 2.5 mL of 0.1 M sodium citrate, 5 mL of 30 mM copper sulfate, 5 mL of 5 mM potassium ferricyanide, and 5 mL of distilled water. The color of the mixing solution was green. The densities of stained nuclei of the hippocampal cells were measured using a Scion image program (Scion Co., Frederick, Md, USA). The sections were viewed at 200x magnification, and the numbers of cells within $100 \times 100\text{-mm}^2$ grids were counted by observers blinded to the experimental groups. Hippocampal area cells were obtained according to the stereotactic atlas of Paxinos and Watson [23]. The cells were counted in three sections per rat within the hippocampal area.

2.9. Total RNA Preparation and RT-PCR Analysis. The hippocampus from three rats in each group was isolated. After decapitation, the brain was quickly removed and stored at −80°C until use. Total RNA was isolated from the brain samples using TRIzol reagent (Invitrogen Co., Carlsbad, Calif, USA) and was used to extract RNA according

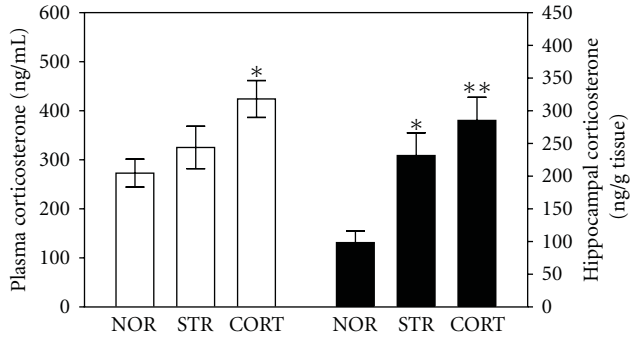

Figure 2: Corticosterone (CORT) concentrations in rat plasma and hippocampus, immediately after CORT injection or immobilization stress for 21 days. The experimental groups were pretreated with nontreated normal (NOR, $n = 4$), restraint-stressed (STR, $n = 4$) and CORT-injected (CORT, $n = 4$) rats. Data were analyzed using a one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* NOR group. Vertical bars indicate SE.

to the supplier's instruction. Complementary DNA was synthesized from total RNA with reverse transcriptase (Takara Co., Shiga, Japan). Brain-derived neurotrophic factor (BDNF) and cAMP-response element-binding protein (CREB) mRNA expression levels were determined by the reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed using a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, Mass, USA). The operating conditions were as follows: for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec; for BDNF, 27 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec; for CREB, 27 cycles of denaturation at 95°C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 30 sec. All primers were designed using published mRNA sequences and primer design software (Primer 3; The Whitehead Institute for Biomedical Research, Cambridge, Mass, USA; http://www.genome.wi.mit.edu/), offered through the web site. The following sequences were used: for GAPDH (409 bp), (forward) 5′-ATC CCA TCA CCA TCT TCC AG-3′ and (reverse) 5′-CCT GCT TCA CCA CCT TCT TG-3′; for BDNF (153 bp), (forward) 5′-CAG GGG CAT AGA CAA AAG-3′ and (reverse) 5′-CTT CCC CTT TTA ATG GTC-3′; for CREB (183 bp), (forward) 5′-TAC CCA GGG AGG AGC AAT AC-3′ and (reverse) 5′-GAG GCA GCT TGA ACA ACA AC-3′. The PCR products were separated on 1.2% agarose gels and stained with ethidium bromide, and the density of each band was analyzed using an image-analyzing system (i-Max, CoreBio System Co., Seoul, Korea). Complementary DNA expression levels were determined by calculating the relative density of each BDNF or CREB band to GAPDH.

2.10. Statistical Analysis. All measurements were performed by an independent investigator blinded to the experimental conditions. Results in figures are expressed as mean ± standard error of means (SE). Differences within or between normally distributed data were analyzed by analysis of variance (ANOVA) using SPSS (Version 13.0; SPSS, Inc., Chicago, Ill, USA) followed by Tukey's *post hoc* test. Statistical significance was set at $P < 0.05$.

For statistical analysis of water maze data, the effect of training on the acquisition of the water escape task was assessed using a one-way ANOVA with a repeated-measure factor of sessions (number of days) followed by the appropriate Tukey's *post hoc* analysis. For the probe trial in the water maze, within-group differences in the time spent in each quadrant and immunohistochemical data and PCR analysis were also analyzed by one-way ANOVA followed by Tukey's *post hoc* test.

3. Results

3.1. Experiment 1

3.1.1. Corticosterone (CORT) in the Blood and Brain Tissues. The ELISA analysis demonstrated that restraint-stress exposure for 21 days and CORT administration for 21 days significantly increased the plasma CORT concentration in the rats by 19.2% ($P = 0.593$) and 55.4% ($P < 0.05$), respectively, compared with rats in the nontreated NOR group [$F(2, 11) = 4.324$; $P < 0.05$]. Additionally, exposure to both restraint stress and exogenous CORT administration induced significant increases in the hippocampal CORT concentration by 135.64% ($P < 0.05$) and 190.2% ($P < 0.01$), respectively, compared with the nontreated NOR group [$F(2, 11) = 9.951$; $P < 0.01$] (Figure 2). The CORT concentration of the blood and the hippocampus in the CORT group was higher than that in the nontreated NOR group and was closely associated with that in the STR group. There was no significant difference in CORT concentration between the CORT induction by psychological stress (STR group) and the physiological CORT injection (CORT group). In these results, exogenous CORT-induced memory impairment was exploited to develop a chronic stress model in the rats.

Also, CORT concentration differed among the five groups after acupuncture stimulation [$F(4, 19) = 7.791$; $P < 0.01$]. The plasma levels of CORT in the CORT-HT groups lower than that in the CORT group ($P = 0.190$), while the CORT group had significantly higher CORT levels compared to the CON group ($P < 0.05$) (Figure 3).

3.2. Experiment 2

3.2.1. Effect of Acupuncture in the Visible-Platform Trial of the Morris Water Maze Test. To exclude the possibility of impairing the animals' vision and changing the motivation to escape the water due to the acupuncture stimulation, a cued version of the MWM test was performed, and the swimming time to reach the visible platform was measured as illustrated in Figure 4(a). When trained to a visible platform, there were no significant differences between groups [$F(4, 30) = 0.116$; $P = 0.976$]. As shown in Figure 4(a), the latency to find the visible platform did not differ among groups on the first,

second, or third trials. On the second and third trials, the latency was markedly reduced in all groups compared with the first trial. All rats, irrespective of grouping, were able to locate the visible platform more rapidly as the trial number increased.

3.2.2. Effect of Acupuncture in the Hidden-Platform Trail of the Morris Water Maze Test. The rats in the CON group rapidly learned the location of the submerged hidden platform and reached it within 20 s on day 6 of the trials (Figure 4(b)). The acupoint-stimulated groups also showed a reduction in escape latency throughout the training period, and the CORT group showed a marked retardation in escape latency reduction due to CORT-induced impairment of learning and memory. Analysis of the training data by repeated-measures ANOVA showed that escape latency differed significantly among the groups when the times were averaged over all sessions [$F(4, 30) = 13.745$; $P < 0.001$]. During the experiment, escape latency decreased over time [$F(5, 150) = 143.875$; $P < 0.001$]. Additionally, a significant interaction between experimental groups and time [$F(20, 150) = 1.788$; $P < 0.05$]. Tukey's post hoc test revealed that rats in the CORT-HT group had significantly reduced swimming latency compared with those in the CORT group ($P < 0.01$ on day 5 and $P < 0.05$ on day 6; Figure 4(b)). The CORT group was not significantly different from other groups in terms of mean swimming speed, calculated by dividing the total swim distance by the latency [$F(4, 30) = 0.936$; $P = 0.457$] (Figure 4(d)). Total distance traveled in each group was closely associated with escape latency in this task (data not shown). On the basis of these results, the HT7-acupoint-stimulated rats showed improved acquisition in the hidden-platform trial, reaching the platform with lower latency than the CORT-injected and/or nonacupoint-stimulated rats.

3.2.3. Effect of Acupuncture in the Probe Trial of the Morris Water Maze Test. To examine the spatial memory of rats, we analyzed their performance in the probe test on day 7 by comparing the percentage of time spent swimming to the expected position of the platform (Figure 4(c)). The time spent swimming around was significantly reduced in the rats that swam to the target area where the platform had been located [$F(4, 34) = 10.765$; $P < 0.001$]. The chronic administration of CORT severely impaired spatial performance in the MWM ($P < 0.001$). Rats in the CORT-HT group spent more time around the platform area than did those in the CORT group ($P < 0.05$). The stimulation of HT7 acupoint significantly attenuated the CORT-induced deficit in learning and memory demonstrated in the water maze task. This study also indicated that the swimming latency in the rats receiving acupuncture stimulation to the acupoint HT7 was higher than that in the rats receiving stimulation to a nonacupoint (CORT-TA) and another acupoint (CORT-TE) as controls. No difference in swimming latency time was observed between the CORT-TA ($P = 0.881$) or CORT-TE groups ($P = 0.247$) compared with the CORT group. Therefore, the HT7-acupoint-stimulated rats also showed a significant amelioration in the memory retention test: they spent more time in the quadrant where the platform was formerly located and swam over the former location of the platform more frequently.

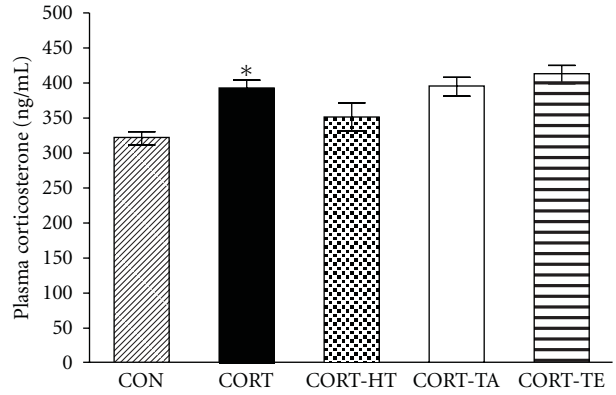

Figure 3: Effect of acupuncture on plasma CORT concentrations induced by repeated CORT injection in the rats. The experimental groups were pretreated with vehicle saline-injected group, instead of CORT (0.9% NaCl, s.c., CON group, $n = 4$), CORT-injected and nontreated group (5 mg/kg, s.c., CORT group; $n = 4$), CORT-injected and Sinmun (HT7) acupoint-stimulated group (CORT-HT group; $n = 4$), CORT-injected and Waiguan (TE5) acupoint-stimulated group (CORT-TE group; $n = 4$), and CORT-injected and nonacupoint- (on the tail) stimulated group (CORT-TA group; $n = 4$). Data were analyzed using a one-way ANOVA followed by Tukey's *post hoc* test. $^*P < 0.05$ *versus* CON group. Vertical bars indicate SE.

3.2.4. Effect of Acupuncture in the Open-Field Test. In an analysis of open-field test results by a parametric one-way ANOVA, no significant differences were observed between groups in terms of memory deficit-related locomotor activity or the total number of line crossings in the open-field test (Figure 5). The statistical differences in observed locomotor activity and the total number of line crossings between groups were $F(4, 34) = 0.794$; $P = 0.539$ and $F(4, 34) = 1.220$; $P = 0.323$, respectively. This indicates that acupuncture stimulation to HT7 acupoint did not affect psychomotor function as measured by the rats' performance in the MWM test.

3.2.5. Effect of Acupuncture on Septal-Hippocampal Choline Acetyltransferase. The possibility that the deficits in septal-hippocampus-dependent learning and memory of the chronic exposure to CORT are associated with cholinergic deficits was examined histologically following completion of the behavioral tasks using ChAT immunohistochemistry. The number of septal-hippocampal cholinergic neurons immunohistochemically stained for ChAT was counted in the septohippocampal fibers, represented by medial septum or hippocampus. The results of number of ChAT-stained septohippocampal cholinergic neurons are shown in (Figure 6). The brains of the CORT group showed significant decreased ChAT positive cells in the medial seputm compared with the CON group ($P < 0.05$). Comparison of

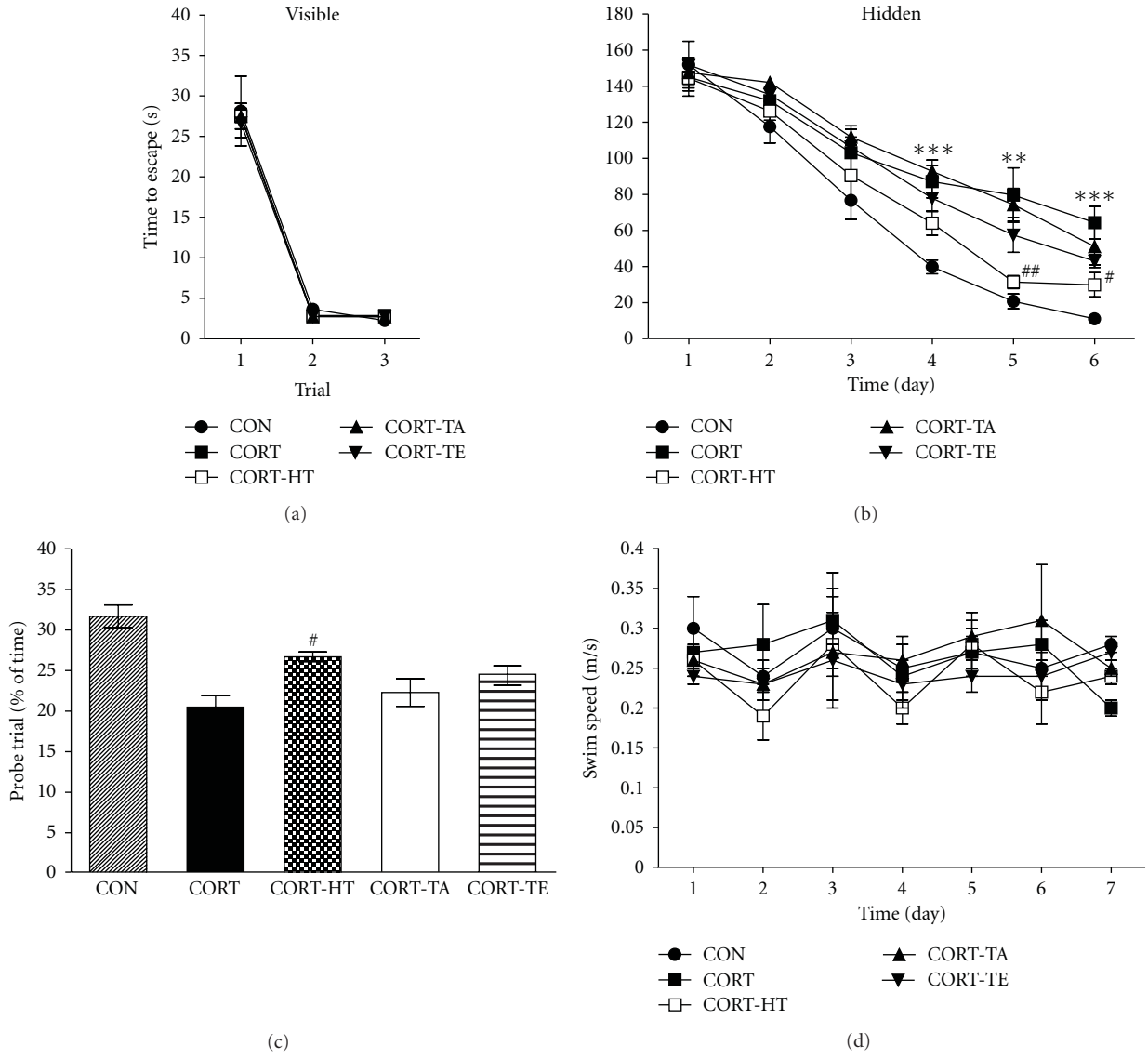

Figure 4: Time to escape (latency) during acquisition trials of visible platform (a), hidden platform (b), probe trial (c), and swim speed (d), during the Morris water maze test. The experimental group were pretreated with vehicle saline, instead of CORT (0.9% NaCl, s.c., CON group, $n = 7$) once daily for 21 consecutive days. The control group were pretreated with CORT (5 mg/kg, s.c., CORT group; $n = 7$) once daily for 21 consecutive days. The other groups were pretreated with CORT-injected and Sinmun (HT7) acupoint-stimulated group (CORT-HT group; $n = 7$), CORT-injected and Waiguan (TE5) acupoint-stimulated group (CORT-TE group; $n = 7$), and CORT-injected and nonacupoint (on the tail)-stimulated group (CORT-TA group; $n = 7$) every second day for 5 min before the CORT injection, respectively. Data were analyzed using a repeated-measures ANOVA followed by Tukey's *post hoc* test. $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* the CON group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* the CORT group. Vertical bars indicate SE.

the numbers of ChAT-immunoreactive neurons by a one-way ANOVA revealed a significant differences among groups [$F(4, 79) = 2.869$; $P < 0.05$] (Figure 7). The number of ChAT-immunoreactive neurons in the medial septum area was 55.19 ± 3.58 (100.0 ± 10.53%) in the CON group, 34.25 ± 3.92 (62.06 ± 7.11%) in the CORT group, 54.50 ± 6.33 (98.75 ± 11.47%) in the CORT-HT group, 44.31 ± 7.99 (80.29 ± 14.48%) in the CORT-TA group, and 48.88 ± 3.18 (89.68 ± 5.83%) in the CORT-TE group. The number of ChAT-immunoreactive neurons was significantly increased in the medial septum region in the CORT-HT group ($P < 0.05$), compared with the CORT group (Figure 7). The brains of the CORT group showed significant decreased ChAT positive cells in the hippocampal CA1 area, one of the target areas of septohippocampal cholinergic neurons, compared with the CON group ($P < 0.01$). Comparison of the numbers of ChAT-immunoreactive neurons by a one-way ANOVA revealed a significant difference among groups [$F(4, 79) = 4.893$; $P < 0.01$] (Figure 7). The number of ChAT-immunoreactive neurons in the hippocampal CA1 area was 92.63 ± 7.40 (100.0 ± 8.56%) in the CON group, 67.13 ± 5.62 (72.47 ± 6.06%) in the CORT group, 86.19 ± 6.00

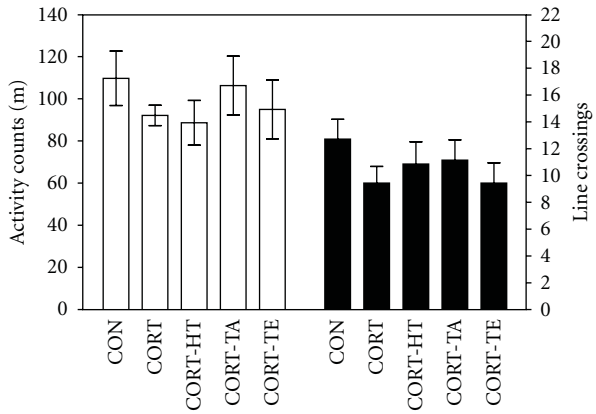

Figure 5: Activity counts of locomotor activity (a) and the total number of line crossings (b) in the open-field test.

(93.05 ± 6.48%) in the CORT-HT group, 72.69 ± 2.59 (78.48 ± 2.80%) in the CORT-TA group, and 70.88 ± 5.47 (82.23 ± 6.35%) in the CORT-TE group. The number of ChAT-immunoreactive neurons was significantly increased in the hippocampal CA1 region in the CORT-HT group ($P < 0.05$), compared with the CORT group (Figure 7). The ChAT-immunoreactivity loss in the CORT group was remarkably restored by the HT7 acupoint stimulation, and the ChAT-immunopositive neuron numbers were closely similar to those in the CON group.

3.2.6. Effect of Acupuncture on Hippocampal Acetylcholinesterase. The density of AchE-immunopositive fibers in the CA1 area of the rat hippocampus was significantly reduced by repeated injections of CORT in the CORT group compared with the saline-injected vehicle group (CON group) (Figure 8). The AchE-positive neuron density in the CA1 was 11.31 ± 0.80 (100.0 ± 4.34%) in the CON group, 7.25 ± 0.40 (64.09 ± 3.56%) in the CORT group, 9.38 ± 0.62 (82.87 ± 5.47%) in the CORT-HT group, 8.38 ± 0.68 (74.03 ± 5.98%) in the CORT-TA group, and 8.13 ± 0.55 (71.82 ± 4.90%) in the CORT-TE group [$F(4, 79) = 9.205$; $P < 0.001$]. The AchE-reactive neuron loss in the hippocampal CA1 area due to chronic exposure to exogenous CORT was significantly restored by the HT7 acupoint stimulation ($P < 0.05$). The density of AchE-reactive neuron in the HT7 acupoint-stimulated group (CORT-HT group) was closely similar to that in the CON group.

The density of AchE fiber in the CA3 region of the hippocampus was also markedly reduced by repeated injections of CORT in the CORT group compared with the CON group (Figure 8). The AchE neuron density in the CA3 region was 11.00 ± 0.79 (100.0 ± 6.23%) in the CON group, 8.31 ± 0.78 (75.57 ± 7.08%) in the CORT group, 9.75 ± 1.01 (88.64 ± 9.22%) in the CORT-HT group, 9.19 ± 0.50 (83.52 ± 4.56%) in the CORT-TA group, and 7.88 ± 0.48 (71.59 ± 4.38%) in the CORT-TE group [$F(4, 79) = 3.588$; $P < 0.05$]. Tukey's post hoc test showed that the HT7 acupoint stimulation restored the loss of AchE reactive neurons in the CA3 area of the hippocampus, although the change was not statistically significant. The exogenous CORT-induced decreases in AchE-immunoreactive neuron densities were not significantly recovered in the CORT-HT group ($P = 0.526$) compared with the CORT group.

3.2.7. Effect of Acupuncture on BDNF and CREB mRNA Expression in the Hippocampus. The effect of acupuncture stimulation to the HT7 acupoint on BDFN and CREB mRNA expression levels in the rats with CORT-induced hippocampus lesions was investigated by RT-PCR analysis (Figure 9). The BDNF and CREB mRNA expression levels were normalized against glyceraldehydes-3-phophate dehydrogenase (GAPDH) mRNA, an internal control. The BDNF mRNA expression in the rat hippocampus in the CORT group was significantly decreased compared to that in the CON group ($P < 0.001$). The reduced expression of BDNF mRNAs in the CORT groups was significantly restored by the HT7 acupoint stimulation in the CORT-HT group ($P < 0.05$), and the restored level was similar to that of normal rats in the CON group [$F(4, 14) = 18.830$; $P < 0.001$]. The CREB mRNA expression in the rat hippocampus in the CORT group also decreased compared with that in the CON group ($P < 0.05$). The reduced expression of CREB mRNAs in the CORT group was also significantly restored by the HT7 acupoint stimulation in the CORT-HT group ($P < 0.01$), and the restored level was similar to that of normal rats in the CON group [$F(4, 14) = 7.531$; $P < 0.01$].

4. Discussion

In the present study, acupuncture stimulation to the HT7 acupoint significantly improved learning and memory retention in the MWM and increased ChAT and AchE immunoreactivities in the hippocampus areas of chronic CORT-induced memory impairment male rats. Interestingly, 5-min acupuncture stimulation prior to CORT administration was enough to modulate CORT-induced neurochemical and behavioral responses (data not shown). In addition, only the acupuncture stimulation to the HT7 acupoint elicited significant responses, compared with another acupoint on a different meridian, TE5, or to a nonacupoint on the tail. These results indicate that stimulation of the acupuncture point spreads throughout the body at a rapid rate, and its effect is highly point specific, at least for modulating CORT-induced memory impairments. Furthermore, acupuncture to HT7 was capable of attenuating a complex behavioral syndrome and protecting the hippocampus from deficits.

In traditional oriental medicine, the Sinmun (HT7) is a specific acupoint located on the heart channel, which is also called the "spirit gate" to the pathway that is used clinically to treat mental, psychosomatic, and cognitive disorders [21, 24]. As a comparable control acupoint, we also performed the stimulation to another acupoint, the Waiguan (TE5) acupoint, on a different (large intestine) meridian, and the triple-energizer channel, which is known to treat immune depression and pain/neuropathy of the arm [21]. Although many studies have been tried to elucidate the effects of acupuncture on various diseases, study of the effect of

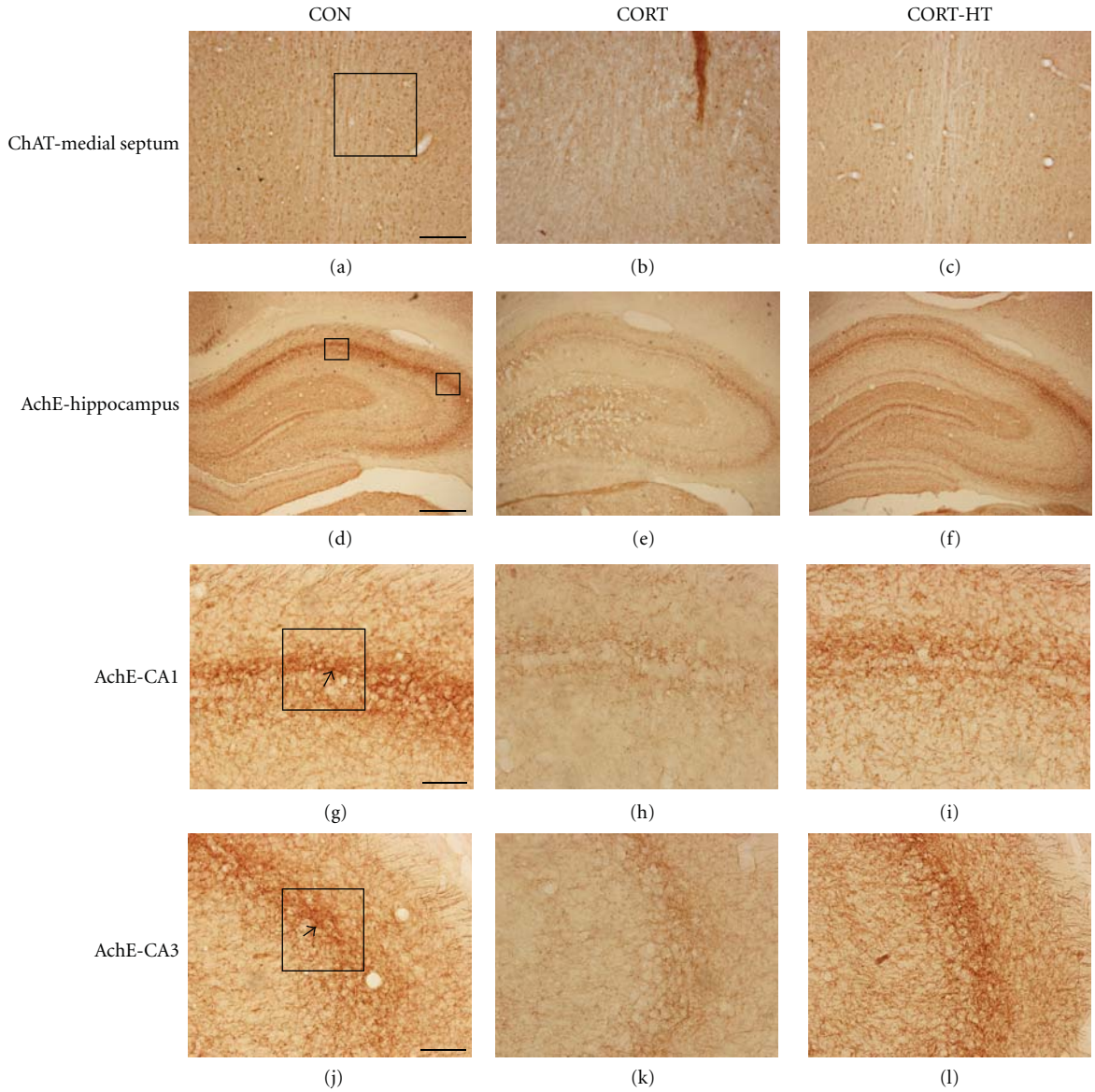

Figure 6: Representative photographs showing the distribution of choline acetyltransferase (ChAT) immunostaining cells in medial septum and acetylcholinesterase (AchE) reactive cells in hippocampus of CORT-induced memory impairment or CORT-injected and Sinmun (HT7) acupoint-stimulated rats. Lower magnification of the small box in panel (d) and high magnification of the big box in panel (g and j) of the same fields of AchE- stained nuclei of the hippocampus. Scale bar represents 100 μm (a), 200 μm (d) and 50 μm (g and j), respectively.

acupuncture on exogenous CORT-induced cognitive deficits and their behavioral and neurochemical responses has not been reported previously.

Many studies very well recognized that dysregulation of the HPA axis by chronic stress or elevated levels of circulating CORT produces hyperactivity of sympathetic adrenomedullary system, such as CORT, corticosteroid-binding-globulin, ACTH, norepinephrine (NE), and epinephrine (E) [25, 26]. In this study, memory impairments induced by repeated injections of CORT were exploited to develop a chronic stress model in rats. The chronic administration of high-dose CORT increased plasma and hippocampal CORT concentrations in the rats, in line with chronic stress models [10]. Accordingly, in animal models, forced sustaining of high CORT levels can affect animal cognition by reducing memory capacity under experimental conditions, and this might be closely associated with the progression or exacerbation of chronically stressful conditions in humans [27, 28]. Therefore, our results showed that the chronic administration of high-dose CORT also produced severe deficits in the performance of cognitive-function tests and decreased the ChAT and AchE activities in the hippocampus, implying neurodegeneration in the brain.

In the CORT-treated rats, reduced spatial cognitive abilities were associated with a significantly increase in plasma and cerebral CORT levels as compared to control group. In present study, we found that acupuncture at HT7 decreased the CORT release in plasma after chronic

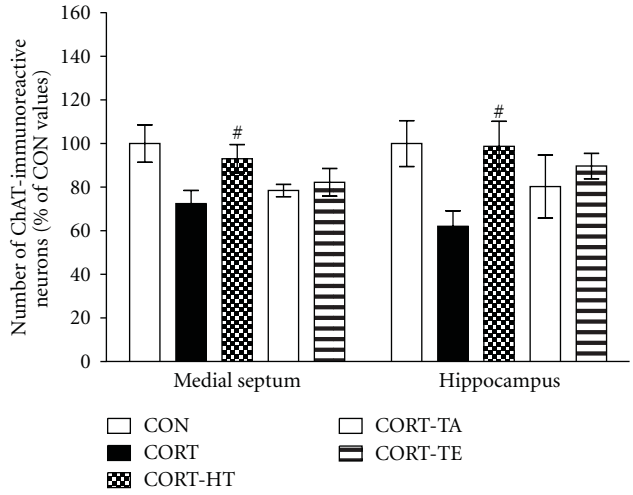

Figure 7: The percentage (±SE) values of the mean number of choline acetyltransferase- (ChAT-) stained septohippocampal cholinergic neurons after the Morris water maze task. Immunohistochemical data were analyzed via a separate one-way ANOVA followed by Tukey's *post hoc* test. $^{**}P < 0.01$ *versus* the CON group; $^{\#}P < 0.05$ *versus* the CORT group. Vertical bars indicate SE.

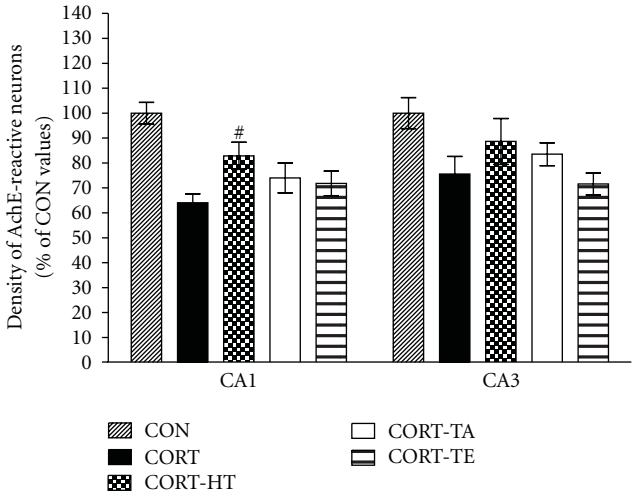

Figure 8: The percentage (±SE) values of the immune-staining density of acetylcholinesterase in different hippocampal areas after the Morris water maze task. Immunohistochemical data were analyzed via a separate one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P < 0.05$ and $^{***}P < 0.001$ *versus* the CON group; $^{\#}P < 0.05$ *versus* the CORT group. Vertical bars indicate SE.

CORT administration. Our findings may help to explain that stimulation at HT7 acupoint may affect the hippocampus to received biochemical and behavioral signals induced by reduced CORT level in plasma. Therefore, acupuncture at HT7 may modulate the dysregulation of HPA axis, which means acupuncture could influence secretion of CORT, thereby normalizing behavioral and neurochemical response. The effects of acupuncture stimulation might possess a relative specificity on acupoints. The correlation observed between brain cholinergic neurons and improved memory abilities were associated with interaction between CORT concentrations and acupuncture in the control of cognitive processes linked to cholinergic neurons. Accordingly, our results suggest that rewarding effect of acupuncture stimulation of HT7 on exogenous CORT-induced cognitive deficits might be the restoration of HPA axis hyperactivity through the decrease of endogenous CORT levels in the CNS, as noted in some studies [29, 30]. Also, cumulative analgesic effects of acupuncture of HT7 by affecting HPA axis activity were probably associated with regulating hypothalamic beta-endorphin [30].

To assess spatial learning and memory in rats, the MWM is more advantageous than other conventional mazes such as the T maze and the radial-arm maze. Training for spatial memory can be easily achieved after several acquisition trials, and the task does not require strong motivating traces or conditions such as scent, punishments, and food and water deprivation. The MWM is a hippocampus-dependent memory task, frequently used for demonstrating cognitive deficits and to examine permanent spatial learning capability and reference memory in rodents [31]. Animals encode spatial working information during the learning step, which serves to guide future memory retrieval. Our findings that the memory deficits, induced by chronic administration of CORT, produced impaired behavioral performance in the MWM are consistent with previous findings [19]. In previous studies, the hidden platform trials were designed mainly to measure acquisition of spatial memory, and the probe trial was used to evaluate retention.

In terms of average swim speed and rest time, which are indices of motor function, the CORT group was not significantly different from HT7-acupuncture group. This indicates that motor impairment was not the main cause of the poor performance of the CORT group in the MWM test. Accordingly, it is evident that acupuncture at the HT7 acupoint significantly improved MWM performance by enhancing spatial working memory.

An open-field test was also performed to rule out any confounding motor impairments, which can influence outcomes in many behavioral tests of depression. No significant individual differences in locomotor activities were observed between groups in the open-field test, suggesting that acupuncture stimulation to the HT7 acupoint had no effect on sensorimotor performance. Accordingly, the changes in behavioral performance in the MWM task were likely due to improved memory instead of differences in sensorimotor function, motor output, or limb flexibility.

Many reports have verified that sustained elevation of circulating GC concentrations produces a variety of cognitive deficits. For instance, rats exposed to daily CORT injections for 8 weeks demonstrated decreased spontaneous alteration in the T maze [18]. Likewise, a 21-day CORT implant that elicited a two- to fourfold increase in serum CORT level impaired acquisition of a passive-avoidance task in rats [32]. In addition, chronic CORT treatment significantly impaired both acquisition in the radial arm maze and accuracy of recall of spatial information in the MWM in rats [19]. In our study, it is likely that poor performance in the MWM task by

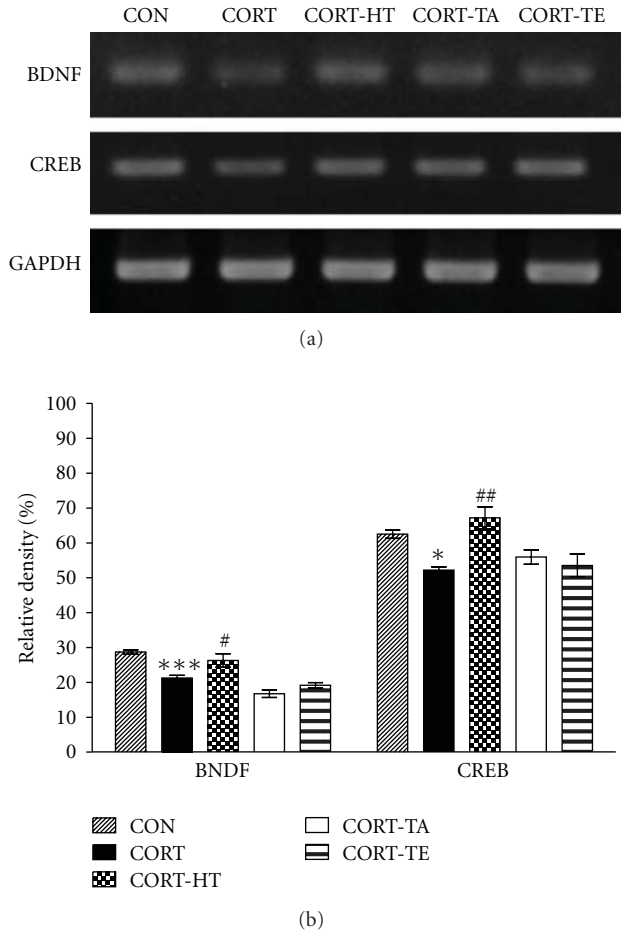

Figure 9: The PCR bands and their relative intensities for brain-derived neurotrophic factor (BDNF) and cAMP-response element-binding (CREB) protein in the hippocampus of rats that received chronic CORT administration. Data were analyzed via separate one-way ANOVAs followed by Tukey's *post hoc* test. $^{*}P < 0.05$ and $^{***}P < 0.001$ *versus* the CON group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* the CORT group. Vertical bars indicate SE.

the rats administered chronic CORT was also attributable to impaired memory [16].

Chronic CORT administration might increase circulating GC levels in the CA1 region of the rat hippocampus, resulting in impairment of long-term potentiation and spatial learning and memory in the MWM [33]. The beneficial effects of acupuncture on CORT-induced learning and memory deficits could be related to an increase in septohippocampal cholinergic function and prevention of degeneration in the cholinergic neuronal population of the septohippocampal fibers in mediating cognitive processes [34]. This effect results in enhanced cognitive performance and abrogation of memory deficit [13]. Several studies have demonstrated the changes in hippocampal neurochemistry in response to chronic administration of exogenous CORT [10, 35]. A prolonged administration of CORT or higher dose of CORT influenced reduction of cholinergic neurons in hippocampus [36]. For example, GC interfered with the storage and inactivation of ACh, via the upregulation of cholinesterase activity [37]. In addition, recent studies have shown that GC receptors on cortical neural stem cells can negatively affected neurogenesis with a subsequent unfavourable functional outcome in the cognitive abilities [38, 39]. Furthermore, deletion adjacent to glucocorticoid-responsive element (GRE) caused constitutive overexpression and anti-AchE hypersensitivity [40], suggesting a physiologically significant role for GC in regulating both neuronal AchE gene expression and anticholinesterase hypersensitivity. Therefore, our results have shown that chronic administration of exogenous CORT induced reduction AchE-stained neurons and ChAT activity in septohippocampal cholinergic neuron, which consistent with those of previous studies [41].

Cholinergic neurons originating in the medial septum (MS) project to the cortex and hippocampus, which play key roles in ACh-associated cognition [42]. Therefore, prolonged administration of CORT reduced spatial cognitive abilities and the number of hippocampal neurons, since the hippocampus received an extensive cholinergic input from the MS area [43]. In the present study, we found that exogenous CORT administration had a reduction in ChAT activity in the MS and hippocampus. The density of AchE-stained neurons in the hippocampal CA1 and CA3 regions was also significantly reduced after chronic CORT administration, which supports the notion of reduced septohippocampal cholinergic neurons. It is relevant to suggest that the reduction in cholinergic neuron loss after acupuncture treatment appears to be associated with improvement of learning and memory, since the CORT-HT group had greater cholinergic markers, such as ChAT and AchE, than the CORT group in the septohippocampal pathway.

The treatment of chronic CORT-induced memory disorders with acupuncture stimulation to the HT7 acupoint might enhances Ach recycling and efficient choline reutilization, and concomitantly induces increases in ChAT and AchE activities. AchE and ChAT belong to a family of enzymatic proteins that are extensively expressed in cholinergic neurons. ChAT is responsible for ACh biosynthesis and is required for cholinergic neurotransmission in the central and peripheral nervous systems. Acetylcholine is rapidly hydrolyzed by AchE; therefore, the duration of ACh action in the synaptic cleft is dependent upon AchE activity [44]. We demonstrated that acupuncture stimulation to the HT7 acupoint protected rats from spatial working memory deficits and attenuated the decrease in AchE and ChAT-immunoreactive neurons in the hippocampus, which is a particularly vulnerable region of the brain.

In the present study, we did not know precisely whether the acupuncture stimulation affects memory improvement through neurochemical changes of cholinergic system or first causes memory improvement leading to the neurochemical changes, because we did not have time-course data of Morris water maze task and cholinergic immunoreactivity in the brain hippocampus. However, CORT levels in the blood were almost restored to the normal level right after the acupuncture stimulation at HT-7 in the CORT-HT group despite little statistical significance (Figure 3). And also time to escape from hidden platform in the CORT-HT group

was getting shorter than other CORT-treated groups as the trials were repeated during the hidden platform training period (Figure 4(b)). These observations implied that the acupuncture stimulation at HT-7 first modulated brain cholinergic system and this effect subsequently elicited the behavioral consequences in the Morris water maze task.

In the acupuncture treatment against various memory-related disorders, the salvage capacity of ACh is enhanced. It also improves cholinergic neurons in the frontoparietal cortex and CA1 region of the hippocampus and continuously induces increases in ChAT and AchE activities, which eventually results in recovery of the entire cholinergic circulation pathway [45]. It is likely that the observed improvement in learning and memory in the HT7-acupuncture rats was associated with the attenuation of hippocampal cell loss.

We thus propose that chronic exposure to CORT triggers dysregulation of the HPA axis, which, in turn, elicits a reduction of ChAT and AchE expression in the hippocampus and eventually causes memory and cognitive decline in the rats. Also, recent studies have shown that chronic CORT-induced HPA axis hyperactivity or excessive increase of GC levels regulated expression of BDNF and CREB and impacted function of the BDNF pathway in the hippocampus [46]. This previous study strongly suggests a close correlation between the reduced expression of BDNF and CREB and HPA axis abnormalities in pathogenesis [46].

On the other hand, recent experimental evidence strongly supports the role of hippocampal BDNF in learning and memory processes, besides its actions on neuronal cell survival and prevention of neurodegeneration [47–49]. There is also sufficient evidence that the CREB regulates the expression of genes involved in neuroplasticity, cell survival, and cognition [50]. The phosphorylated CREBs are able to bind to cAMP response elements of the target genes considered to be involved in memory formation [51]. Thus, BDNF transcription, regulated by CREB, may also be a critical player in the adaptive neuronal responses underlying learning and memory function [52]. Several studies have suggested an association of hippocampal BDNF and CREB with memory performance, particularly in the water maze test [53]. The CORT-induced memory deficits led to a significant reduction of BDNF and CREB expression in the hippocampus, as well as poor performance in the learning and memory tests [17].

In oriental traditional medicine, acupuncture improves reversible malfunctions of the body via direct activation of various brain pathways, and thus contributes to the restoration of normal systemic balance, probably due to regulation of neurotransmitters including Ach [54]. Currently, acupuncture is a relevant therapy in complementary and alternative medicine for managing various cognitive disorders and psychosomatic diseases such as stress, depression, and anxiety [5, 55, 56]. Many recent studies also demonstrated that acupuncture stimulation reduced immobilization-stress-induced elevation of CORT levels and modulated HPA axis function in animals [3]. Acupuncture stimulation to the HT7 acupoint had potential effects on brain function in Alzheimer's disease patients, and ST36 (Zusanli) stimulation exerted a protective effect on cognitive impairment caused by cerebral multi-infarct dementia in rats [57]. The stimulation of acupoints such as Zusanli (ST36) and Xuehai (SP10) alleviated memory impairment induced by cerebral multi-infarction, as evaluated by shortened escape latency and increased swimming time in the target quadrant in rats [58]. These findings suggest that acupuncture stimulation can ameliorate memory-related performance in many behavior tests and modulate cholinergic neurons.

5. Conclusions

The present study demonstrated that memory and cognitive deficits induced by exogenous CORT-induced septohippocampal cholinergic neuron loss were closely related to the degeneration of cholinergic neurons in the rat hippocampus and that acupuncture stimulation to the HT7 acupoint significantly ameliorated learning and memory deficits through recovery of the ACh system. Acupuncture improved performance on the spatial memory test and protected septohippocampal cholinergic neurons from exogenous CORT-induced destruction. The attenuation of impairments of memory and cognition by acupuncture stimulation might be due to the restoration of cholinergic neurochemical abnormalities. It is most likely that acupuncture therapy is strongly effective in protecting against memory-related neuronal degeneration in the brain and retards the progression of memory defects in neurodegenerative diseases.

Acknowledgments

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Review Article

Acupuncture as Treatment of Hot Flashes and the Possible Role of Calcitonin Gene-Related Peptide

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The mechanisms behind hot flashes in menopausal women are not fully understood. The flashes in women are probably preceded by and actually initiated by a sudden downward shift in the set point for the core body temperature in the thermoregulatory center that is affected by sex steroids, β-endorphins, and other central neurotransmitters. Treatments that influence these factors may be expected to reduce hot flashes. Since therapy with sex steroids for hot flashes has appeared to cause a number of side effects and risks and women with hot flashes and breast cancer as well as men with prostate cancer and hot flashes are prevented from sex steroid therapy there is a great need for alternative therapies. Acupuncture affecting the opioid system has been suggested as an alternative treatment option for hot flashes in menopausal women and castrated men. The heat loss during hot flashes may be mediated by the potent vasodilator and sweat gland activator calcitonin gene-related peptide (CGRP) the concentration of which increases in plasma during flashes in menopausal women and, according to one study, in castrated men with flushes. There is also evidence for connections between the opioid system and the release of CGRP. In this paper we discuss acupuncture as a treatment alternative for hot flashes and the role of CGRP in this context.

1. Introduction

Hot flashes are classic menopausal symptoms in women [1, 2]. However, flashes are also reported by 43–77% of men after castration therapy [3–5], and they usually persist for many years and may impair quality of life [4]. Night sweats can be severe enough to cause sleep disturbance. In addition, hot flashes can be associated with anxiety, nausea, tachycardia, and tachypnea, as well as pressure in the head and chest. Hot flashes also occur in men with testicular insufficiency and in "normal aging men," but to a lesser extent [6, 7]. Nearly all reported flashes in women and men after castration therapy are also objectively confirmed by increased cutaneous blood flow, skin conductance, and/or skin-temperature [8–11].

The physiology of hot flashes is not known in detail, but probably involves the core body temperature, neuromodulators, and peripheral vasculature and sweat glands. Vasomotor symptoms have been shown to correlate with the decrease in estrogen production during the menopausal transition and with testosterone decreases after castration therapy with orchiectomy or GnRH Analogues in men with prostate cancer. Estrogens, testosterone, and also other hormones are potent neuromodulators of the central nervous system [12]. Freedman and Subramanian have demonstrated that women with hot flashes have an increased core temperature and a reduced thermoneutral zone compared with women without flashes [13]. It is possible that fluctuations in estrogen concentrations cause changes in other hormones or neurotransmitters in the central nervous system that lead to an alteration in core temperature and a narrower thermoneutral zone in women predisposed to hot flashes [14]. A narrow thermoneutral zone causes reactions that lower central body temperature when the actual central temperature reaches or exceeds the upper limit of the neutral zone. Such reactions are vasodilation causing increased blood flow in the upper trunk, arms, and hands and irradiation of heat, that is, energy

to the surrounding environment and activation of sweat glands, which leads to energy loss by evaporation. Another theory is that decreased endogenous estrogen concentrations also decrease hypothalamic β-endorphins that lead to an instability in the hypothalamic thermoregulatory centre [15, 16]. When the set point in the thermoregulatory center is suddenly lowered, reactions are initiated that decrease body temperature, acting in the same way as when the upper limit of the thermoneutral zone is exceeded. We suggest that testosterone plays a role in men similar to the role played by estrogen in women in this context and thus when testosterone concentrations are lowered as they are after castration therapy, thermoregulation becomes less stable.

The relationship between serotonin and temperature control has long been recognized. Probably both noradrenalin and serotonin may affect the risk of hot flushes via a narrowed thermoneutral zone [13]. Serum levels of serotonin are lower in postmenopausal women than the levels found before menopause, and estrogen therapy has been shown to normalize these levels. Estrogen withdrawal causes a reduction in circulating serotonin, resulting in an upregulation of the 5-HT_{2A} receptor in the hypothalamus [17]. It has thus been suggested that both the concentrations of β-endorphins and serotonin in the hypothalamus decrease with decreasing estrogen concentration [18]. The reduced β-endorphin and serotonin concentrations increase the release of noradrenaline, and this may in turn cause sudden drops in the set point in the thermoregulatory centre in the hypothalamus and elicit inappropriate heat loss [18–21]. According to this hypothesis, any intervention that increases estrogen, β-endorphin, or serotonin concentrations or decreases noradrenalin levels may be expected to reduce hot flashes.

The heat loss during the hot flash and sweating seems to be achieved by activation of cholinergic sweat glands and vasodilation of the skin, and these reactions may be mediated by the potent vasodilator calcitonin gene-related peptide (CGRP) [22]. Endogenous opioids modulate the release of the potent endothelium-dependent vasodilator CGRP at the spinal cord level [23, 24]. When CGRP is administrated intravenously to healthy male volunteers, it produces symptoms very similar to hot flashes, with a dose-dependent increase in cutaneous blood flow [25]. CGRP has been found to increase in plasma during hot flashes in postmenopausal women [26–29] and also, according to one study, in men with flashes who had been castrated due to carcinoma of the prostate [11]. Urinary excretion of CGRP over 24 h is higher in flashing postmenopausal women than in postmenopausal women without hot flashes, and in a group of postmenopausal women with hot flashes CGRP in 24 h urine decreased significantly after 12 weeks of successful treatment with acupuncture [30].

2. Treatment of Hot Flashes

The gold standard for treating hot flashes is estrogen therapy [12] which reduces the frequency and severity of hot flashes by 75% percent compared to placebo according to a meta-analysis from the Cochrane Database System [31]. Since hormonal treatment is at present controversial, largely as a result of results like those from the HERS study and the WHI study, there is a need for other nonhormonal treatment alternatives [32, 33]. Findings from these and other studies have led to more restrictive recommendations from the authorities on the use of estrogens and substantially decreased the use of hormone therapy. Therefore, many women today have climacteric symptoms including flashes but abstain from hormone replacement therapy. Furthermore, numerous women with breast cancer and men with prostate cancer have troublesome hot flashes but should not use sex steroid therapy because of the risk of cancer recurrence. As pointed out by, for example, Borrelli and Ernst, the potential serious side effects from hormone replacement therapy cause a great need for alternative and complementary treatments of hot flashes [34].

Progestagens have been shown to reduce hot flashes by 80–90% [35, 36], but their side effects include weight gain, fluid retention, and mastalgia [35] and should not be given to women with breast cancer.

Tibolone, a synthetic hormone that acts on sex hormone receptors, has also been shown to be as effective as estrogen therapy for treating hot flashes [37, 38]. Tibolone, however, should not be used in women who have had breast cancer because of the risk of recurrence and because it causes other side effects similar to those caused by estrogens [39].

Clonidine, selective serotonin reuptake inhibitors (SSRI), and gabapentin may decrease the frequency of hot flushes and the distress caused by them. The use of phytoestrogens and black cohorsh showed mixed results. The mechanism of action of SSRIs is thought to involve increased serotonin levels and thereby less severe vasomotor symptoms [40]. SSRIs reduce hot flashes by up to 50–60% compared to 80% reduction in women using estrogen [41]. However, recent data suggest that at least some SSRIs are associated with an increased risk of death from breast cancer, probably because SSRIs inhibit cytochrome P450 2DG (CYP2D6), which is necessary for the metabolism of tamoxifen, thus reducing the effect of the tamoxifen given to many women as part of treatment for breast cancer. Paroxetine is the one SSRI that is the strongest inhibitor of CYP2D6 and should therefore not be given to women with breast cancer [42].

Venlafaxine is the SSRI most frequently prescribed as an alternative to estrogens for the treatment of climacteric symptoms. In addition to its function as an SSRI, venlafaxine also acts as a noradrenergic reuptake inhibitor and is therefore called serotonin-noradrenaline reuptake inhibitor (SNRI). It is the most frequently prescribed alternative to estrogens [41] and halves the severity of hot flashes. Even if the effect is significant, it is only marginally better than the effect of placebo [43].

Lifestyle factors seem also to contribute to strengthening climacteric symptoms; greater BMI is a risk factor for hot flashes, as are smoking and high consumption of caffeine and alcohol [44]. Weight loss, regular exercise, and smoking cessation have also been recommended in order to decrease hot flashes. The efficacy of these recommendations has not

been demonstrated, however, because of lack of sufficient clinical trials according to the Cochrane Database System Review [12, 45]. Physically active postmenopausal women have a lower occurrence of vasomotor symptoms, perhaps due to a higher central opioid activity [46, 47]. Acupuncture also seems to be effective in reducing the intensity and frequency of hot flashes in women and in men deprived of sex steroids due to prostate cancer [22, 48–53].

3. Acupuncture

Acupuncture is known as one of the oldest healing systems in the world and is a part of traditional chinese medicine (TCM). Variants of TCM acupuncture, based on the old Taoist theories of Yin and Yang and Qi, are practiced throughout the Western World today [54]. The physiological processes involved in acupuncture treatment are not fully known, but factors of importance may include changes in autonomic nerve functioning [55–57] and may affect hormones such as cortisol [58, 59], oxytocin [60, 61], neuropeptides as β-endorphin [62], serotonin [63, 64], and cytokines [65–67] and alterations in collagen network communication [68, 69]. Acupuncture probably affects serotonin and noradrenalin activity in the central nervous system [70, 71], and thus has the potential to influence the thermoregulatory centre, making it more stable [22]. Acupuncture may also have peripheral effects and cause the release of substance P, vasoactive intestinal peptide, and CGRP [72–74]. Probably the effects of acupuncture are caused by multicomponent, complex interventions. In sham-controlled studies, attempts are frequently made to control the needling effect by controlling the location, insertion depth, stimulation, needle size, and number. However, several other potentially therapeutic acupuncture-specific components may be present in the control group; these include nonspecific components (time, attention, credibility, and expectation) and specific non-needling components such as psychological history, diagnosis, and education and also physiological events like palpation and moxibustion [75].

One of the traditional forms of acupuncture is manual acupuncture (MA), where the needles are inserted in the specific acupuncture points, according to TCM. Often the needles are twirled to evoke the DeQui sensation, characterised by a distinct sensation of distension and numbness [62]. The DeQui sensation is believed to activate A-delta fibers from free nerve endings in the skin or from high-threshold ergoreceptors in the muscle. Electroacupuncture (EA) is derived from MA, with the addition of electric stimulation applied to one or two pairs of the needles, either applied with a high (80–100 Hz) or low frequency (2 Hz). EA has been shown to be more powerful than MA in studies on pain treatment [76, 77]. This stimulation is believed to activate peripheral nerve endings, muscles, and also connective tissue. The nerve stimulation causes afferent signals, which increase, for example, central β-endorphins, and serotonin and probably also activate receptors [78–80].

3.1. Acupuncture and Hot Flashes. Acupuncture has been tried for hot flashes since it seems to increase central β-endorphin activity [62], which would as a result make thermoregulation more stable and in turn decrease vasomotor symptoms [81, 82]. Some studies have shown a decreased activity, measured by fMRI, in the amygdala and hypothalamus, when acupuncture is given [83]. It may be speculated that during an incident of hot flashes there is a high neuronal activity in the hypothalamus and that acupuncture may reduce this activity, perhaps mediated by increased β-endorphin release and decreased noradrenalin activity.

Acupuncture has been found to decrease the number of hot flashes by at least 50% but does not seem to be as effective as estrogen therapy [84]. EA has been associated with a decreased number and intensity of hot flashes in menopausal women, both with and without breast cancer [22, 49–52], and also in men treated by castration due to prostate cancer [48, 53]. While pharmacological studies often use "placebo pills" for treatment of the control groups, it has been more problematic to find a credible but still inert sham technique that may be used in acupuncture studies. Several devices have been tried [85, 86], but these methods do not seem to be totally without effect, probably because they cause neuronal stimulation attributable to local pressure on or beside acupuncture points and induce tactile neuronal stimulation [87, 88]. Earlier studies have shown a better effect of EA on pain in lateral epicondylalgia [77] and low back pain [76] than with acupuncture using superficial needle insertion (SNI). Therefore, Wyon et al. [22] randomised women with hot flashes to either EA or superficial needle insertion (SNI) in the belief that EA would have a superior effect on hot flashes compared to SNI. It was not, however, possible to see any differences in effect of treatment, although, in the EA group, the reduction of hot flashes was sustained for a longer period of time after treatment than in the SNI group. Frisk et al. [53] also randomised between EA and MA in men castrated due to prostate cancer, but they were unable to show any differences between the treatment options regarding reduction in number of hot flashes.

In a systematic review article [89], the effectiveness of acupuncture versus sham acupuncture for treatment of hot flashes was assessed. They found six randomised clinical studies of acupuncture versus sham acupuncture but were not able to show any differences between the effects on hot flashes of acupuncture versus sham acupuncture. They concluded, however, that the sample was too small and that different types of sham-acupuncture were used in the different studies [89].

It is of course of great interest and importance to find out if acupuncture would affect other climacteric symptoms than the hit flashes. There are, however, to our knowledge no studies on the effects of acupuncture on, for example, excessive sweating, anxiety, or other climacteric disorders as primary outcome.

4. Calcitonin Gene-Related Peptide (CGRP)

CGRP is a 37-amino-acid neuropeptide, found predominantly in sensory C and Aδ nerve fibers. It is a well-known

very potent vasodilator of the skin and microvasculature [90] and plays an important role in neurogenic vasodilation of the skin [91]. It can potentiate both acetylcholine-mediated vasodilation and sweating [92]. CGRP has cardiovascular effects, proinflammatory actions, and metabolic effects [93] and often coexists with other peptides in sensory afferents, for example, substance P (SP), cholecystokinin, and dynorphin. Studies indicate that CGRP possibly plays a role in the transmission of nociception in the rat spinal cord, but the exact interactions with other nociceptive neurotransmitters in the spinal cord, such as SP, glutamate, and opioids are unknown [94]. Neuropeptides in the skin are synthesised and released predominantly by a subpopulation of small unmyelinated afferent neurons (C-fibers) designated as C-polymodal nociceptors, which represent about 70% of all cutaneous C-fibres and, to a far smaller extent, by small myelinated Aδ-fibres [95]. Two forms of CGRP have thus far been isolated, CGRP-α and CGRP-β. CGRP-α occurs primarily in sensory neurons, whereas enteric neurons mainly contain CGRP-β. CGRP-α and CGRP-β are suggested to be regulated differently, and they probably act through different receptor subtypes [94]. Two receptor subtypes, CGRP1 and CGRP2, have been identified that are specific plasma membrane receptors. These are G-protein coupled and are able to activate adenylate cyclase and increase in intracellular cAMP that are sufficient to explain many of their effects [94, 96]. Other effects are NO dependent [97].

A wide distribution of CGRP messenger RNA, CGRP immunoreactive (IR) cell bodies, and nerve fibers is seen in the central nervous systems (CNS) of various species including the rat, cat, and human. CGRP-positive cells are also found in various autonomic ganglia but to a lesser extent in sympathetic principal neurones in the stellate and lumbar sympathetic ganglia. Some of the neurones, which contain both CGRP and vasoactive intestinal peptide (VIP), project to the sweat glands in rats [94, 98, 99].

CGRP fiber terminals are heavily concentrated in the dorsal horn of the rat spinal cord. The CGRP-containing axons are largely unmyelinated or small diameter myelinated fibres and constitute almost 30% of the primary afferent axons of the major afferent input to the superficial laminae of the dorsal horn [94]. It has been concluded that highly concentrated CGRP in nerve terminals is supplied by axonal transport from the neurone cell bodies [100].

4.1. CGRP and the Cardiovascular System. Microinjections of CGRP into the central nucleus of the amygdala elicited an increase in arterial blood pressure and heart rate in the rat [94]. In rats, low-intensity spinal cord stimulation induces cutaneous vasodilation that is possibly mediated by peripheral release of CGRP [101], which also increases the heart rate and force of contraction of the heart [94]. In humans, exogenously administered human α-CGRP showed vasodilatory action in the skin [25]. The vasodilation induced by CGRP may be achieved through more than one mechanism. In some tissues vasodilation correlates strongly with a rise in cAMP that is independent of nitric oxide (NO). In contrast, in other tissues (e.g., rat aorta), the effect is suggested to be NO-dependent via an NO-induced increase in cGMP [97]. In microvascular dermal endothelial cells, CGRP and SP have been shown to induce the release of NO [102]. K^+ channels in arterial smooth muscle cells of rabbits are sometimes involved in CGRP-mediated vasodilation [94]. Hence, CGRP can activate various transduction signalling pathways and the vasodilation involves multiple second messengers [94].

4.2. CGRP and Sweat Glands. In the eccrine sweat glands, Zancanaro et al. [103] have found immunoreactivity for CGRP in secretory cells, granulated cells, and to some extent parietal cells. Immunoactivity of CGRP has also been detected in human axons of sudomotor cholinergic nerves stimulating eccrine sweat glands [104] where vasoactive intestinal peptide (VIP) has been shown to coexist [99, 104]. It has previously been reported that CGRP and VIP exert an influence on human sweating under physiological conditions [101]. It was therefore suggested that CGRP-(and SP-) containing neurones are involved in the local vasodilation associated with increased sweat production [103]. Immunoreactivity for NO was seen in myoepithelial cells (i.e., contractile cells within the sweat glands). The presence of CGRP, SP, and NO suggests local function interactions involving NO release, myoepithelial cell contraction, and vasodilation in the sweat glands [103].

4.3. CGRP and the Thermoregulatory Center. CGRP, when injected into the hypothalamus, can increase body temperature [105–108]. A recent study has shown that it decreases the rate of firing of warm-sensitive neurons in the thermoregulatory center, leading to hyperthermia [109]. It is possible that both central and peripheral actions are relevant for hot flushes [110].

4.4. Relationship between Estrogens, CGRP, and the Opioid System. CGRP-IR fibers have been observed in the superficial layers of the spinal dorsal horn with lower numbers of fibers in the deeper laminae of the spinal cord [111], the same location where Blomqvist et al. [112] found colocalisation of estrogen receptor IR and preproenkephalin messenger RNA expression. Estrogen injected subcutaneously in ovariectomized female rats results in a rapid increase in spinal cord enkephalin mRNA levels [113]. This suggests that estrogen influences opioid/enkephalin expression in the lower medulla and spinal cord (preferentially in the superficial layers) and may thereby exert a modulatory effect on sensory and nociceptive processing directly at the spinal and medullary levels [112] especially of relevance when studying pain.

Endogenous and exogenous opioids modulate the release of the potent vasodilator CGRP at the spinal cord level [114, 115]. Gonadal hormones influence the endogenous opioid system [116]. In male castrated rats messenger RNA for the opioid precursor pro-opiomelanocortin is increased in the hypothalamus following testosterone and estradiol supplementation [117]. It has also been shown that there is a positive correlation between cerebrospinal β-endorphin concentrations and estrogen concentrations in plasma [118],

and estrogen affects hypothalamic β-endorphin activity in rats [119].

In ovariectomised rats treated with estrogen (implanted silastic capsule), CGRP immunoreactivity and methionine-enkephalin immunoreactivity increased in the medial preoptic nucleus and the periventricular preoptic nucleus of the hypothalamus [120]. These findings show a connection between estrogens, enkephalins, and CGRP in the central nervous system very close to the thermoregulatory center. Thus, estrogens may affect CGRP production and release both directly and indirectly via opioids.

5. Acupuncture and CGRP

Since CGRP, like other neuropeptides, has a short half-life in the circulation system [121] and is degraded by neutral endopeptidase, tryptase, and chymase [122–124], much of its action cannot be measured in the blood, because degradation products circulate in the blood. However, we suggest that 24-hour urinary measurement of CGRP is a more reliable measurement of total amount of CGRP released into the circulation. Wyon and coworkers found a higher 24 hour urinary excretion of CGRP in women with vasomotor symptoms compared to excretion observed three months later after successful acupuncture therapy [22]. Twenty-four-hour CGRP excretion in urine was higher in postmenopausal women with flashes than in postmenopausal women without flashes and in fertile women [30]. In contrast, in men treated by castration due to prostate cancer, no changes were seen in urinary 24-hour excretion of CGRP three months after castration compared to before castration, neither in the group as a whole nor in the men who developed hot flashes [125]. Men treated successfully for hot flushes with acupuncture did not display a change in their 24 h urine CGRP excretion statistically [53].

Borud and co-workers could not find changes in CGRP excretion during acupuncture therapy of hot flashes, but this study included no 24-hour measurements of CGRP and was therefore not really suitable to answer the question if acupuncture therapy for hot flashes affects CGRP [126].

6. Conclusions and Suggestions for the Future

The effect of acupuncture on hot flashes in women and men [22, 48–53, 84] is probably multifactorial, and placebo-controlled studies are difficult to achieve, since there are so many components to be controlled for [75]. In the studies on acupuncture and hot flashes, efforts have been made to control the needling components, but in most cases no difference is seen between treatment groups whereas the within-group changes are evident [22, 53]. According to the hypothesis of the mechanisms of hot flashes, any intervention that increases estrogen, endorphin, or serotonin concentrations or decreases noradrenalin activity may be expected to reduce hot flashes in menopausal women. Exogenous estrogen seems to have an effect on many systems [31], probably affecting all neurotransmitters involved. Alternative treatments for hot flashes may affect one or perhaps several but not all of the systems involved; that is, acupuncture affects the β-endorphin levels [62] and also affects serotonin and noradrenalin activity in the central nervous system [70, 71]. Theoretically, by combining different alternative treatments, for example, SSRI and acupuncture, a synergistic effect of these treatments would appear with a better effect on hot flashes than any single treatment alternative would have by itself—except from that induced by estrogen treatment. Randomised studies are required to investigate this.

Whether or not acupuncture has a direct effect on the release of CGRP in peripheral nerve endings remains to be investigated, and it is possible that other neurotransmitters, such as substance P, neurokinin A, neuropeptide Y, and adrenomedullin [110], are also involved in the pathogenesis of hot flashes. However, there is evidence that CGRP is involved in hot flashes in women and men with prostate cancer [11, 26–29], and a suggested treatment could therefore include a CGRP antagonist. There are a number of CGRP receptor antagonists in various stages of preclinical or clinical development, all of which are intended to treat acute episodes of migraine [127, 128]. Hopefully, these may become available in the future and tried as treatment alternatives for hot flashes.

Recently, accumulating neuroimaging studies of humans have shown that acupuncture can modulate a widely distributed brain network. For example, the hypothalamus presented saliently intermittent activations during an fMRI session, both during needling and a prolonged period thereafter [129]. These new techniques may in the future be able to measure activity during hot flashes and perhaps also make it possible to study neurotransmitters involved in this process.

What the real effect is of the needling component of acupuncture on hot flashes remains to be investigated. More randomised trials are needed if this is to be investigated satisfactorily, and in these not only the needling effect must be controlled for but also for the nonneedling components as well as for other nonspecific components as described by Langevin et al. [75].

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Brain-Modulated Effects of Auricular Acupressure on the Regulation of Autonomic Function in Healthy Volunteers

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Auricular acupuncture has been described in ancient China as well as Egypt, Greece, and Rome. At the end of the 1950s, ear acupuncture was further developed by the French physician Dr. Paul Nogier. The goal of this study was to develop a new system for ear acupressure (vibration stimulation) and to perform pilot investigations on the possible acute effects of vibration and manual ear acupressure on heart rate (HR), heart rate variability (HRV), pulse wave velocity (PWV), and the augmentation index (AIx) using new noninvasive recording methods. Investigations were performed in 14 healthy volunteers (mean age ± SD: 26.3±4.3 years; 9 females, 5 males) before, during, and after acupressure vibration and manual acupressure stimulation at the "heart" auricular acupuncture point. The results showed a significant decrease in HR ($P \leq 0.001$) and a significant increase in HRV total ($P = 0.008$) after manual ear acupressure. The PWV decreased markedly (yet insignificantly) whereas the AIx increased immediately after both methods of stimulation. The increase in the low-frequency band of HRV was mainly based on the intensification of the related mechanism of blood pressure regulation (10-s-rhythm). Further studies in Beijing using animal models and investigations in Graz using human subjects are already in progress.

1. Introduction

The Chinese have provided an ancient explanation of traditional acupuncture based on the principle of energy flow around the body in channels called meridians. This energy flow, called "Qi," can be out of balance. Inserting acupuncture needles or stimulating acupoints using acupressure can reestablish harmony.

Using technology from western medicine, one can clearly measure the effects of acupuncture and acupuncture-like stimulation in the brain and periphery [1–3]. Computer-based monitoring of heart rate (HR) and heart rate variability (HRV) as well as innovative pulse wave analysis allow for diagnosis and prognosis concerning the functional state of the arteries and the heart, which is modulated by different centres of the brain [4]. Pulse wave velocity (PWV) is widely recognised as a direct marker of arterial stiffness. The augmentation index (AIx) is used more often as a parameter of wave reflection [5, 6].

Auricular acupuncture has been described in ancient China as well as Egypt, Greece, and Rome [7]. At the end of the 1950s, ear acupuncture was further developed by the French physician Dr. Paul Nogier. He systematically demonstrated that different regions of the ear and specific organs have definite functional relationships and dependencies. Due to these relationships, needling and/or stimulation of one or more ear acupuncture point can be performed to treat specific organ functions. Ear acupuncture points are also relevant for diagnostics in the field of auricular medicine. According to Nogier, a change in skin resistance at certain areas of specific ear acupuncture points is present in particular organic diseases [8–11].

The goal of this study was to develop a new system for acupressure (vibration stimulation) and to perform pilot

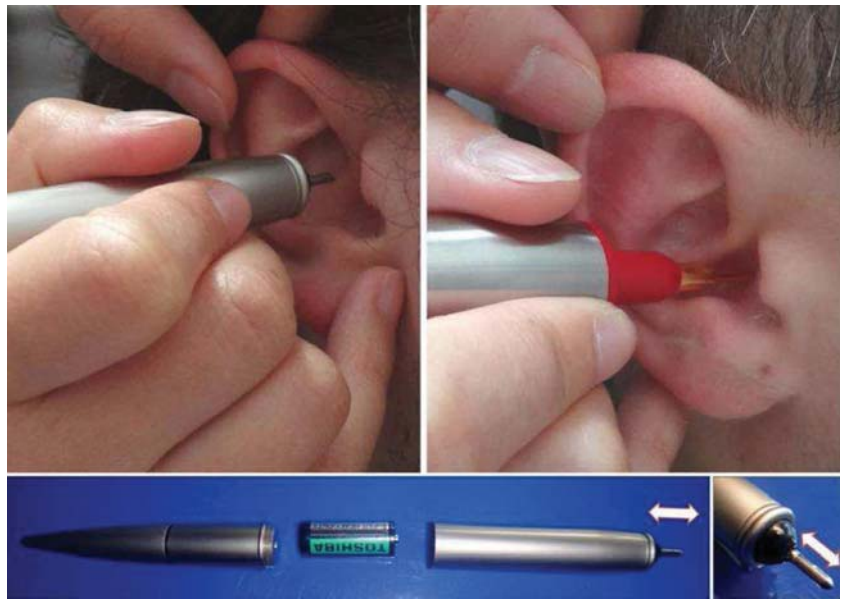

Figure 1: New instruments for acupressure (vibration) stimulation used in ear acupuncture at the "heart" acupoint.

investigations on possible acute effects of vibration and ear acupressure at the "heart" ear acupoint on HR, HRV, PWV and AIx in a group of healthy volunteers using new noninvasive recording methods.

2. Methods and Subjects

2.1. A New System for Ear Acupressure (Vibration Stimulation). Two different methods were used for stimulating the "heart" ear acupoint. The first method uses a pen with a special electronic device inside. With this pen, mechanical vibration stimuli can be administered at a frequency of about 30 Hz. The tip of the equipment is made of stainless steel (material no. 4301) and has a diameter of 2 mm and a length of 7 mm (see Figure 1, left and bottom). The vibration starts once the contact pressure reaches about 1 N (100 g bearing pressure). The second method uses a commercially available point locator (Biegler GmbH, Mauerbach, Austria) from which the battery has been removed in order to avoid acoustic stimuli (see Figure 1, right). This pen was used for manual acupressure (without vibration).

2.2. Recording Systems and Evaluation Parameters. An HRV medilog AR12 (Huntleigh Healthcare, Cardiff, UK, and Leupamed GmbH, Graz, Austria) system was used for electrocardiographic (ECG) monitoring. The system is designed for a monitoring period of more than 24 hours. The sampling rate of the recorder is 4096 samples per second. Therefore, R-waves can be detected accurately. All raw data are stored digitally on a 32-MB compact flash memory card. After removing the card from the portable systems, the data can be read by an appropriate card reader connected with a standard computer. The R-peak time resolution is 244 microseconds, and the P and T time resolution is 1,953 microseconds. The dimensions of the HRV recorder are $70 \times 100 \times 22$ millimeters, and the weight is about 95 grams with batteries [12, 13].

HRV is measured as the percentage of change in sequential chamber complexes called RR-intervals in the ECG. The registration of HRV is performed using three electrodes (Skintact Premier F-55; Leonhard Lang GmbH, Innsbruck, Austria) on the chest (cf. Figure 2). HRV can be quantified over time by registering the percentage changes in the RR-intervals in the time domain as well as the changes in the frequency range by analysis of the ECG power spectra. The HRV parameters are recommended by the task force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology [14]. With new software (Huntleigh Healthcare, Cardiff, UK), the HRV is analysed and displayed in a novel way to evaluate the function of the autonomic nervous system. The mean HR, the total HRV, and the LF (low frequency)/HF (high frequency) ratio of the HRV served as the evaluation parameters [14]. This innovative analysis demonstrates how well the human body reacts to acupuncture [12].

The methods for determining arterial stiffness and wave reflection parameters were noninvasive. The measurements were performed with a cuff applied to the brachial artery (cf. Figure 2). Arteriograph (TensioMed, Budapest, Hungary) is

Figure 2: The measurements took place in the lab of the TCM Research Center in Graz at the Medical University of Graz (with permission of all medical doctors and volunteers).

a new, noninvasive system that uses an entirely novel method to determine PWV and AIx. Signals can be detected from an upper arm cuff, even if it is overinflated by 35–40 mmHg beyond the systolic blood pressure, despite a completely closed brachial artery. For further explanations, see [5, 6].

AIx describes the influence of the reflected pulse wave on systolic pressure (in percent of blood pressure amplitude) [6]. PWV describes the stiffness of the aortic vascular wall. It is considered a direct measure of (aortic) arterial stiffness [6].

2.3. Volunteers, Acupuncture, and Procedure. Within this study, 14 healthy volunteers (9 females, 5 males) with a mean age ± standard deviation (SD) of 26.3 ± 4.3 years (range 19–34 years), a mean height of 169.9 ± 6.6 cm, and a mean weight of 63.4 ± 10.7 kg were investigated. The measurement profile and measurement times (a–d) are shown schematically before, during, and after ear vibration and ear acupressure stimulation in Figure 3.

None of the volunteers were taking any medication. All volunteers were informed about the nature of the investigation as far as the study design allowed. The study was approved by the local ethics committee, and all volunteers gave their written informed consent.

The volunteers laid on a bed in our lab (see Figure 2). Room temperature was kept constant at 25°C. Four measurement periods—one before ear vibration (a), one immediately after 30 sec ear vibration (b), one in a second control section (c), and one immediately after 30 sec manual acupressure using a special instrument (d)—were compared (see Figure 3).

Acupressure stimulation was performed at the "heart" acupoint. This acupoint is one of the most important ear acupuncture points and is commonly used in patients with hypertension [15]. The "heart" auricular acupuncture point is located in the middle of the ear cavity [15–18].

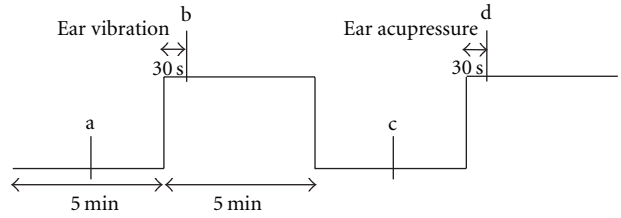

Figure 3: Measurement profile. Ear vibration and manual ear acupressure were performed for 30 sec each. The measurement points for wave reflection and arterial stiffness are indicated with a–d.

2.4. Statistical Analysis. Data were analysed using Friedman repeated measures ANOVA on ranks (SigmaPlot 11.0, Systat Software Inc., Chicago, Ill, USA). Post hoc analysis was performed with Tukey test. The level of significance was defined as $P < 0.05$.

3. Results

Figure 4 shows the mean HR and HRV total (total heart rate variability) from the ECG recordings during two control measurements (b and c) as well as during and after ear vibration at the "heart" ear acupoint (b) and manual ear acupuncture at the same point (d). There was a significant decrease of HR ($P \leq 0.001$) during both stimulation sections. At the same time, HRV total increased significantly ($P = 0.008$) only during manual ear acupressure; however, it also increased insignificantly during ear vibration.

Furthermore, the biosignal monitoring during acupressure (vibration) and manual acupressure showed substantial increases in the LF frequency band (Figure 5(b)). The LF/HF ratio alterations were insignificant (Figure 5(a)).

A typical example from the new software analysis is shown in Figure 6. In this person (27-year-old male subject),

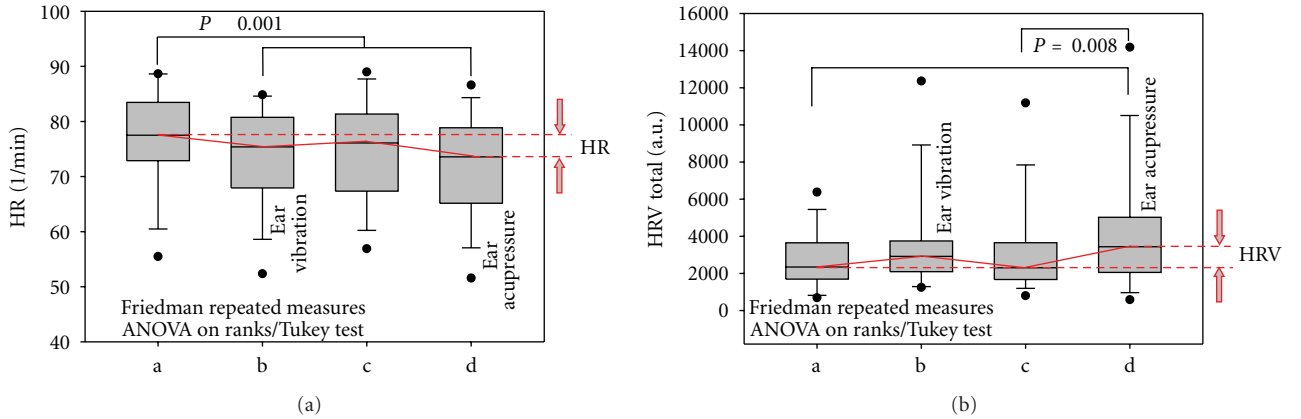

Figure 4: (a) mean heart rate (HR). (b) total heart rate variability (HRV total). Box plot illustrations of 14 healthy volunteers are shown. Note the significant differences. The ends of the boxes define the 25th and 75th percentiles with a line at the median and error bars defining the 10th and 90th percentiles. The different measurement phases and points (a–d) are indicated (cf Figure 3).

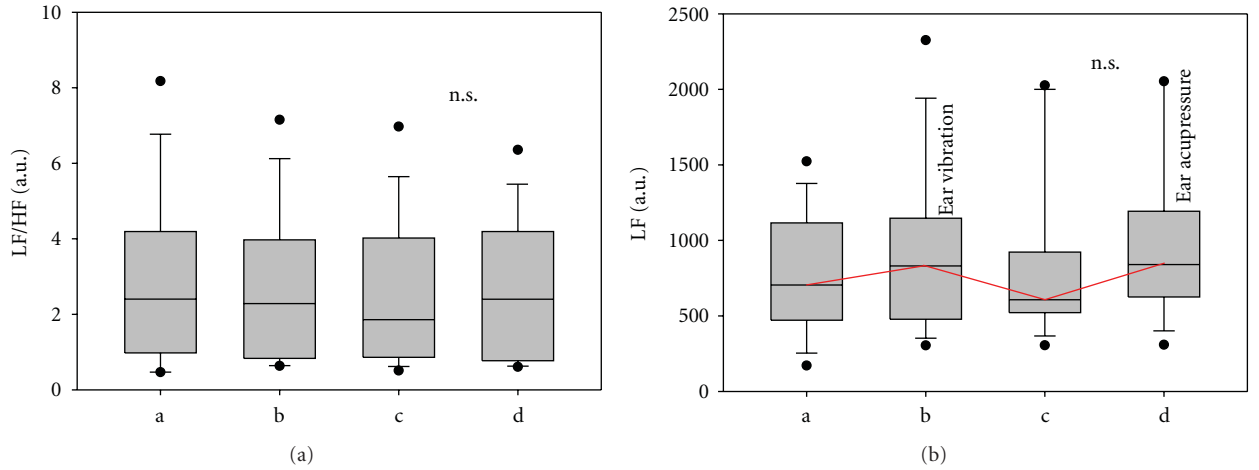

Figure 5: (a) LF (low frequency)/HF (high frequency) ratio. (b) the LF (low frequency) band of HRV. Note that the median of the LF parameter increases during ear acupressure vibration and during manual ear acupressure in 14 subjects. For further explanation, see Figures 3 and 4.

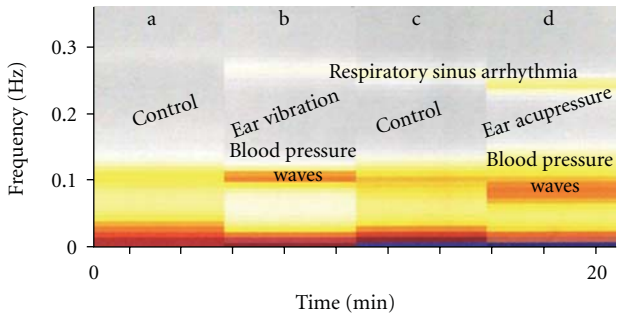

Figure 6: Frequency analysis of heart rate variability. Note the appearance of the influence of blood pressure modulation (~0.1 Hz) during "b" (ear acupressure vibration) and "c" (manual acupressure). For further explanation, see Figure 3.

strong influences of blood pressure waves (~0.1 Hz) appear in the frequency analysis of the HRV during and after the stimulation phases. Additionally, the influence of the respiratory sinus arrhythmia is demonstrated (~0.27 Hz). The analysis of the blood pressure is shown in Figure 7. No significant changes caused by stimulation were found.

Figure 8(a) summarises the preliminary results of the parameter AIx for the 14 participants in this pilot study. The AIx values increased during acupuncture vibration; however, statistical significance was not reached. The velocity of the pulse wave between the aortic root and the bifurcation of the aorta in m/s is demonstrated in the same Figure 8(b). There was a continuous insignificant decrease in PWVao (aortic pulse wave velocity) during the recording procedure.

4. Discussion

Auricular acupuncture is used for various autonomic disorders in clinical practice in western and eastern medicine. Recently, there is a growing focus on the important role

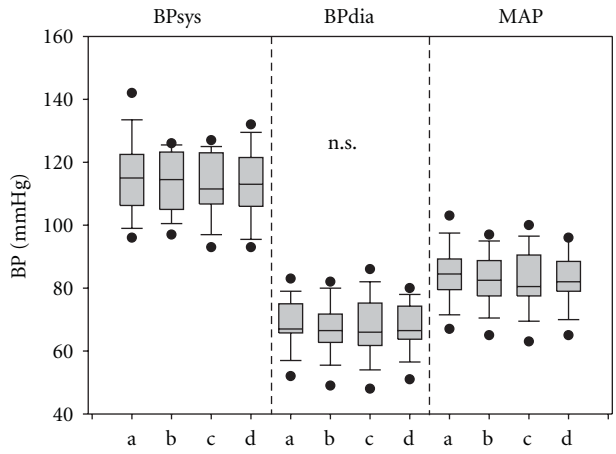

Figure 7: Systolic blood pressure (BPsys), diastolic blood pressure (BPdia), and mean arterial pressure (MAP) of the 14 healthy volunteers during the different phases (a–d). For further explanation of the box plots, see Figure 4.

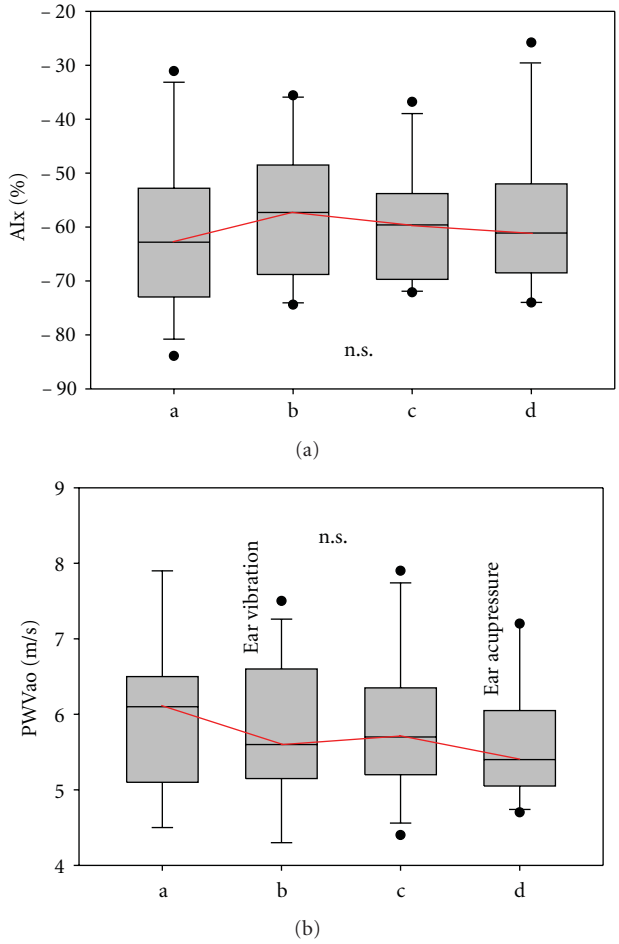

Figure 8: (a) brachial augmentation index (AIx) in %, describing the influence of the reflected pulse wave on systolic pressure (in percent of pulse pressure) of the 14 healthy volunteers during measurement phases a–d (see Figure 3). (b) PWVao (aortic pulse wave velocity in m/s) in the same subjects. For further explanation of the box plots, see Figure 4.

of the brain, and, therefore, there is also a need to explain how acupuncture and acupuncture-like stimulations affect the cerebral autonomic function. There is strong evidence from previous animal and human studies that acupuncture impacts the autonomic nervous system. There are two important publications from Gao et al. [17, 18] describing experiments in animal models. The first study [17] aims to examine the effects of acupuncture stimulation at different auricular areas on cardiovascular and gastric responses. In male anesthetised Sprague-Dawley rats, stimulation with manual acupuncture was performed. The authors found that the biggest depressor response was evoked from an area that corresponds to the "heart" stimulation point in humans that was used in our present investigation. The results from Gao et al. [17] also show that similar patterns of cardiovascular and gastric responses could be evoked by stimulation of different areas of the auricle. Their results do not support the theory of a highly specific functional map in the ear. Rather, there is a similar pattern of autonomic changes in response to auricular acupuncture with variable intensity depending on the area of stimulation [17]. Due to these previous results, we used two different active stimulation methods applied at the same acupoint, and we did not perform acupressure at a control point localised closed to the stimulation area.

The second study from Gao et al. was published recently in 2011 in Brain Research [18] and, as already mentioned above, showed that auricular acupuncture induces cardiovascular inhibition, increases the response of cardiac-related neurons in the nucleus tractus solitaries, and evokes cardiovascular inhibition through the baroreceptor reflex mechanism. Acupuncture-like stimulation was repeated in 58 male Sprague-Dawley rats in the area of the "heart" auricular point. In contrast to our investigation in humans, the authors of this study recorded invasive arterial pressure and HR to detect the cardiovascular response induced by auricular acupuncture. They could clearly show that acupuncture at the "heart" auricular point regulates cardiovascular function by activating the cardiac-related and depressor neurons in the nucleus tractus solitaries in a manner similar to the baroreceptor reflex in cardiovascular inhibition [18].

Experimental studies concerning ear acupuncture-like stimulation at the "heart" acupoint in humans are rare. In a Chinese study, the authors investigated 30 patients with hypertension. A comparison of the hypotensive short-term effects between the "heart" ear point and another point of ear needling showed that there was a marked hypotensive effect associated with stimulation of the "heart" point [15].

In 1993, Zhou [16] investigated the effect of auriculoacupuncture plus needle embedding in the "heart" point on left cardiac, humoral, and endocrine function. Twelve patients with heart failure complicated by dilating cardiomyopathy were divided randomly into an auriculoacupuncture group ($n = 7$) and controls ($n = 5$). Left cardiac function and plasma levels were measured. The results of that study indicated that auriculoacupuncture plus needle-embedding in the "heart" acupoint could improve the left cardiac

function in patients with heart failure complicated by dilating cardiomyopathy and that the function of an acupoint is distinctly different from that of a nonacupoint [16].

Concerning our present study, it is important to point out that the RR-intervals in the ECG are controlled by the blood pressure control system, which is influenced by the hypothalamus and, in particular, by the vagal cardiovascular centre in the lower brainstem [6, 14]. Calculation of the ECG power spectra is thought to provide an understanding of the effects of the sympathetic and parasympathetic systems on HRV. Some of the frequency bands in the spectrum of the HRV could be interpreted as markers of physiological relevance. Several of the associated mechanisms are thermoregulatory, which can be found in the very low frequency band, blood pressure and respiratory effects [6, 14]. The following influences can be distinguished for different ranges of HRV: (a) respiratory sinus arrhythmia (approx. 0.15–0.5 Hz), including central nervous system respiratory impulses and interactions with pulmonary afferents; (b) the so-called "10-s-rhythm" (approx. 0.05–0.15 Hz), which describes the natural rhythm of active cardiovascular neurons in the lower brainstem (the circulatory centre and its modulation by feedback with natural vasomotor rhythms via baroreceptor feedback); (c) longer wave HRV-rhythms (approx. <0.05 Hz), such as effects from the renin angiotensin system and temperature regulation as well as metabolic processes [4, 14].

In the present study, HR decreased significantly and HRV total increased during both ear acupressure and ear vibration (compare Figure 4(b)). Manual acupressure had more of an effect on HRV than the application of vibration stimuli. The analysis of the LF frequency band also showed a marked increase during stimulation. This increase is mainly based on the intensification of the related mechanism of the blood pressure regulation (10-s-rhythm). Figure 6 clearly demonstrates the appearance of this influence.

The present study also includes a new application of the innovative oscillometric technique for measuring arterial stiffness in the field of acupuncture. As of May 2011, only six scientific articles concerning "arterial stiffness and acupuncture," "wave reflection and acupuncture," and "pulse wave velocity and acupuncture" could be found in the scientific literature [6, 19–23].

Scientists from Austria, China, Japan, Mexico, and Taiwan (alphabetical order) performed these studies.

The PWV and AIx increase in somewhat different ways in parallel with the aging process, and they provide different information regarding the arterial vascular status [23, 24]. Both parameters provide extensive information on the arterial vascular system, and the prognostic significance of arterial stiffness is expected to be high [5]. The results from our pilot study conducted in 14 healthy volunteers regarding the acute effects of auricular acupressure on human arterial stiffness and wave reflection showed a minor and insignificant increase in the brachial AIx after acupressure vibration (see Figure 8(a)) and a decrease in the aortic PWV immediately after acupressure vibration or manual acupressure stimulation (see Figure 8(b)).

However, there are some limitations of this pilot study. The number of subjects was small ($n = 14$), and there was no control group with a control acupuncture point. As mentioned at the beginning of the discussion section, previous results from a study by Gao et al. [17] showed that it is difficult to identify an ear placebo point for such investigations Therefore, based on the results of this pilot study and of other previous studies [6, 19–23], we intend to conduct a larger study to confirm or refute these preliminary findings. Our hypothesis is that ear acupressure (manual acupressure or acupressure vibration stimulation) can influence the autonomic nervous system. We believe that it is possible that these ear stimulation methods may cause measurable, reproducible physiological alterations, especially of HR, HRV, and blood pressure, as well as changes in the parameters of human arterial stiffness and wave reflection. These latter responses have only been used in two studies [6, 19] on needle body acupuncture. The present study is the first investigation of noninvasive parameters in humans using ear acupuncture-like stimulation. Therefore, further investigations are necessary. With reference to the present study, differences between the effects of needle acupuncture and acupressure on the parameters mentioned above also a matter of future research.

Ear acupuncture has been used for medical treatment for thousands of years. A large amount of empirical data is available, but the quantification of the effects on the brain and the periphery has not previously been possible. Using modern biomedical techniques, changes in vital parameters can now be quantified in a noninvasive way. Modernisation of acupuncture at the Medical University of Graz [1–4, 6, 25–39] has been achieved, and research on this topic is underway.

5. Conclusion

The following conclusion can be drawn from the results of this study: HR decreases and HRV total increases significantly during ear acupressure and/or ear acupressure vibration. The velocity of the pulse wave between the aortic root and the bifurcation of the aorta decreases markedly (yet insignificantly), whereas the augmentation index increases immediately after acupressure vibration and manual acupressure at the "heart" auricular acupoint. Our hypothesis as stated in the Discussion will require future investigation for verification.

Acknowledgments

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and Moxibustion" on May 31th, 2011 in Beijing organised by the China Academy of Chinese Medical Sciences Beijing and the TCM Research Center at the Medical University of Graz. X.-Y., Gao, L. Wang, and G. Litscher contributed equally in this paper.

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Research Article

Electroacupuncture at PC6 (Neiguan) Improves Extracellular Signal-Regulated Kinase Signaling Pathways Through the Regulation of Neuroendocrine Cytokines in Myocardial Hypertrophic Rats

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Electroacupuncture (EA) therapy has been widely accepted as a useful therapeutic technique with low or no risk in the clinical prevention of cardiac hypertrophy. However, the signaling transduction mechanism underlying this effect remains unclear. The current study investigates the effects of EA on the signaling pathways of myocardial hypertrophy (MH) in rats. Up to 40 3-month-old Sprague-Dawley (SD) rats were randomly divided into normal, model, PC6 (Neiguan), and LI4 (Hegu) groups, with ten rats in each group. All the rats except for the normal group received 3 mg/kg·d of isoprinosine hydrochloride (ISO) injection into the back skin. The rats in the PC6 and LI4 groups received EA for 14 days. On the 15th day, electrocardiograms were recorded, and the ultrastructure of the myocardial cells was observed. The myocardial hypertrophy indices (MHIs), electrocardiograph (ECG), ultrastructure observation, levels of plasma angiotensin II (Ang II) and endothelin (ET), as well as protein expression of extracellular signal-regulated kinase (ERK), and phosphorylation extracellular signal regulating kinase (p-ERK) in the left ventricular myocardial tissue were measured. The results indicated that EA can improve cardiac function in MH rats by modulating upstream neuroendocrine cytokines that regulate the ERK signaling pathways.

1. Introduction

In long-lasting pathologic emergency cases, myocardial hypertrophy (MH) may lead to coronary heart disease, congestive heart failure, stroke, and so on and can easily cause patient death or sudden death [1]. On a cellular and molecular level, the pathogenesis of MH may divide into three links, namely, extracellular hypertrophic stimulation, intracellular signal transduction, and intranuclear gene transcriptional activation, and eventually trigger hypertrophic myocardial cell changes. The intracellular signaling pathway is the coupling link of exocellular stimulus and nuclear gene activation. Thus, this signaling pathway has been proven as a sally port for researching the pathogenesis of MH, with extensive prospects for its application [2].

Angiotensin II (Ang II) and endothelin (ET) play a central part in MH, myofibrosis cordis, and cardiovascular reconstruction process. Lines of evidence showed that Ang II could enhance the expression of immediate early gene IEG (c-fos, c-jun, c-myc, etc.), terminal myocardial cell genes (skeleton actin, atrial natriuretic polypeptide), and transforming growth factor β gene in rat myocardial cells, confirming that the upregulation of these genes induces ventricular hypertrophy in the rat model [3]. Borges et al. [4] revealed that Ang II could facilitate MH and cardiovascular reconstruction, depending mainly on local secretion and release. ET is polypeptide that causes vasoconstriction, with a slow, but long-lasting and wide effect [5]. ET-1 causes strong coronary arterial constriction and has positive chronotropic action and positive inotropic action; therefore, it plays an important part in myocardial infarction [6]. These two neuroendocrine cytokines play an essential role in MH and act as upstream factors of extracellular signal-regulated kinase (ERK) signaling pathways [7].

ERK plays a key role in MH reaction, including gene expression and protein synthesis increase [8], which is not only referred to myocardial signal transduction and growth, but also closely related to MH myocytes and apoptosis [9]. Recent animal studies indicate that ERK activation is a key factor in regulating cardiac hypertrophy [10]. As we know, ERK signaling pathways mediate extensive biological effects [11], involving cell proliferation, cell apoptosis, inflammatory responses, oxidative stress, and even influences of the form and evolution of tumors. Research has confirmed that ERK1/2 is closely related to MH and that ERK is activated through ERK cascade (Ras/Raf/MEK/ERK) [12]. Therefore, the ERK signaling pathway was chosen as the research pointcut to explore the influence of electroacupuncture (EA) on MH, as well as to provide evidence from basic experiments to clinical research.

Acupuncture, undeniably the most well-known complementary and alternative medical treatment, has been proven as one of the most popular therapies in the world. Multiple researches confirmed that acupuncture could play a stable hypotensive effect achieve improvement or normalization of contractile function and diastolic values, a decrease of energy loss, and reversal of MH [13]. It has been well documented that acupuncture can effectively improve symptoms of angina, palpitation, and so forth. and improve the left cardiac function in coronary heart disease patients to inhibit MH [14], but the signaling pathways mechanism of the remarkable transformation caused by the treatment remains unknown.

Neiguan (PC6), a classical and experimental acupuncture point, has been recorded in ancient Chinese medical literature for thousands years and is preferred effectively in treating cardiovascular disorders. We learned that acupuncture had a more positive effect in increasing the glucose metabolic level in a stroke-injured area in the brain than nonacupoint stimulus and blank controls. In the present study, a real acupoint Hegu (LI4) located near PC6 was selected as the control point to compare its therapeutic differences with PC6.

Acupuncture indeed improves the cardiac function in MH, and this effect may have a close relation with ERK signaling pathway through regulating the role of neuroendocrine cytokines. The aim of the current study is to investigate whether EA could mediate ERK signaling pathway through regulating Ang II and ET. Two acupoints, PC6 and LI4, were also compared to observe the distinctive difference between them.

2. Materials and Methods

2.1. Animals and Model. Female Sprague-Dawley rats, 3-month-old, weighing 170–180 g, were randomly divided into four groups: normal, model, PC6, and LI4 groups, with ten rats in each group. The rats received free food and water under a controlled temperature (24 ± 1°C), with 12 hours of artificial light per day.

Following the method by Yin et al. [15], rats were continuously injected every morning with 3 mg/kg of isoprinosine hydrochloride (ISO) into the back skin for 14 days, while rats in normal group were treated with an equal volume of physiologic saline, and rats were observed for behavior. The experimental procedures were carried out in accordance with the guidelines for the care and use of Laboratory Animals published by National Institutes of Health of the United States.

2.2. EA. The PC6 rats were given improvised clothing and subjected to acupuncture on PC6 after an injection of ISO. The LI4 rats were treated in the same way, except that the acupoint was instead of LI4. The acupoints were referred from the "Map of the Experimental Animal Acupuncture Points," formulated by the Experimental Acupuncture Institute of China Association of Acupuncture and Moxibustion. The acupuncture needle, 15 mm long and 0.3 mm in diameter, was penetrated 2-3 mm into the subcutis. The bilateral acupoints were interconnected with a Hans acupoint nerve stimulator (HANS) EA apparatus, and the following stimulus parameters were selected: continuous-wave at 2 Hz and 1 mA, for 20 min a day.

2.3. Electrocardiograph (ECG) Recording Electrode Resettlement. A BL-420 physiological function experiment system (Chengdu TME Technology Co, Ltd, China) was used to record the standard II-lead ECG. Negative and positive stainless steel electrodes were placed horizontally beneath the skin of the left and right forelimbs, and the reference electrode was placed beneath the skin of the right hind limb. Recording was performed at a resolution ratio of 500 nv/mv and a chart speed of 50 ms/div; 10 cardiac cycles were included into the calculation.

2.4. Ultrastructure Observation. The central ventricular muscle of the left ventricular free wall (LVFW) was chopped into 1 mm × 1 mm × 1 mm pieces, fixed in 2.5% glutaraldehyde for 48 h and 1% osmium tetrachloride for 1.5 h, dehydrated, embedded, sectioned, and examined under a Hitachi H-600 transmission electron microscopy (Japan's Hitachi Company, Japan).

2.5. Myocardial Hypertrophy Index (MHI). The body weights of the rats, as well as those of their left ventricle and the whole heart, were recorded; then their MHI, left ventricular weight index (LVWI), and the heart weight index (HWI) were calculated.

2.6. Radioimmunoassay (RIA) Method. The plasma levels of Ang II and ET were determined with RIA in a full-automatic Gc-911 RIA counter (Zhong Jia Science and Technology Industry Company, China). At the end of the experiment, rats were executed, blood was collected, and plasma was frozen and stored until assayed. Plasma Ang II and ET activity was detected with an intrarun coefficient of variation under 10%; the interrun coefficient of variation under 15% (Beijing North Institute of Biological Technology, China). For analysis, we get log10-dose reference system into use.

2.7. Western Blotting. The expression of ERK and phosphorylation extracellular signal regulating kinase (p-ERK) in left ventricle tissue was measured with western blotting. About 100 mg left ventricular myocardial tissues was collected after being departed from heart on ice. The tissues were homogenized in a tissue lysis buffer as turn into lysates. Then the lysates were centrifuged at 12,000 g for 10 minutes, and the supernatant was transferred into another tube to be tested. Protein concentrations were determined using the BCA protein assay (Pierce). Equivalent amounts of protein (30 μg/lane) were resolved electrophoretically by SDS-polyacrylamide gels (10%) and transferred onto PVDF membranes. Nonspecific reactivity was blocked in 5% skim milk in PBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween-20) for 1 h at 6–8°C. Afterwards, membranes were incubated with ERK and P-ERK antibodies (1 : 200; Santa Cruz, Calif, USA) overnight at 4°C, and followed by secondary antibodies for 1 hour at room temperature. Protein was visualized by using the enhanced chemiluminescence system (ECL, Beyotime Institute of Biotechnology, Jiangsu, China).

2.8. Statistical Analysis. Data were expressed as mean ± SD and analyzed with one-way ANOVA using SPSS13.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. EA Modulated ECG after ISO Injection on Rats. Rats were observed for behavior, strong heartbeat, and tachypnea (above the standard 66–114 c.p.m., some of them could reach 150 c.p.m.) at 10 min after drug injection, and burnout sleepiness and weakness at 30 min after drug injection. ECG-R-R and ECG-ST were subjected to analysis (Figure 1). Compared with those in the normal group, the R-R interval and ST segment were significantly higher in the model group ($P < 0.05$). These two parameters tend to be normal in the PC6 and LI4 groups compared with the model group ($P < 0.05$). However, there was no statistically significant difference between the two EA groups ($P > 0.05$).

3.2. Effect of EA on Cardiac Cell Ultrastructure. MH showed a series of structural changes, including cardiomyocyte hypertrophy, interstitial connective tissue hyperplasia, and coronary circulation capillaries decrease [16]. In the current study, the rats in the model group mainly manifested six differences from the normal group (Figure 2), including mitochondria swelling, cell apoptosis, endothelial cell interstitial hyperplasia, muscle plasma nets expansion, outsync contraction of contraction band, and intercalated disc deformation. With reference to normal group, compared EA group with model group, EA significantly reduced cardiac muscular tissue injury.

3.3. EA Treatment Downregulated MHI. LVWI and HWI can reflex the MH level, providing an indication of the level of damage and the restoration of cardiac function. Compared with the normal group, the LVWIs and HWIs in the model group were significantly higher ($P < 0.01$). Compared with the model group, the two parameters were lower in the EA groups ($P < 0.05$). In addition, those in the LI4 group were superior to those in the PC6 group ($P < 0.05$), as shown in Figure 3.

3.4. EA Therapy Decreased Two Vasoconstrictors (Ang II and ET). Figure 4 shows that the Ang II and ET levels in the model group were significantly higher than in the normal group ($P < 0.05$) whereas these two parameters in EA groups were lower than those in the model group ($P < 0.05$). However, there was no statistically significant difference between these two EA groups ($P > 0.05$), as shown in Figure 4.

3.5. Effect of EA Downregulated ERK and p-ERK Protein Expression. The expression of ERK and p-ERK in the model group were significantly higher than that in the normal group ($P < 0.05$), whereas these two parameters in EA groups were lower than in the model group ($P < 0.05$). However, there was no statistically significant difference between the two EA groups ($P > 0.05$), as shown in Figure 5.

4. Discussion

The World Health Organization lists approximately 40 diseases wherein acupuncture treatment is effective [17]. Modern research has extensively analyzed the beneficial effects of acupuncture on the cardiovascular system [18]. Growing evidences showed that acupuncture effect is closely related to neural mechanism, and it has been well documented that PC6 can effectively improve symptoms of postoperative nausea, vomiting [19], labor pain [20], and obese diabetic [21] via nervous system. To the best of our knowledge, the current study is the first time to report that EA can improve MH by regulating the ERK signaling pathways through adjusting upstream neuroendocrine cytokines (Ang II and ET). The present study is based on neural endocrine system to discuss the role of EA on MH.

As classical and preferred acupoints in the long history of China, the "song of eight major points (Ba Zong Xue Ge)" [22] states that PC6 and LI4 as "for heart and chest, it is point Neiguan, for face and mouth, Hegu controls," which can be combined with clinical practice. Generally, the use of PC6 in acupuncture is advisable for the treatment of symptoms of heart and chest diseases, such as palpitation, stuffy chest, angina, nausea, and vomiting. The use of LI4 in acupuncture is also advisable for the treatment of symptoms of such diseases as toothache, facial pain, headache, nasal obstruction, and eye redness. PC6 is often preferred over LI4 for its improved efficacy in treating cardiovascular diseases. However, the current study found that LI4 could have a better effect than PC6, especially with statistically significant difference in the MHI experiment. These findings may be different from those of previous researches. In recent studies, acupuncture induced a significant decrease in the LF/HF ratio and a significant increase in HF power; manual acupuncture at both LI4 and SP6 (Point Sanyinjiao)

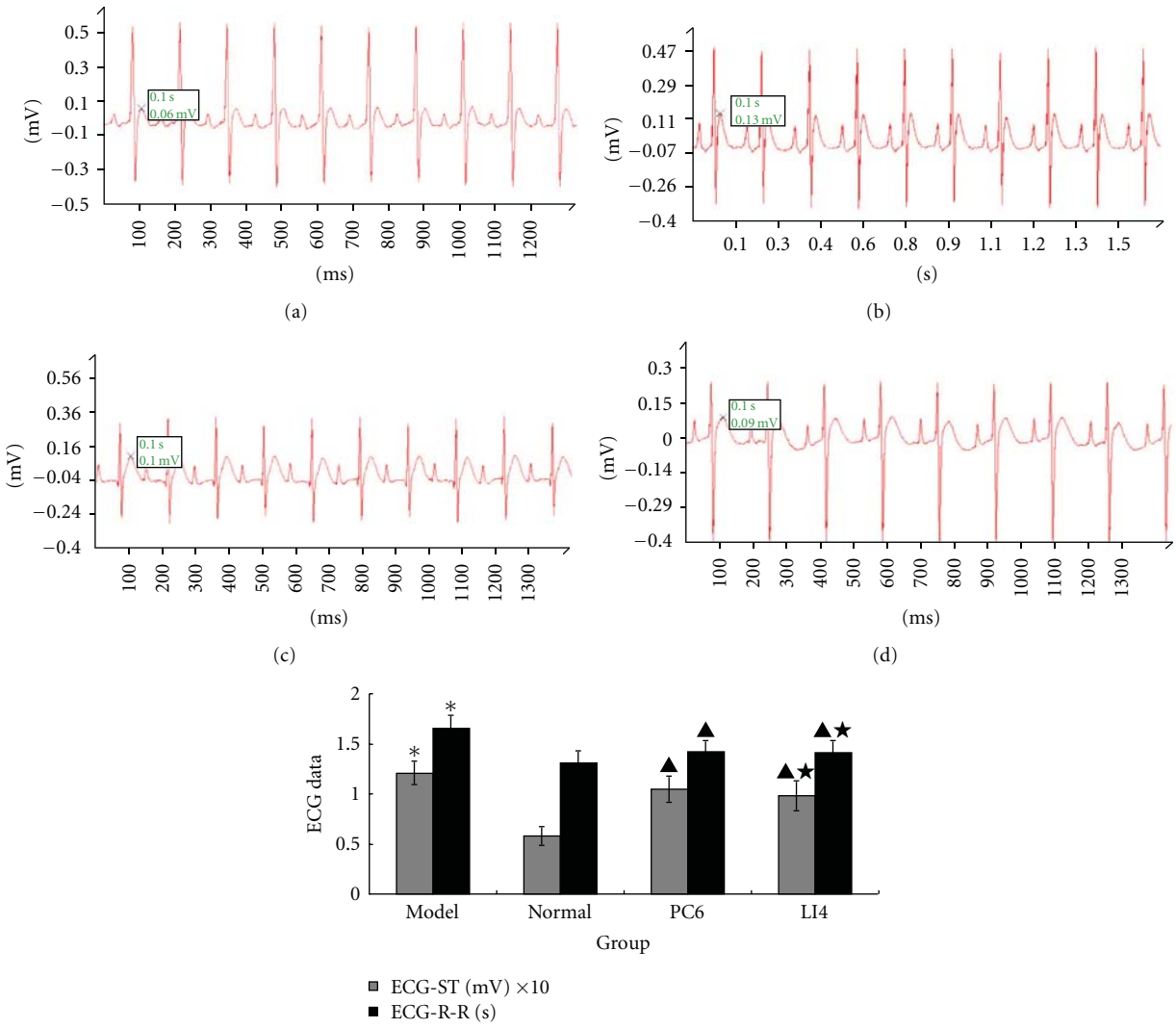

Figure 1: Comparison of the ECG-R-R and ECG-ST in rats for each group ($n = 10$ each group). ECG images are examples of four groups, involving 10 cardiac cycles in each figure. We can find out the changes in these pictures, normal group example ((a), R-R interval is 1.31 ms and ST amplitude is 0.06 mV), model group example ((b), R-R interval is 1.68 ms, and ST amplitude is 0.13 mV), PC6 group example ((c), R-R interval is 1.42 ms, and ST amplitude is 0.1 mV) and LI4 group example ((d), R-R interval is 1.42 ms, and ST amplitude is 0.09 mV). R-R interval prolonged and ST amplitude heightened in (b) while these two values diminished in (c, d). $^{*}P < 0.05$ versus normal; $^{\blacktriangle}P < 0.05$ versus model; $^{\bigstar}P > 0.05$ versus PC6.

acupoints may play a role in the treatment of dysmenorrhea with autonomic nervous system involvement [23]. Li et al. [24] also found that acupuncture on LI4 or PC6 could regulate heart rate. This may provide exact evidence that LI4 is useful in regulating the heart rate. In addition, study of Nayak et al. suggested that electrostimulation application by point surface electrodes at LI4, ST36 (Zusanli), HT7 (Shenmen), and LR3 (Taichong) points performed sedation of critically ill patients in the intensive care unit [25] thus, it can be suggested that LI4 application in painful diseases is more extensive than PC6. However, this unexpected result was only observed once, and further evidence was not obtained in the current study. Hence, this will be investigated further in future studies.

In the following respect, this study stimulated the acupoints with low-current and low-frequency (1 mA, 2 Hz) EA. Recent research showed that low-frequency (2 Hz) EA activated many more somatic afferents than high-frequency stimulation such as 10 and 20 Hz. Ten minutes of stimulation by 2 Hz EA on healthy volunteers can evaluate in terms of heart rate variability, pulse rate variability, and skin conductance response [26]. Similarly, thirty minutes of low-current, low-frequency (0.3–0.5 mA, 2 Hz) significantly inhibited the gastric-cardiovascular pressor reflex, whereas a similar period of EA at 40 or 100 Hz did not alter the response [27]. Current topic was designed to discuss brain stem responses to different frequencies at pericardial acupoints located over the median nerve, and researchers

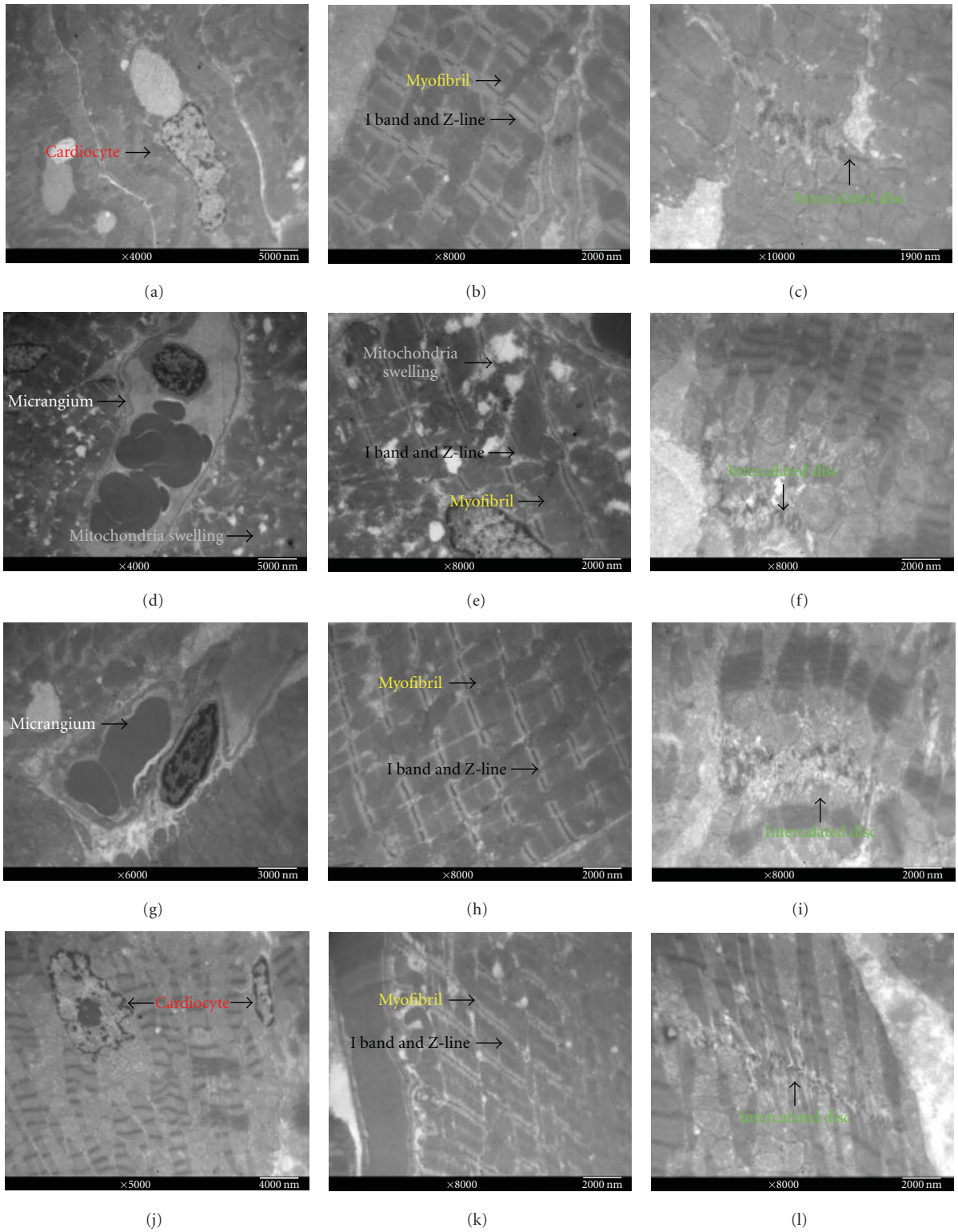

Figure 2: The ultrastructure of myocardium in rats for each group ($n = 10$ each group). Ultrastructure features in normal group ((a) ×4000, (b) ×8000, (c) ×10000), model group ((d) ×4000, (e) ×8000, (f) ×8000), PC6 group ((g) ×6000, (h) ×8000, (i) ×8000), and LI4 group ((j) ×5000, (k) ×8000, (l) ×8000). Cardiocyte and micrangium ((a, d, g, j), ×4000~6000), myofibril, I band, and Z-line ((b, e, h, k), ×8000), intercalated disc ((c,f,i, l), ×8000~10000). Some changes of the ultrastructure: mitochondria swelling ((d, e), cell apoptosis (j), endothelial cell interstitial hyperplasia (d), muscle plasma nets expansion (e), out-sync contraction of contraction band ((e, j), and intercalated disc deformation ((f, i, l). With reference to normal group, compared EA group with model group, EA significantly reduced cardiac muscular tissue damage.

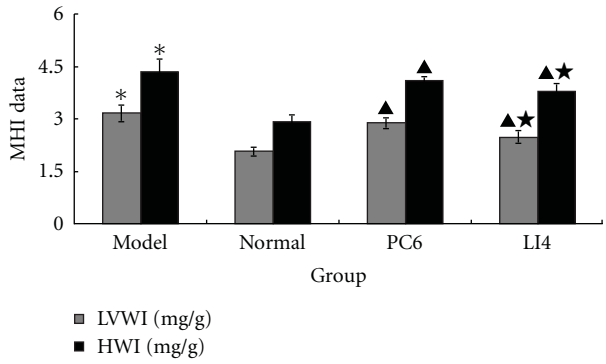

Figure 3: Effects of EA on LVWI, HWI in rats with MH induced by ISO ($n = 10$ each group). MHI: myocardial hypertrophy index, LVWI: left ventricular weight index, and HWI: heart weight index. LVWIs and HWIs in the model group were significantly higher compared with the model group. EA lowered the value, and LI4 was superior to PC6. $^{*}P < 0.01$ versus normal; $^{\blacktriangle}P < 0.05$ versus model; $^{\bigstar}P < 0.05$ versus PC6.

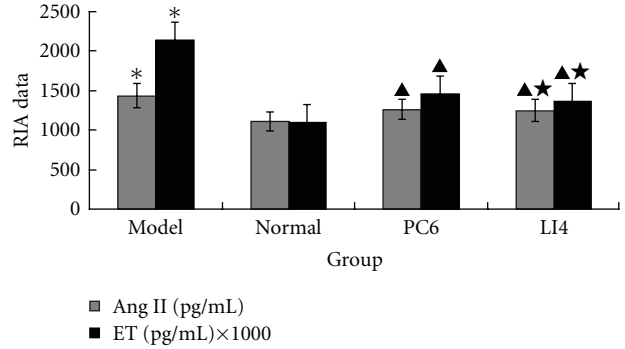

Figure 4: Effects of EA on the ratios of circulating Ang II, ET in rats for each group ($n = 10$ each group). The plasma Ang II, ET level of model group was significantly increased compared with normal group. EA decreased these cytokines, but there was no statistically significant difference between PC6 group and LI4 group. $^{*}P < 0.05$ versus normal; $^{\blacktriangle}P < 0.05$ versus model; $^{\bigstar}P > 0.05$ versus PC6.

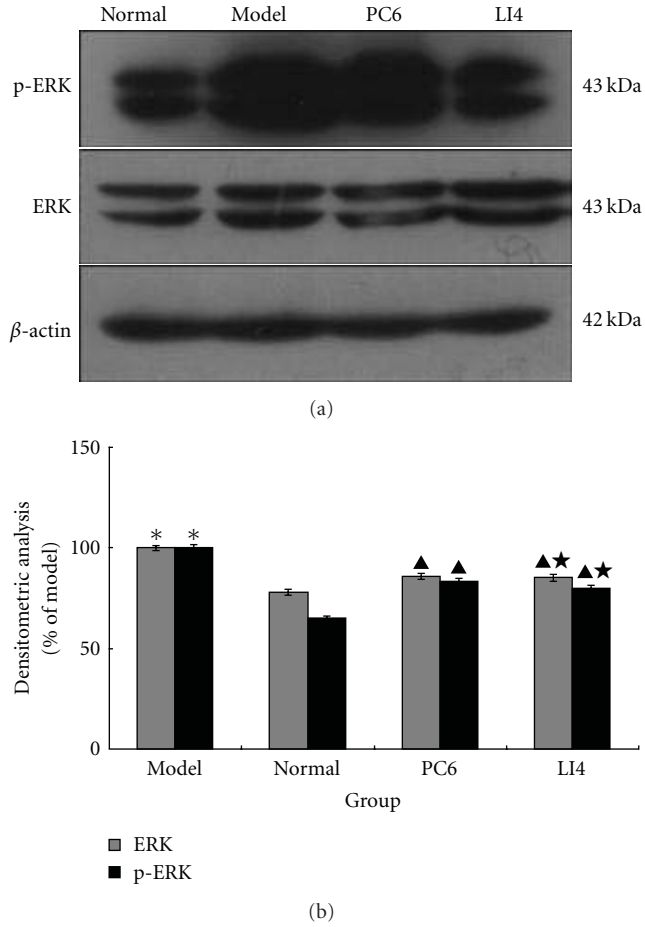

Figure 5: Effects of EA on the expression of ERK1/2 and p-ERK in cardiac muscular tissue in rats for each group ($n = 10$ each group). Western blotting was performed on protein extracts from left ventricular myocardial tissue. Quantitative analysis revealed a significant increase in model group while decrease in EA groups. However, there was no statistical significance difference between PC6 group and LI4 group. $^{*}P < 0.05$ versus normal; $^{\blacktriangle}P < 0.05$ versus model; $^{\bigstar}P > 0.05$ versus PC6.

discovered that premotor sympathetic cardiovascular neurons that receive convergent input from the splanchnic and median nerves during low-frequency EA and manual acupuncture were inhibited similarly for prolonged periods by low-frequency [28].

ECG is often regarded as the basic indicator for judging cardiac function. An increase in the QRS interval [29, 30] and a changing ST-T interval [31] provide an accurate diagnosis of MH. Considerable evidence documents that changes of the ST-segment is an effective indicator of the severity of myocardial ischemia [32]. The current study found that the R-R intervals were elongated and ST-T amplitudes were evidently increased in the model group and that EA could regulate the values. This indicates that EA on PC6 or LI4 can be medicative for MH.

Our experimental results showed that Ang II and ET participate in the formation of MH and that EA can improve MH by regulating the role of neuroendocrine-cytokines. The development of MH induced by hemodynamic overload is very likely initiated by mechanical stress. However, the involvement of growth promoting factors (e.g., TGF-b and VEGF), hormones (such as Ang II and ET-1), and cytokines (for instance CT-1) cannot be foreclosed [33]. As a main active metabolite of renin-angiotensin system, Ang II plays a key role in promoting MH and myocardial fibrosis [34]. Previous literature has shown that endogenous Ang II can enhance MH due to ISO in rats [35]. Ang II in cardiac interstitial tissue is produced in the transformation of chymase and has a cardioactive effect on myocardial and sympathetic nerve endings that regulate cardiac pressure chronotropic action and promote MH [36]. As the strongest vasoconstrictor yet discovered, ET participates in the pathogenesis of various cardiac diseases, such as myocardial ischemia and hypertension [37].

The mitogen-activated protein kinases (MAPKs) signal pathway, including extracellular signal-regulated kinase

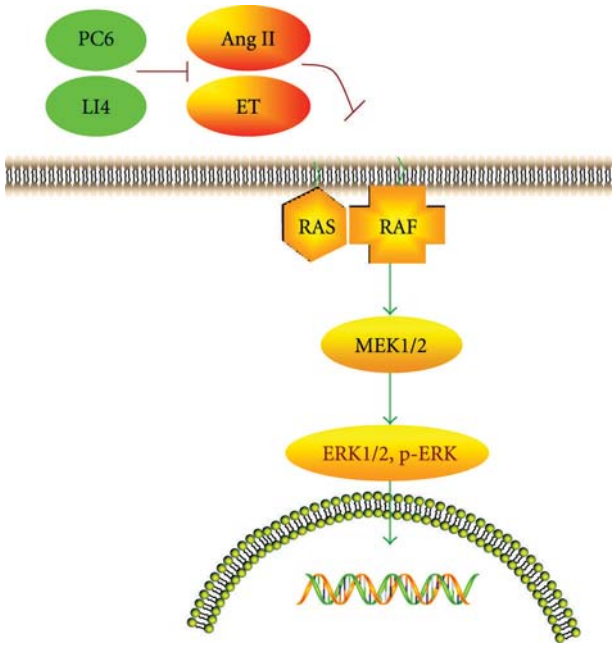

Figure 6: EA improves ERK signaling pathways through the regulation of neuroendocrine cytokines in myocardial hypertrophic rats. The proposed hypothetic mechanism of acupuncture affecting the heart acupuncture indeed improves the cardiac function in MH, and this effect has a close relation with ERK signaling pathway through regulating the role of neuroendocrine cytokines.

(ERK)1/2, c-Jun NH2-terminal kinase (JNK), and p38 kinase, has been recognized as a central mechanism underlying development of many types of cardiovascular disease, including cardiac failure and hypertrophy [38]. As the latest and the most important pathway, ERK is mainly activated by the Ras/Raf/MEK/ERK cascade. This pathway involves a module of four protein kinases, ERK, MEK, Raf, and Ras. As a low-molecular-weight GTPase, the small (21-kDa) guanine nucleotide-binding protein Ras plays a central role in the regulation of cell growth and division and it acts as upstream molecule to regulate raf [39]. There are three isozyme in Raf family, Raf-1, A-Raf, and B-Raf [40]. Raf-1 has been reported to be activated by Ras.GTP acts as a key protein kinase to activate dual protein kinase MEK [41]. MEK in turn activates ERKs by phosphorylating their threonine and tyrosine residues [42]. ERK exists in two isoforms in mammalian cells, ERK1 and ERK2. P-ERK is a homocysteine thiolactone that is eventually transported into nuclear and acts on downstream transcription factors (ELK, AP-1, NF-κB, and etc.) and then regulates related protooncogenes (such as c-fos, c-myc, c-jun, jun-B, and Egr-1), which lead to MH. ERK is not only an important regulating factor in the nervous system and in cell division, but also essential for the signaling pathways of various cytokines (ET-1, NA, Ang II, etc.) that promote MH [43]. The ERK signaling pathways can be stimulated upon G protein coupled receptor occupation by binding of hormones (by binding of ET-1 [44] and Ang II [45]). The current study found that EA could inhibit the expression of ERK and p-ERK protein through mediating various cytokines to improve cardiac function in MH.

5. Conclusion

In summary, acupuncture improves cardiac function in MH, and this effect is closely related with the ERK signaling pathways through the regulation of neuroendocrine cytokines (Figure 6). EA stimulation of LI4 may be more effective than stimulation of PC6 in MH rats. This contrasts with previous research and could be a singular phenomenon that needs further verification.

Conflict of Interests

All authors manifest that there is no conflict of interests.

Acknowledgments

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Research Article

Effect of Electroacupuncture on Activation of p38MAPK in Spinal Dorsal Horn in Rats with Complete Freund's Adjuvant-Induced Inflammatory Pain

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Activation of mitogen-activated protein kinases (MAPKs), especially p38 MAPK, plays an important role in the development of central sensitization related to persistent inflammatory pain. Electroacupuncture (EA) is well known to relieve persistent inflammatory pain. However, little is known about relationship between EA and p38 MAPK. Inflammatory pain rat model was induced by intraplantar injection of complete Freund's adjuvant (CFA). Male adult SD rats were randomly divided into the saline group, CFA group, and CFA + EA group. EA (constant saquare wave, 2 Hz and 100 Hz alternating frequencies, intensities ranging from 1 to 2 mA) was applied to bilateral "Zusanli" (ST 36) and "Kunlun" acupoints (BL 60) for 30 min, once per day. The paw edema and paw withdrawal threshold (PWT) were measured at preinjection and days postinjection 1, 3, and 14. Spinal p-p38MAPK- immunoreactivty (p-p38MAPK-IR) cells were detected by immunohistochemistry at postinjection day 3 and 14. EA significantly inhibited paw edema at postinjection days 14 and increased PWT at postinjection days 3 and 14. Moreover, the increasing number of spinal p-p38MAPK-IR cells which was induced by CFA injection was suppressed by EA stimulation. These results indicate that anti-inflammatory and analgesic effect of EA might be associated with its inhibition of spinal p38 MAPK activation and thereby provide a potential mechanism for the treatment of inflammatory pain by EA.

1. Introduction

The mitogen-activated protein kinases (MAPKs) are important for intracellular signal transduction and play pivotal roles in mediating the generation and maintenance of pain [1]. To date, six distinct groups of MAPKs have been characterized in mammals: extracellular-signal-regulated kinase (ERK), p38, c-jun N-terminal kinase (JNK), ERK7/8, ERK3/4, and ERK5 [2]. Recent data demonstrate that MAPKs, p38 in particular, can be activated in the spinal cord by peripheral and spinal noxious stimuli [3]. It is also shown that phosphorylated p38 (p-p38) MAPK, the active form of p38 MAPK, increases in spinal cord after peripheral inflammation which is induced by CFA [4], bee-venom [5], formalin [3, 6], or capsaicin [7]. Moreover, intrathecal administration of a p38 MAPK inhibitor into spinal cord has been shown to effectively reduce pain behavior associated with the peripheral inflammation [3, 8–10].

Electroacupuncture (EA), as a traditional complementary and alternative medicine approach, has been used for several decades in the treatment of many acute and chronic inflammatory diseases [11]. Accumulative evidence demonstrates that EA significantly inhibits paw inflammation and hyperalgesia in a rat model [12–14]. However, it has been reported that EA may produce differential effects under healthy and pathological conditions [15]. The underlying mechanisms of EA analgesia are still not completely understood, and the influence of EA on p38 MAPK activation in spinal cord which is associated with inflammatory pain is unclear. Injection of complete Freund's adjuvant (CFA) into a rat's hind paw provides a very good animal model to study the mechanism of inflammatory pain [16]. Thus,

we used a rat CFA model to observe the anti-inflammatory and analgesic effect of EA and to investigate whether EA may relieve inflammatory pain by suppressing the activation of spinal p38 MAPK in CFA-inflamed rats.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley rats weighing 180–220 g were provided by the Department of Animal Sciences of our university. They were housed five per cage with food pellets and water ad libitum. Prior to experimental manipulation, rats were allowed to acclimate to the housing facilities for one week and maintained on a 12 : 12 h light-dark cycle and a constant room temperature of 25 ± 2°C. All rats in this study were used strictly in accordance with the National Institutions of Health Guide for the Care and Use of Laboratory Animals in order to minimize the number of animals used and their suffering.

2.2. Establishment of Model and Experimental Groups. Inflammatory pain rat model was induced by an injection of 100 μL CFA (Sigma, USA) into the plantar surface of right hind paw. The rats were separated randomly into 3 groups: (1) the saline group with saline injection (same volume as used for the CFA injection); (2) the CFA group with CFA injection, immobilization, and without EA stimulation; (3) the CFA + EA group with CFA injection, immobilization but treated with EA. The rats of the CFA group were also immobilized gently by assistants' hands for 30 min every day, which simulate holder stress in rats of CFA + EA group. Ten rats for each group were included for assessment of inflammation and behavioral test. Three rats were included at each survival time point for immunohistochemical analyses.

2.3. EA Stimulation. Rats were loosely immobilized by assistants' hands. Four stainless steel acupuncture needles of 0.25 mm in diameter were inserted a depth of 5 mm into bilateral "Zusanli" (ST36, 5 mm lateral to the anterior tubercule of the tibia) and "Kunlun" (BL60, at the ankle joint level and between the tip of the external malleolus and tendo calcaneus) acupoints. It has been showed that these two acupoints were good at treating inflammatory pain [17]. The two ipsilateral needles were connected with the output terminals of the HANS Acupuncture point Nerve Stimulator (LH-202H, Huawei Co., Ltd., Beijing, China). The EA parameters were set as follows: constant square wave current output (pulse width: 0.6 ms at 2 Hz, 0.2 ms at 100 Hz); intensities ranging from 1 to 2 mA (each intensity for 15 min, totaling 30 min); to 2 Hz and 100 Hz alternating frequencies (automatically shifting between 2 Hz and 100 Hz stimulation for three seconds each). The stimulation was given for 30 min, once per day, and started at day 1 after injection when the assessment of inflammation and behavioral test have been finished.

2.4. Assessment of Inflammation. Paw volume (to assess the severity of inflammation) was measured by a water displacement plethysmometer (LYS-7A, Shandong Medical Instrument Factory, China). The hind paw was immersed in a chamber containing electrolyte solution up to the boundary between hairy and nonhairy skin, and the volume displacement was determined electronically. Paw volume was measured in duplicate before CFA/saline injection (as basal paw volume) and 1, 3, and 14 days after CFA/saline injection. Paw edema was calculated as follows: paw edema (ml) = $V_t - V_0$, where V_t is the paw volume after CFA or saline injection and V_0 is basal paw volume.

2.5. Behavioral Test. The paw withdrawal threshold (PWT) was used to assess the inflammatory pain. Rats were placed on a metal mesh table and adapted to the new environment. The mechanical stimulus was delivered to the plantar surface of right hind paw from below the floor of the test chamber by an automated testing device (dynamic plantar aesthesiometer, Ugo Basile, Italy). A steel rod (diameter of 0.5 mm) was pushed against the hind paw with ascending force. The force went from 0 to 50 g over a 20 s period. When the animal withdrew its hind paw, the mechanical stimulus was automatically stopped, and the force at which the animal withdrew its paw was recorded as PWT. Withdrawal responses were taken from four consecutive trials with at least 1min between trials and averaged.

2.6. Immunohistochemistry. Animals were deeply anesthetized with 10% choral hydrate (0.35 mL/100 g, ip) and transcardially perfused with 150 mL cold sterilized saline followed by 500 mL cold, fresh 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The L_4–L_6 segments were removed and postfixed in the same fixative for 6 h at 4°C before transfer to 15–30% sucrose for cytoprotection. Transverse 30 μm thick sections were cut on a cryostat and stored as free-floating sections in 0.1 M PBS (containing 30% sucrose, 30% ethylene glycol) at −20°C. Immunochemistry was performed on free-floating spinal cord section. Endogenous peroxidase activity was quenched with 15 min incubation in 3% H_2O_2 at 37°C. Sections were blocked in 5% normal goat serum for 30 min at room temperature and then transferred to primary antibody solution containing rabbit anti rat p-p38MAPK (1 : 200, CST, USA) for overnight at 4°C. Sections were washed and placed in biotinylated secondary antibody (goat antirabbit, 1 : 400) for 1 h at 37°C. Following incubation with HRP-conjugated avidin (1 : 400) for 1 h at 37°C, sections were incubated with DAB substrate for 40 seconds. Sections were mounted on glass slides, dehydrated through an ascending series of alcohols, cleared with xylene, and coverslipped. Images were captured from ipsilateral dorsal horn at 20 × magnification using a Leica CCD camera. In a given area (200 μm × 150 μm) that is located in superficial spinal cord (laminae I-II), the number of p-p38 MAPK positive cells was counted automatically using Image Pro Plus 6.0 under blinded conditions. A minimum of five tissue sections/animal with a minimum of three animals/group were counted.

2.7. Statistical Analysis. Data are expressed as mean ± standard deviation (SD). The differences among groups were

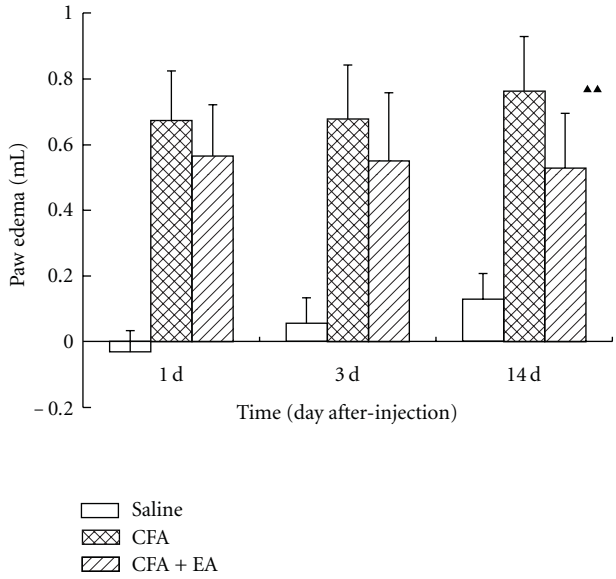


Figure 1: Effect of electroacupuncture (EA) on ipsilateral paw edema at different time points in complete Freund's adjuvant (CFA) rats. Values are mean ± SD, $n = 10$ animals per experimental group. $^{**}P < 0.01$ versus saline group; $^{\blacktriangle\blacktriangle}P < 0.01$ versus CFA group at corresponding time points.

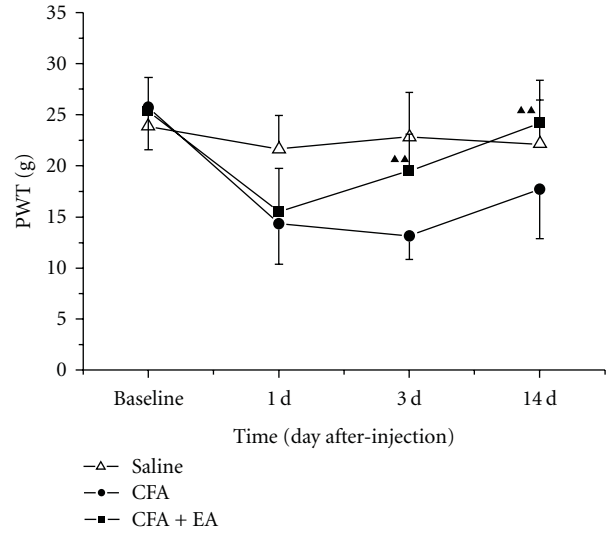


Figure 2: Effect of EA on ipsilateral paw withdrawal thresholds (PWTs) at different time points in CFA rats. Values are mean ± SD, $n = 10$ animals per experimental group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus saline group; $^{\blacktriangle\blacktriangle}P < 0.01$ versus CFA group at corresponding time points.

assessed by one-way analysis of variance (ANOVA) and followed by LSD post hoc test for the normal distribution and homogeneity of variance data. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of EA on Ipsilateral Paw Edema in CFA Rats. Basal paw volumes in the saline, CFA, and CFA + EA group were 1.455 (±0.129), 1.448 (±0.161), 1.555 (±0.089) mL, respectively ($P > 0.05$). As shown in Figure 1, the paw edema in CFA group and CFA + EA group was higher than that in saline group at all time points ($P < 0.01$). The paw edema in CFA group increased gradually whereas that in CFA + EA group decreased slightly. Compared with the CFA group at corresponding time points, the paw edema in the CFA + EA group markedly decreased at postinjection day 14 ($P < 0.01$).

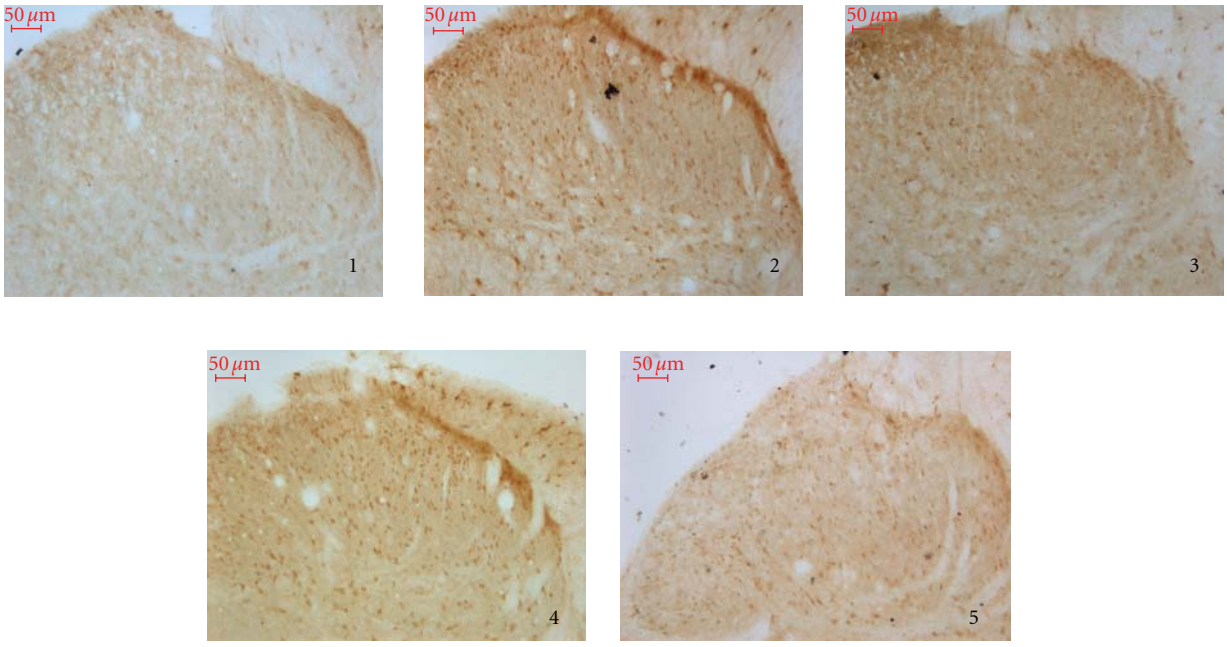
3.2. Effect of EA on Ispilateral PWTs in CFA Rats. As shown in Figure 2, no difference in basal PWTs among groups was observed before saline or CFA injection. However, at day 1, 3, and 14 after CFA injection, the PWTs in the CFA group were obviously lower than those in the saline group ($P < 0.01$ or $P < 0.05$). After EA stimulation, the rats' PWTs were increased significantly as compared with the CFA group at postinjection days 3 and 14 ($P < 0.01$).

3.3. Effect of EA on Phosphor-p38MAPK-Immunoreactivity (p-p38MAPK-IR) Expression in the Ipsilateral Spinal Dorsal Horn in CFA Rats. CFA injection induced an increase in number and intensity of p-p38MAPK-IR cells in the spinal dorsal horn ipsilateral to inflammation. However, a low basal constitutive expression of p-p38MAPK-IR was also observed in the saline group (Figure 3(a)). The number of p-p38MAPK-IR cells was detected increasingly at day 3 after CFA injection, and the significant level was maintained until 14 days ($P < 0.01$). After EA stimulation, the number of p-p38MAPK-IR cells in ipsilateral spinal dorsal horn was decreased markedly as compared with the CFA group at postinjection days 3 and 14 ($P < 0.01$) (Figure 3(b)).

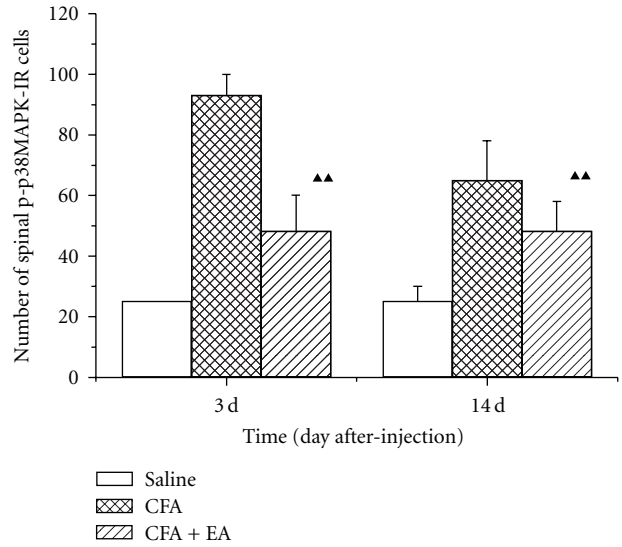
4. Discussion

The present study demonstrates, for the first time, that the anti-inflammatory and analgesic effects of EA were associated with the activation of p38MAPK in spinal dorsal horn. In the CFA-induced inflammatory pain model, the paw edema and the number of p-p38MAPK-IR cells in ipsilateral spinal dorsal horn were significantly increased, while the pain threshold was decreased. Treatment with EA obviously ameliorated CFA-induced paw edema and hyperalgesia and decreased the number of p-p38MAPK-IR cells in spinal dorsal horn, indicating that the p38MAPK signaling pathway might play an important part in the anti-inflammatory and analgesic effect of EA.

Recent reports have demonstrated that activation of p38 MAPK within the spinal cord has been implicated in a variety of enhanced pain states [8, 18–24] and plays a pivotal role in creation of persistent pain. Increasing activation of p38 MAPK in spinal cord has been observed not only at peripheral nerve injury caused by chronic constriction injury (CCI) [8, 23] and spinal nerve ligation (SNL) [18–20], but also at peripheral inflammation induced by CFA [4], bee-venom [5], formalin [3, 6], or capsaicin [7]. It has been reported



(a)



(b)

Figure 3: Effect of EA on phosphor-p38MAPK-immunoreactivty (p-p38MAPK-IR) expression in the ipsilateral spinal dorsal horn induced in CFA rats. (a) Representative sections of p-p38MAPK-IR cells in the ipsilateral spinal dorsal horn at postinjection days 3 and 14. (section 1, saline group; sections 2 and 4, CFA group, sections 3 and 5, CFA + EA group; sections 2 and 3, postinjection day 3; sections 4 and 5, postinjection day 14; Bars = 50 μm; section thickness = 30 μm). (b) Quantification of p-p38MAPK-IR showing that EA suppressed p-p38MAPK-IR expression in the ipsilateral spinal dorsal horn. Values are mean ± SD, $n = 3$ animals per experimental group. $^{**}P < 0.01$ versus saline group; $^{▲▲}P < 0.01$ versus CFA group at corresponding time points.

that p-p38 MAPK levels in lumbar enlargements increased in individual rats between days 8 and 17 after CFA immunization by western blot analysis. Moreover, it is shown that numerous cells stained positively for p-p38 MAPK in the dorsal horn of CFA-immunized rats, whereas the control rats contained only a few scattered p-p38MAPK-positive cells [4]. There was a rapid increase in phosphorylated p38 MAPK in spinal cord following intrathecal administration of substance P or intradermal injection of formalin. Immunocytochemistry also revealed that phosphorylated-p38-MAPK-immunoreactive cells were predominantly present in laminae I–IV of the dorsal horn [3]. Consistent with these previous studies in the inflammatory pain model, we have shown that activation of p38 MAPK in ipsilateral spinal dorsal horn was

detected on day 3 after CFA injection and was maintained for two weeks.

Accumulating evidence has shown that activation of p38 MAPK in the dorsal horn, which contributes to the pathogenesis of pain, was expressed exclusively in microglia, but not in neurons or astrocytes. Gu et al. have shown a marked increase of the level of p-p38 MAPK in the microglia of dorsal horn in CCI model [23]. Moreover, similar results have also been found in the SNL model [18–20]. Svensson et al. have reported that activated p38 MAPK is located in spinal microglia, but not in neurons or other glia after intrathecal administration of SP [3]. In contrast to the present findings with peripheral nerve injury and central inflammatory stimulation, Hua et al. have shown that after carrageenan paw injection, the increased p-p38 immunoreactivity was seen primarily in microglia but also in a small population of neurons [25]. Taking these results together, we speculate that it remains to be investigated how p38 MAPK activation in spinal microglia contributes to CFA-induced pain sensitization.

Acupuncture therapy, especially EA, has been accepted worldwide mainly for the treatment of acute and chronic pain [26–29]. In the present study, we observed that EA has suppressed paw edema induced by intraplantar CFA. This is supported by previous studies reporting that EA significantly inhibits edema compared to sham EA control in CFA-injected rat [12, 14, 30]. A previous study reported that a single or repetitive EA could reduce mechanical hyperalgesia, but not thermal hyperalgesia, in CFA-inflammatory pain rats [31]. Consistent with this, we also found that EA increased paw withdrawal threshold in CFA rats at postinjection days 3 and 14. Although great progress has been made in recent years in investigating analgesic mechanisms of acupuncture, the influence of acupuncture on signal molecules and signal pathways remains elusive. A recent study has reported that pre-EA significantly decreased p-p38 MAPK protein expression in the spinal dorsal horn of rats suffering from visceral pain [32]. Our present study demonstrated for the first time that repeated EA significantly suppressed CFA-induced activation of p38MAPK in spinal dorsal horn at postinjection days 3 and 14. Further investigation is required to determine the exact effect of the MAPK signal pathway on the spinal cord of rats suffering from the peripheral inflammatory pain.

In conclusion, EA has anti-inflammatory and analgesic effect on CFA-induced inflammatory pain, which might be associated with its inhibition of spinal p38 MAPK activation. Spinal p38 MAPK, exactly p38 MAPK in microglia, and other members of the p38 MAPK signal transduction pathway represent promising targets for the treatment of inflammatory pain by EA.

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Research Article

Does Acupuncture Needling Induce Analgesic Effects Comparable to Diffuse Noxious Inhibitory Controls?

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Diffuse noxious inhibitory control (DNIC) is described as one possible mechanism of acupuncture analgesia. This study investigated the analgesic effect of acupuncture without stimulation compared to nonpenetrating sham acupuncture (NPSA) and cold-pressor-induced DNIC. Forty-five subjects received each of the three interventions in a randomized order. The analgesic effect was measured using pressure algometry at the second toe before and after each of the interventions. Pressure pain detection threshold (PPDT) rose from 299 kPa (SD 112 kPa) to 364 kPa (SD 144), 353 kPa (SD 135), and 467 kPa (SD 168) after acupuncture, NPSA, and DNIC test, respectively. There was no statistically significant difference between acupuncture and NPSA at any time, but a significantly higher increase of PPDT in the DNIC test compared to acupuncture and NPSA. PPDT decreased after the DNIC test, whereas it remained stable after acupuncture and NPSA. Acupuncture needling at low pain stimulus intensity showed a small analgesic effect which did not significantly differ from placebo response and was significantly less than a DNIC-like effect of a painful noninvasive stimulus.

1. Introduction

Acupuncture is frequently used in pain therapy and has a clinically relevant effect on several types of chronic pain disorders [1–3]. However, the mechanism of action of acupuncture remains unclear, and several theories are currently being discussed. Involvement of the endogenous opioid system [4, 5] as well as spinal or supraspinal mechanisms (e.g., gate-control [6, 7], long-term depression [8, 9] or diffuse noxious inhibitory controls [10, 11]) are thought to account for acupuncture-induced pain relief.

The concept of diffuse noxious inhibitory controls (DNIC) has attracted particular attention in the past years. Under normal conditions, pain after application of an experimental nociceptive stimulus is attenuated by a conditioning nox-ious stimulus to a remote body region [12]. According to experimental animal studies, this effect could contribute to acupuncture analgesia [10, 11]. However, animal experimental settings are different from clinical routine in humans regarding the intensity of acupuncture stimuli [13]. The analgesic effect might therefore be different in animals and humans.

There are several further considerations that question the involvement of DNIC in acupuncture. Firstly, patients suffering from diseases with typically impaired DNIC (e.g., fibromyalgia [14], osteoarthritis of the hip [15], irritable bowel syndrome [16], or temporomandibular disorder [17]) still benefit from acupuncture therapy [18–21]. Secondly, classic DNIC-inducing tests in humans, such as the cold pressor test, ischemic tourniquet test and thermal heat test are noninvasive but very painful, whereas acupuncture is invasive but usually not very painful.

To date, several trials in humans have demonstrated an increase in pain thresholds after acupuncture [22, 23] or a decrease in pain ratings [24], but this has, to our knowledge, never been compared to a classical DNIC-inducing test paradigm and to placebo.

The aim of the present study was to compare the analgesic effects of acupuncture needling without stimulation and cold-pressor-induced DNIC, using nonpenetrating sham acupuncture (NPSA) as a control condition.

2. Materials and Methods

2.1. Design. This was a randomized, blinded, crossover study. All subjects underwent three interventions on the same day: acupuncture needling, NPSA and cold pressor test. The three tests were performed in a randomized order according to a computer-generated randomization list. The interstimulus interval was set at 10 minutes. In the light of only short duration of stimuli, we considered that wash-out period sufficient.

The study was approved by the local ethics committee and was carried out at the Department of Anesthesiology and Pain Therapy of the University Hospital of Bern. Written informed consent was obtained from all participants.

2.2. Volunteers. Forty-five healthy pain-free volunteers were recruited from hospital staff, among medical students and by word of mouth. Minimal age of 18 years and no previous acupuncture experience were required. Exclusion criteria were any ongoing pain, current intake of analgesic drugs, antidepressants or anticonvulsants, vascular disorders of the hand or the foot to be tested, any neurological disorders, diabetes mellitus, alcohol or drug abuse, and pregnancy.

2.3. Acupuncture and Nonpenetrating Sham Acupuncture (NPSA). Acupuncture and NPSA were performed at LI4 (large intestine 4, Hegu), an acupoint which is commonly used for acupuncture analgesia [25]. LI4 is situated between thumb and index finger at the highest point of the first dorsal interosseus muscle. For acupuncture, a 0.3×30 mm needle (asia-med, Suhl, Germany) was used. For NPSA, the so-called Streitberger needle [26] (asia-med, Suhl, Germany) was used, which has been evaluated in several studies [27, 28]. The blunt needle tip does not penetrate the skin but instead moves back inside its shaft when slight pressure is applied. When the needle tip touches the skin, the subject feels a pricking sensation. The subjects were told that two different types of needles were used but were unaware of one of them being a non-penetrating needle.

Subjects were blinded to the acupuncture and NPSA needles by placing a plastic ring over the acupuncture point and fixing it with a plaster (Durapore Surgical Tape, 3 M, Minn, USA). The needles were placed through the plaster in the middle of the plastic ring which served as a holder for the nonpenetrating needle. Blinding of the acupuncturist was not possible. The needles were left in place for 5 minutes without further stimulation and the subjects rated the perceived pain intensity on a 0–10 numeric rating scale (NRS) immediately after needle removal, with 0 = no pain and 10 = worst pain imaginable.

2.4. Assessment of DNIC. Volunteers immersed their hand in ice-saturated water ($1.5 \pm 1^{\circ}$C) for a maximum of two minutes. The water was constantly recirculated in order to avoid laminar warming around the hand. If the pain was considered intolerable before 2 minutes had elapsed, subjects withdrew their hand and the elapsed time was noted. Maximal perceived pain intensity was rated on a 0–10 NRS immediately after hand withdrawal.

2.5. Pressure Algometry. Pressure pain detection threshold (PPDT) at the ipsilateral second toe was measured by a second investigator who was blinded by a curtain drawn across the patient so that he could not see whether acupuncture or NPSA was in progress. An electronic algometer (Somedic AB, Horby, Sweden) with a probe area of 1 cm^2 was used. Pressure was increased from 0 to a maximum of 1000 kPa at a rate of 30 kPa/s. The subjects were instructed to stop the measuring at the moment when the pressure sensation turned to pain (detection threshold) by pressing the button. First, several measurements were performed on the opposite foot for training purposes. Then two assessments of PPDT were made at the second toe, the mean of which represented the baseline threshold. Subsequent assessments of PPDT were made after each of the three interventions immediately after removal of the acupuncture needles or hand withdrawal from the ice water (time 0) and after 2 and 5 minutes. Figure 1 depicts the time flow of the experiments.

2.6. Data Analysis. Assuming that NPSA does not induce a considerable analgesic effect, sample size was calculated based on the difference between acupuncture and NPSA at time 0. Approximately 8% PPDT elevation after acupuncture at LI4 was found in the study of Zaslawski et al. [23] Expecting a similar effect in our population and assuming a standard deviation of 18%, examination of 43 subjects would provide 80% power. We tested 45 subjects in order to account for possible higher variability.

Based on the two measurements before the interventions, the arithmetical mean was calculated and considered the baseline pressure pain threshold. This baseline value was subtracted from each of the subsequently obtained measurements and a two-way repeated measures ANOVA was performed on these differences. Differences to baseline instead of absolute values were chosen for easier depiction. The two factors investigated were (i) intervention with the three levels "acupuncture", "NPSA" and "DNIC test", and (ii) time with the levels "0 minutes", "2 minutes" and "5 minutes."

3. Results

Forty-five healthy volunteers were enrolled (23 females, 22 males) with a mean age of 24.2 years (SD 5.7). Female subjects were aged 24.6 years (SD 7.5), while male subjects were aged 23.9 (SD 3.0). There was no significant age difference between males and females ($P = 0.67$). PPDT at baseline was 299 kPa (SD 112) and rose to 364 kPa (SD 144), 353 kPa (SD 135), and 467 kPa (SD 168) immediately after acupuncture, NPSA, and DNIC test, respectively. The absolute values for PPDT assessments at every time point

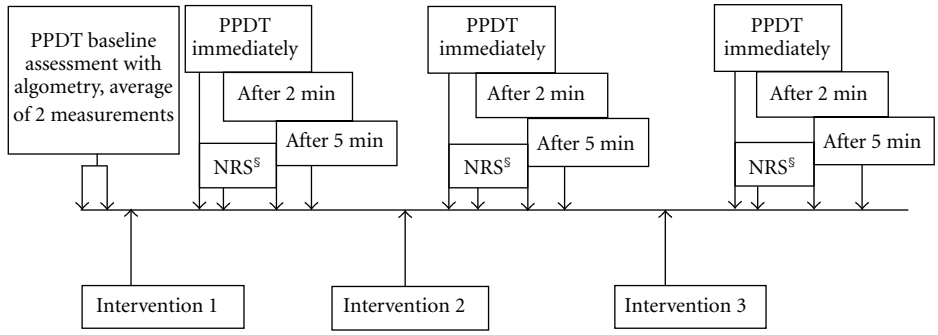

Figure 1: Time line of the experiment. PPDT: pressure pain detection threshold, NRS: numeric rating scale. *Acupuncture, nonpenetrating sham acupuncture (NPSA), and DNIC test were given in randomized order. The duration of acupuncture and NPSA was 5 minutes, and the maximal duration of the DNIC test was 2 minutes. §The subjects were asked to rate the pain intensity during the intervention on a 0–10 NRS (0 = no pain, 10 = worst pain imaginable).

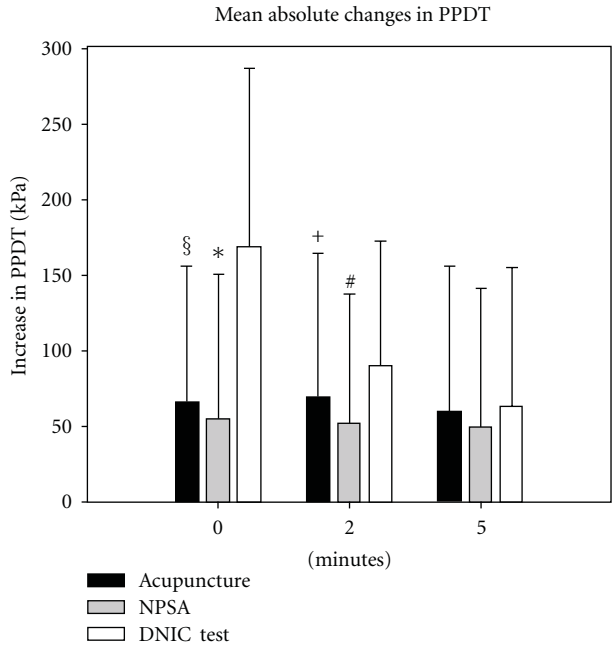

Figure 2: Comparison of pain threshold elevation at the end of acupuncture, NPSA, and DNIC test. PPDT: pressure pain detection threshold, NPSA: nonpenetrating sham acupuncture, DNIC: diffuse noxious inhibitory control. Acupuncture and NPSA are significantly different from DNIC test at 0 and 2 minutes. §$P < 0.001$, *$P < 0.001$, +$P = 0.05$, #$P < 0.001$.

are presented in Table 1. PPDT after each of the three interventions was significantly different from the baseline threshold at all time points ($P < 0.001$).

Figure 2 displays the absolute changes in PPDT during the five-minute posttest period after each of the interventions. We found a significantly higher PPDT-increase in the DNIC test compared to acupuncture, and no significant difference between acupuncture and NPSA. Comparison between the three interventions using the Student-Newman-Keuls method for all-pairwise comparisons showed statistically significant differences between DNIC test and

Table 1: Absolute values of pressure pain detection thresholds after each of the interventions during the five-minute observation interval. All values are presented as mean (SD). NPSA: nonpenetrating sham acupuncture, DNIC: diffuse noxious inhibitory control.

	baseline	0 minutes	2 minutes	5 minutes
Acupuncture	298.9 (111.7)	364.1 (143.7)	367.4 (143.4)	357.9 (135.9)
NPSA	298.9 (111.7)	353.2 (135.3)	350.6 (133.4)	349.9 (135.6)
DNIC test	298.9 (111.7)	467.0 (169.7)	388.6 (147.4)	361.2 (142.2)

acupuncture at 0 and at 2 minutes ($P < 0.001$ and $P = 0.05$, resp.) as well as between DNIC test and NPSA at 0 and at 2 minutes (both $P < 0.001$). There was no statistically significant difference between any of the interventions after 5 minutes.

There was a trend for greater PPDT changes after acupuncture than after NPSA throughout all measurements, but the difference was not significant at any of the time points ($P = 0.353$ at 0 minutes, $P = 0.15$ at 2 minutes, and $P = 0.399$ at 5 minutes).

The measurements after DNIC test displayed a significant decrease over time, with $P < 0.001$ for both 0 versus 2 and 0 versus 5 minutes and $P = 0.013$ for 2 versus 5 minutes. There was no time dependency for PPDT change after both acupuncture and NPSA.

The pain intensity during each of the three different procedures was rated on a 0–10 NRS. Verum acupuncture was rated as 2.4 (SD 1.5), NPSA was rated as 1.1 (SD 0.9), and ice water was rated as 7.1 (SD 1.5). These differences were statistically significant ($P < 0.001$).

4. Discussion

The present study showed a similar increase in pressure pain thresholds after acupuncture and NPSA, but neither the magnitude nor the time profile was similar to the DNIC effect evoked by the more painful cold-pressor test.

Unlike the classic DNIC response, which was short lasting and decreasing over time, acupuncture produced a constant and only moderate pain threshold elevation. Moreover, our study showed no significant difference in analgesic effect between acupuncture and NPSA in healthy, pain-free subjects, although there was a trend for greater increase in PPDT after acupuncture than after NPSA. This remains to be verified in a patient population, since healthy volunteers possibly react differently to acupuncture treatment than pain patients do: studies by Napadow et al. have shown that patients with carpal tunnel syndrome responded to acupuncture with more pronounced fMRI signal increase in the hypothalamus and signal decrease in the amygdala compared to healthy controls [29, 30]. In a pain patient population with possibly altered DNIC, a difference between verum acupuncture and NPSA might be more pronounced.

It has been shown in animal studies that acupuncture activates neuronal pathways which are involved in DNIC [31]. However, findings of animal studies seem not to be transposable to human acupuncture since the experimental settings largely differ from clinical routine-acupuncture [32], especially in terms of pain intensity [13]: DNIC-like effects in animals were observed at high pain intensities, whereas acupuncture in humans is usually performed with low pain intensities which might be insufficient to induce DNIC. The subjects in the present study received only a single needle without stimulation, whereas in animal studies, usually several needles are simultaneously applied with electrical stimulation.

A study by Treister et al. [33] has shown that the amount of endogenous analgesia after either noxious or innocuous conditioning stimuli (water immersion at 12°C and 25°C, resp.) is highly correlated to the NRS reported for the corresponding stimulus. Consequently, the average NRS of 2.4 ± 1.5 experienced by our subjects might have been too low to induce a DNIC-like effect as observed after the DNIC test (NRS 7.5 ± 1.5). Stronger stimulation of the needle by manual or electrical stimulation might increase the NRS and evoke a DNIC-like effect, as shown in a study by Barlas et al. [34]: they observed a significantly higher PPDT after acupuncture with strong electrical stimulation than after acupuncture with weak stimulation or NPSA. Strong electrical stimulation was defined as "to tolerance, but subnoxious," whereas weak stimulation was "strong but comfortable." The weak stimulation resulted in similar PPDT values as NPSA. Conceivably, acupuncture needles can be intensely stimulated, until the pain is strong enough to induce a DNIC response, but under therapeutic conditions (i.e., no or weak stimulation, low NRS), relevant contribution of DNIC to acupuncture analgesia is questionable. Hence our findings do not imply that acupuncture and DNIC are mechanistically different, but they suggest that results from experimental animal studies may not necessarily apply to clinical acupuncture therapy in humans.

Apart from the intensity of the conditioning stimulus, the time profile is another important characteristic of a DNIC response: it is most intense during application of the conditioning stimulus [35] and usually decreases to baseline within 5–10 minutes [36–38]. Although our results show an increase in PPDT after acupuncture, there is no observable decrease during the following 5 minutes, as would be expected if it evoked a DNIC effect.

Possible carry-over effects between treatments represent the major limitation to this study. Although the treatment order was randomized and the interstimulus interval was twice as long as the duration of acupuncture, carry-over effects cannot be completely ruled out. This might certainly be an issue to be addressed in the future.

5. Conclusions

Acupuncture at low pain stimulus intensity did not produce a DNIC-like effect comparable to a classical, painful DNIC test and its effect did not significantly differ from the one induced by NPSA. Our results showed that the penetration of an acupuncture needle by itself, though noxious, seems not to induce an analgesic effect mainly mediated by DNIC.

Conflict of Interests

The authors have no conflict of interests to declare.

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