

# Spinal cholinergic mechanism of the relieving effects of electroacupuncture on cold and warm allodynia in a rat model of neuropathic pain

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Received: 31 December 2008 / Accepted: 12 March 2009 / Published online: 3 April 2009  
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**Abstract** This study was performed to determine whether spinal cholinergic systems mediate the relieving effects of electroacupuncture (EA) on cold and warm allodynia in a rat model of neuropathic pain. For neuropathic surgery, the right superior caudal trunk was resected at the level between the S1 and S2 spinal nerves innervating the tail. Two weeks after the injury, the intrathecal (i.t.) catheter was implanted. Five days after the catheterization, the rats were injected with atropine (non-selective muscarinic antagonist, 30 µg), mecamlamine

(non-selective nicotinic antagonist, 50 µg), pirenzepine ( $M_1$  muscarinic antagonist, 10 µg), methoctramine ( $M_2$  antagonist, 10 µg) or 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) ( $M_3$  antagonist, 10 µg). Ten minutes after the injection, EA was applied to the ST36 acupoint for 30 min. The cold and warm allodynia were assessed by the tail immersion test [i.e., immersing the tail in cold (4°C) or warm (40°C) water and measuring the latency of an abrupt tail movement] before and after the treatments. The i.t. atropine, but not mecamlamine, blocked the relieving effects of EA on cold and warm allodynia. Furthermore, i.t. pirenzepine attenuated the antiallodynic effects of EA, whereas methoctramine and 4-DAMP did not. These results suggest that spinal muscarinic receptors, especially  $M_1$  subtype, mediate the EA-induced antiallodynia in neuropathic rats.

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**Keywords** Neuropathic pain · Allodynia · Electroacupuncture · Spinal cord · Cholinergic · Muscarinic

## Introduction

Neuropathic pain, characterized by spontaneous burning pain, hyperalgesia (an increased response to a stimulus that is normally painful) and allodynia (pain as a result of a stimulus that does not normally provoke pain), remains one of the most difficult clinical pain syndromes to treat. The mechanisms underlying this pain are complex and appear to involve both peripheral and central components of the nervous system [3]. Numerous studies have attempted to elucidate pathophysiological mechanisms or drug effects against neuropathic pain in patients and experimental animal models [39, 43]. However, the mechanisms are still

unclear, and therapeutic outcomes of conventional analgesics have been observed as variable [10, 38, 43].

Acupuncture has been used for thousands of years in East Asia to treat various maladies, especially pain, with few side effects. It is now viewed as an alternative method of medicine in Western countries [7, 27]. Electroacupuncture (EA) is a modified acupuncture technique that, as its name implies, utilizes electrical stimulation, and its analgesic effects on different types of acute pains and persistent inflammatory pains have been shown in rodents and humans [2, 4, 20, 28, 35, 49]. In the last 2 decades, several clinical studies have also reported the beneficial effects of acupuncture or EA on neuropathic pain [1, 14, 46]. Moreover, recent studies have shown that EA relieves the behavioral signs of hyperalgesia [9, 11] and allodynia [23, 25, 29, 30] in rat models of neuropathic pain. It is well known that the analgesic effects of EA are mediated by descending pain inhibitory systems [15, 35, 48], which mainly involve spinal opioid, adrenergic, serotonergic and cholinergic receptors [12, 13, 39]. Our previous studies demonstrated that spinal opioidergic, adrenergic and serotonergic systems mediate antiallodynic effects of EA in neuropathic rats [29, 30]. However, the spinal cholinergic mechanism of EA-induced antiallodynia remains to be elucidated.

It has been shown that cholinergic receptors are present in the superficial and deep dorsal horn of the spinal cord, and activation of spinal nicotinic or muscarinic acetylcholine receptors produces analgesia [12]. In this study, we investigated whether spinal nicotinic or muscarinic receptors play a role in the relieving effects of EA on cold and warm allodynia in the rat tail model of neuropathic pain [40], using intrathecal (i.t.) administration of selective antagonists.

## Materials and methods

### Experimental animals

Young adult male Sprague–Dawley rats [Sam:TacN(SD) BR, 200–220 g, 6 weeks,  $n = 192$ ] were housed in groups of four, with water and food available ad libitum. The room was maintained with a 12-h light/dark cycle and kept at  $23 \pm 2^\circ\text{C}$ . All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Kyung Hee University and were conducted in accordance with the guidelines of the International Association for the Study of Pain [53].

### Neuropathic surgery and behavioral test

Neuropathic surgery and behavioral test for cold and warm allodynia were performed as previously described [30, 40].

Briefly, during isoflurane anesthesia, the right superior caudal trunk was exposed, freed from the surrounding tissues and transected at the level between the S1 and S2 spinal nerves that innervate the tail in rats. The cold and warm allodynia signs were tested by immersing the tail in cold ( $4^\circ\text{C}$ ) and warm ( $40^\circ\text{C}$ ) water, respectively. Each animal was lightly immobilized in a plastic holder, and its tail was drooped for proper application of cold and warm water stimuli. Following the tail immersion, the latency to an abrupt tail movement was measured with a cutoff time of 15 s. The tail immersion test was repeated five times at 5-min intervals. When calculating the average latency, the cut-off time was assigned to the normal responses. The average latency was taken as a measure for the severity of allodynia; a shorter latency was interpreted as more severe allodynia.

### EA stimulation

For EA stimulation, a pair of stainless steel acupuncture needles (0.25 mm in diameter and 4 cm long) was inserted (5 mm in depth) into the left “Zusanli” acupoint (ST36), which is located in the anterior tibial muscle, 5 mm lateral and distal to the anterior tubercle of the tibia, and into the point 5 mm distal from the first needle. EA stimulation at this point is known to produce analgesic effects in various types of pain, including neuropathic pain [4, 9, 20, 28–30]. Cathodal and anodal leads from an electrical stimulator were connected to the acupuncture needle at the ST36 and the other needle, respectively, and train-pulses (constant rectangular current, 2 Hz, 0.5-ms pulse duration) were then applied for 30 min. In order to exclude the stress effect that might be induced by EA stimulation itself, we used the intensity of the muscle twitch threshold (0.2–0.3 mA) to not make rats squawk during EA stimulation. Although this intensity is quite moderate when compared with other studies (1.0–3.0 mA), it is sufficient to produce a significant antiallodynic effect. For the control, needles were inserted at the same location without electrical stimulation (plain acupuncture, PA).

### Implantation of intrathecal catheter

Fourteen days after the neuropathic surgery, under isoflurane anesthesia, the rats were implanted with PE-10 tubing by a procedure modified from Yaksh and Colleagues [37]. A PE-10 tubing (12.5 cm) that had a knot 8 cm from the tip was inserted into the spinal subarachnoid space through an incision made on the atlanto-occipital membrane. The tip of the tubing lay in the region of the lumbar enlargement, which was examined before

actual pharmacological experiments by observing the motor paralysis of the animal's hind limb following i.t. injection of lidocaine and was anatomically confirmed with a methylene blue dye injection after the experiments. The animals were allowed to recover for 5 days. If any signs of the spinal cord or root damage were observed, the animal was discarded.

## Drugs

The following drugs were used:  $\alpha$ -(hydroxymethyl)benzene acetic acid 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester tropine tropate (atropine, non-selective muscarinic antagonist, 30  $\mu$ g), *N*,2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride (mecamylamine, non-selective nicotinic antagonist, 50  $\mu$ g), 5,11-dihydro-11-[(4-methyl-1-piperaziny) acetyl]-6H-pyrido[2,3-b][1, 4]benzodiazepin-6-one dihydrochloride (pirenzepine,  $M_1$  receptor antagonist, 10  $\mu$ g), *N*, *N'*-bis[6[[[(2-methoxyphenyl)methyl]amino]hexyl]-1,8-octane diamine tetrahydrochloride (methocetramine,  $M_2$  receptor antagonist, 10  $\mu$ g) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP;  $M_3$  receptor antagonist, 10  $\mu$ g). All chemicals were dissolved in normal saline (0.9% NaCl) and obtained from Sigma Chemical Co., USA. The doses of antagonists were selected based on previously published studies that showed pharmacological antagonism of individual receptor subtypes [6, 22, 24, 44, 45]. Drugs or normal saline were injected into the i.t. space in a volume of 10  $\mu$ l, and the tubing was flushed with 10- $\mu$ l normal saline after drug injection.

## Experimental protocol

Five days after the implantation of i.t. catheters, the tail immersion test for cold or warm allodynia was performed, and one of the cholinergic antagonists or normal saline was injected intrathecally. Ten minutes after the injection, EA was applied to the ST36 for 30 min, and then the tail immersion test was performed again immediately after the end of EA stimulation. When assessing the effect of the antagonists without EA on allodynia, the tail immersion test was performed 10–30 and 40–60 min after the injection, respectively. Different rats were used in each group of different sets of the experiments. All behavioral tests were performed under a blind design for the type of drugs injected.

## Statistical analysis

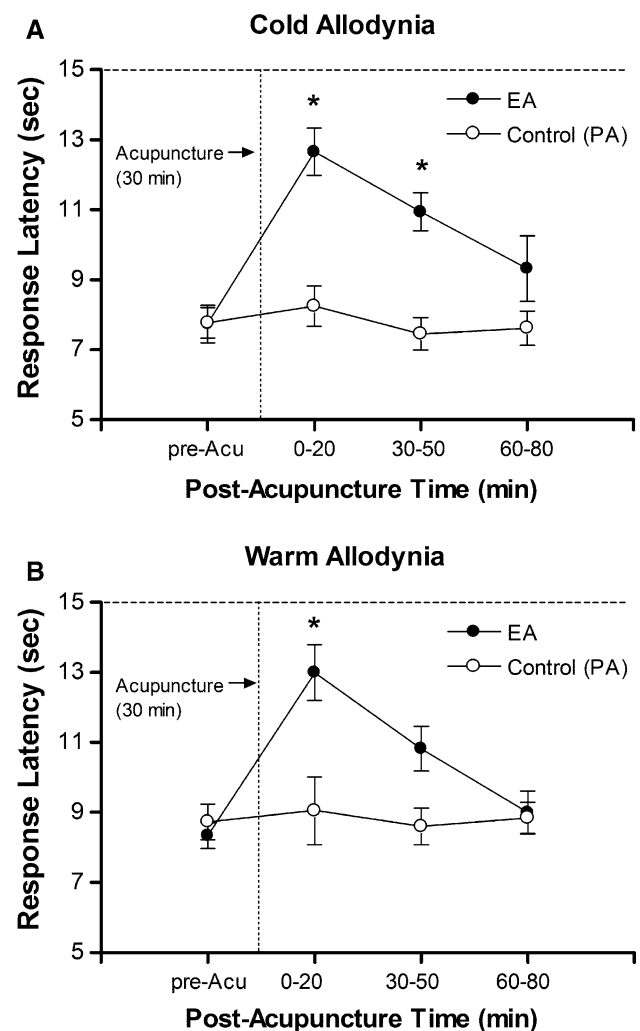
All data are presented as mean  $\pm$  SEM. For the statistical analysis, the two-way repeated measures analysis of variance followed by Bonferroni test was used. In all cases,  $P < 0.05$  was considered significant.

## Results

### Effects of EA on cold and warm allodynia in neuropathic rats

Preceding the nerve injury, the rats did not show an abrupt tail movement in response to the cold or warm water stimuli. After the neuropathic surgery, the rats showed an increased sensitivity to cold or warm water stimuli. We interpreted this as a sign of allodynia. The allodynia sign appeared 1 day after the nerve injury, with maximal allodynia being observed in the 2nd week [30, 40].

The relieving effects of EA stimulation on cold and warm allodynia are shown in Fig. 1. The tail response latency to cold and warm water stimuli increased



**Fig. 1** The effects of EA on cold and warm allodynia in neuropathic rats. The behavioral tests for cold (a) and warm (b) allodynia were performed before and after the EA stimulation. Data are presented as mean  $\pm$  SEM. \* $P < 0.05$ , EA versus control (PA) by the Bonferroni test following the two-way repeated measures ANOVA. Each group,  $n = 6$

maximally at 0–20 min after the EA stimulation. This increased response latency gradually decreased and returned to the baseline latency at 60–80 min after the EA. In contrast, the control (PA) group did not show any change in response latency. Similar to the previous report [30], there was a statistically significant difference in response latency between the EA and control (PA) groups for up to 50 min after the EA stimulation in the cold allodynia test ( $P < 0.05$ ; Fig. 1a). In the warm allodynia test, a significant difference in response latency between the two groups was observed at 0–20 min after the EA ( $P < 0.05$ , Fig. 1b).

#### Effects of non-selective muscarinic and nicotinic antagonists on EA-induced antiallodynia

To determine whether spinal nicotinic or muscarinic receptors mediate the relieving effects of EA on cold and warm allodynia, the effects of i.t. administration of atropine and mecamylamine on EA-induced antiallodynic action were examined (Fig. 2). Each of the drugs had no significant effects on cold (Fig. 2a) and warm allodynia signs (Fig. 2b) when administered intrathecally at the same dose without EA stimulation ( $P > 0.05$ , vs. saline). The relieving effects of EA on cold (Fig. 2a) and warm allodynia (Fig. 2b) were completely blocked by the i.t. pretreatment of non-selective muscarinic receptor antagonist atropine

(cold  $P < 0.05$ , warm  $P < 0.01$ , vs. saline), but not by the nicotinic receptor antagonist mecamylamine ( $P > 0.05$ , vs. saline).

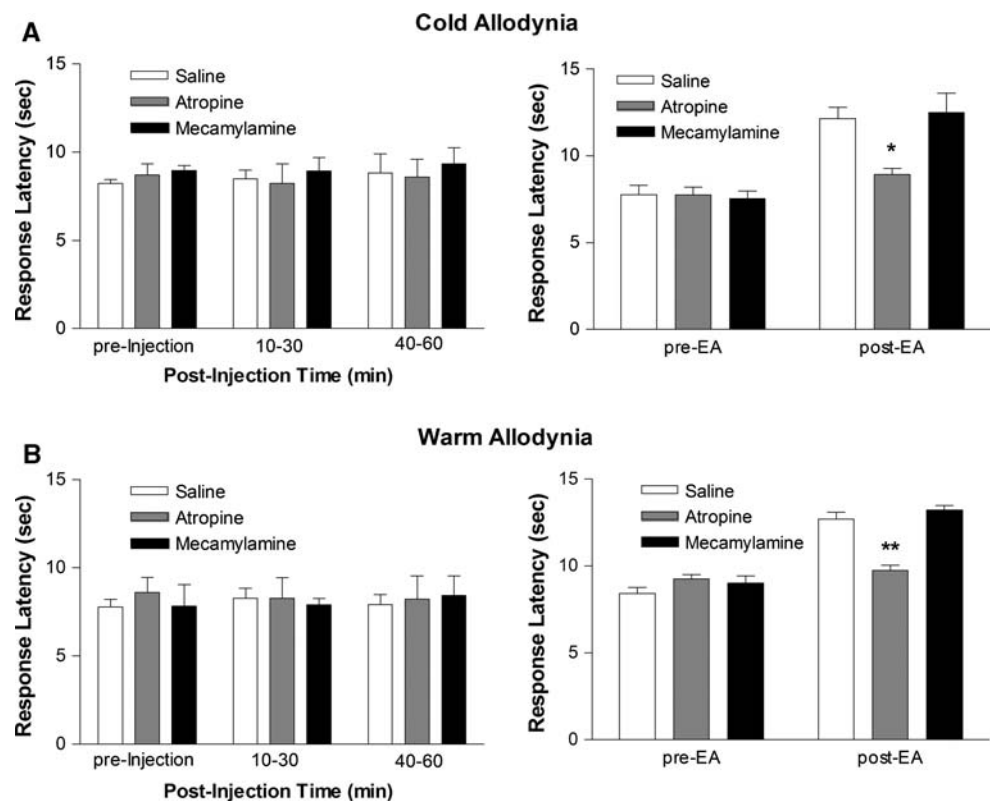
#### Effects of muscarinic receptor subtype antagonists on EA-induced antiallodynic action

To further determine which muscarinic receptor subtype plays a major role in the antiallodynic action of EA, selective antagonists against  $M_1$ ,  $M_2$  and  $M_3$  muscarinic receptors were used (Fig. 3). The i.t. injection of pirenzepine ( $M_1$  muscarinic receptor antagonist) completely blocked the relieving effects of EA on cold (Fig. 3a;  $P < 0.05$ , vs. saline) and warm allodynia (Fig. 3b;  $P < 0.01$ , vs. saline), whereas methoctramine ( $M_2$  muscarinic antagonist) and 4-DAMP ( $M_3$  muscarinic antagonist) did not ( $P > 0.05$ , vs. saline). None of the antagonists showed any significant effects on allodynia signs when administered intrathecally at the same doses without EA stimulation ( $P > 0.05$ , vs. saline).

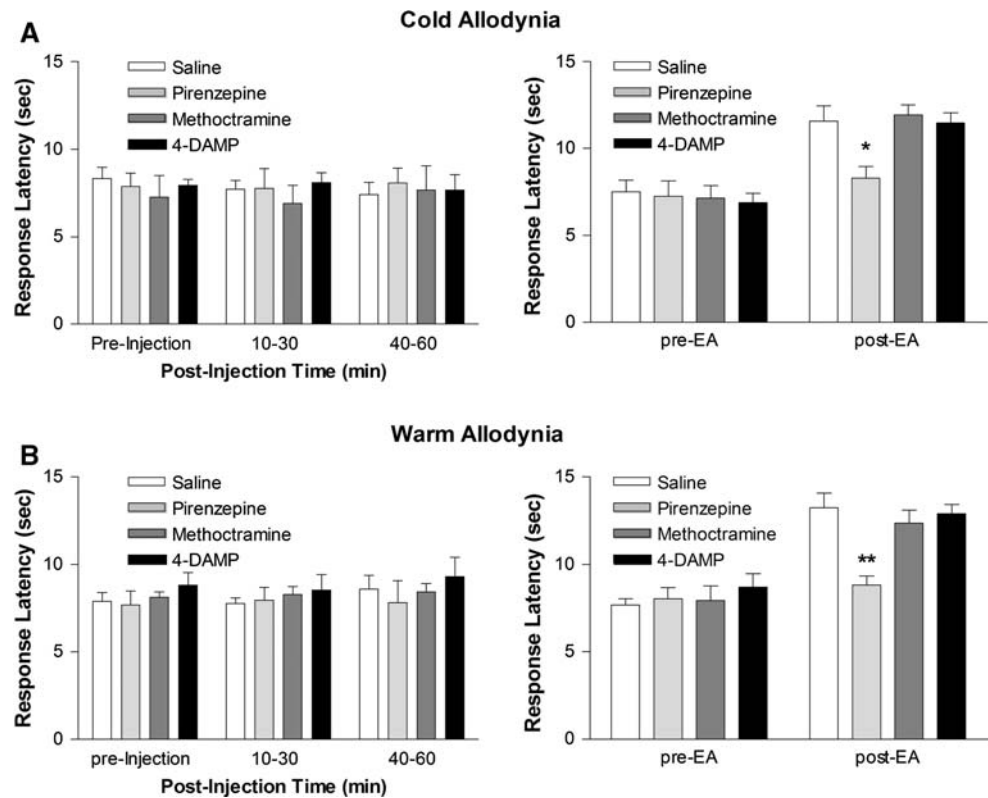
#### Discussion

Considerable evidence indicates that acupuncture or EA increases experimental pain thresholds in animals as well as in humans and that EA is more effective than PA

**Fig. 2** The effects of muscarinic and nicotinic receptor antagonists on EA-induced antiallodynic action. Atropine, non-selective muscarinic receptor antagonist. Mecamylamine, non-selective nicotinic receptor antagonist. Data are presented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , versus saline by the Bonferroni test following the two-way repeated measures ANOVA. Each group,  $n = 6$



**Fig. 3** The effects of muscarinic receptor subtype antagonists on EA-induced antiallodynic action. *Pirenzepine*: M<sub>1</sub> muscarinic receptor antagonist; *Methoctramine*: M<sub>2</sub> muscarinic receptor antagonist; *4-DAMP*: M<sub>3</sub> muscarinic receptor antagonist. Data are presented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , versus saline by the Bonferroni test following the two-way repeated measures ANOVA. Each group,  $n = 6$



[20, 21, 28, 49]. Previous studies also have shown that EA can relieve several types of persistent pain, such as inflammatory and ankle sprain pain [2, 32–34]. Interestingly, several clinical studies have reported that acupuncture or acupuncture-like stimulations, including EA, transcutaneous electrical nerve stimulation (TENS) and percutaneous electrical nerve stimulation, is effective for the neuropathic pain of malignancy [14] and diabetic neuropathy [1, 19], and phantom limb pain [5, 16]. Furthermore, animal studies by other researchers and by our own team demonstrated that EA significantly relieved mechanical and heat hyperalgesia [9], mechanical allodynia [23, 25, 29] and cold allodynia [30] in the rat models of neuropathic pain. In line with those studies, the present study showed that EA significantly relieved the behavioral signs of cold allodynia as well as warm allodynia in a rat model of neuropathic pain. Taken together, it can be proposed that EA may be a useful alternative therapeutic option to the conventional analgesics for the management of neuropathic pain.

The involvement of descending pain inhibitory systems is the most known mechanism of EA-induced analgesia [15, 20, 35, 48]. It is widely accepted that descending inhibition is translated into antinociception in the spinal cord mainly by activation of opioidergic, adrenergic, serotonergic and cholinergic receptors [12, 13, 39]. Results from our previous studies indicate that the antiallodynic

effects of EA are mediated by activation of spinal  $\mu$ - and  $\delta$ -opioid receptors,  $\alpha_2$ -adrenergic receptor, and 5-HT<sub>1A</sub> and 5-HT<sub>3</sub> receptors [29, 30], which is generally consistent with other studies using different pain models [4, 33, 47, 51]. However, there has been little information on the spinal cholinergic mechanism of EA-induced analgesia, especially in the neuropathic pain.

In this study, an i.t. administration of non-selective muscarinic, but not nicotinic, receptor antagonist blocked the relieving effects of EA on cold and warm allodynia. This suggested that the EA-induced antiallodynia in a rat model of neuropathic pain is mediated by activation of spinal inhibitory receptors, including cholinergic muscarinic receptors. Similarly, some previous studies reported that systemic administration of atropine prevented the analgesic effects of EA [2, 50]. Cholinergic innervation of the spinal dorsal horn is primarily intrinsic, and both the nicotinic and muscarinic receptors are located in the superficial and deep dorsal horn, in which nociceptive information is transmitted and modulated [8, 12, 39]. However, the role of spinal nicotinic receptors in antinociception is highly controversial, and a large majority of studies indicates that antinociceptive and antiallodynic effects of cholinergic drugs are mediated mainly by the muscarinic receptors, but not by the nicotinic receptors [12, 24, 26, 41, 45].

Currently, five classes of muscarinic receptor have been identified (i.e., M<sub>1–5</sub>). The subtypes implicated in the spinal



nociceptive transmission and modulation are mainly  $M_1$ ,  $M_2$  and  $M_3$  [12, 26, 42, 52]. The data presented here indicate that EA stimulation activates spinal  $M_1$  muscarinic receptors to relieve cold and warm allodynia signs. This finding is in agreement with prior pharmacological studies showing that  $M_1$  receptor subtype mediates spinal antinociception and antiallodynia [17, 22, 24, 31, 42, 45]. However, neither  $M_2$  nor  $M_3$  receptor antagonists reversed the antiallodynic effects of EA in this study. In fact, the effects of  $M_2$  and  $M_3$  receptor antagonists on analgesic actions of cholinergic agents have been reported as variable [18, 24, 26, 36, 42]. These conflicting findings may be due to the different pain models, testing methods and drugs used. For example, both the spinal  $M_1$  and  $M_3$  receptors are involved in analgesic effects of cholinergic agonists, cholinesterase inhibitors and TENS in the acute and inflammatory pain [42, 45], whereas only spinal  $M_1$  receptor mediates the antiallodynic effects of neostigmine (cholinesterase inhibitor) and clonidine ( $\alpha_2$ -adrenergic agonist) in the neuropathic pain [24, 31].

This study did not include an active control group (e.g., non-acupoint EA stimulation) because it was not intended to determine the acupoint (ST36)-specific effect of EA on neuropathic pain. In addition, our previous studies, using the same rat model of neuropathic pain, have shown that EA stimulation at a non-acupoint does not produce a significant antiallodynic effect [25, 29, 30]. Under our experimental conditions (neuropathic model and EA stimulation parameters), the relieving effect of EA on mechanical allodynia was weaker and shorter than its effect on cold allodynia [29, 30]. The present study thus focused on the effect of EA on cold and warm allodynia, not on mechanical allodynia. However, since the effect of EA on mechanical allodynia was statistically significant [29], it is also of interest to investigate the spinal cholinergic mechanism of EA on mechanical allodynia.

In conclusion, the data from this study clearly show that the antiallodynia produced by EA is mediated by spinal muscarinic, but not nicotinic receptors, and indicate that the  $M_1$  muscarinic receptor subtype plays an important role in mediating the EA-induced antiallodynia in a rat model of neuropathic pain. This study provides evidence for mediation by spinal cholinergic receptors in the relieving effects of EA on cold and warm allodynia in neuropathic rats. To date, we have revealed the involvement of spinal  $\mu$ - and  $\delta$ -opioid [29],  $\alpha_2$ -adrenergic, 5-HT<sub>1A</sub> and 5-HT<sub>3</sub> [30], and  $M_1$  muscarinic receptors in the EA-induced antiallodynia. Since a functional interrelationship exists among the opioidergic, noradrenergic, serotonergic and cholinergic systems in the spinal dorsal horn and supra-spinal level [12, 39], further studies are needed to determine how those receptors interact and to which degree

each receptor system contributes independently to EA-induced antiallodynia.

**Acknowledgments** This work was supported by the Acupuncture, Moxibustion and Meridian Research Project of Korea Institute of Oriental Medicine in 2006–2007 (K06070). Sun Kwang Kim was supported by the Graduate Research Scholarship from Graduate School of Kyung Hee University in 2007.

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