J Acupunct Meridian Stud 2015;8(4):175–182

Available online at www.sciencedirect.com

Journal of Acupuncture and Meridian Studies

journal homepage: www.jams-kpi.com

RESEARCH ARTICLE

Acupuncture Suppresses Morphine Craving in Progressive Ratio Through the GABA System

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Available online 9 April 2015

Received: Feb 2, 2015
Revised: Mar 26, 2015
Accepted: Apr 2, 2015

KEYWORDS
acupuncture;
GABA;
morphine;
progressive ratio;
self-administration;
SI5

Abstract
Previous studies revealed that acupuncture suppressed both morphine self-administration and morphine-seeking behavior after abstinence. Based on these results, this study examined whether acupuncture attenuated morphine-craving under a progressive ratio (PR) schedule and investigated the possible neuronal mechanism. Male Sprague-Dawley rats were trained to self-administer morphine (0.5 mg/kg) at a fixed ratio for 9 days, and rats who achieved stable infusion were switched to a PR schedule. When animals had taken no more morphine for 1 hour, the number of infusions was defined as the break point (BP). After PR training, animals that had established a stable BP received acupuncture the next day. Acupuncture was applied for 1 minute immediately before the test session. Bicuculline (1.0 mg/kg) and SCH 50911 (2.0 mg/kg) were given 30 minutes prior to acupuncture. The c-Fos levels in the ventral tegmental area (VTA) and the nucleus accumbens (NAc)

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pISSN 2005-2901 eISSN 2093-8152
http://dx.doi.org/10.1016/j.jams.2015.04.001

were examined. Acupuncture at SI5 reduced the BP significantly. Moreover, the effects of acupuncture were blocked by either bicuculline or SCH 50911. Immunofluorescence revealed that acupuncture at SI5 decreased c-Fos expressions in the VTA and the NAc. This study demonstrates that acupuncture at SI5 is effective for the treatment of morphine-craving and that this effect is mediated via the GABA pathway.

1. Introduction

Morphine is a representative pain killer widely used as an analgesic [1]. This analgesic effect of morphine is caused by the enhanced release of dopamine (DA) at the nucleus accumbens (NAc) of mesolimbic system in midbrain. However, enhancement of DA release in the NAc tends to induce a reinforcing effect when repeatedly used, driving a participant into abuse and dependence [2]. Therefore, morphine use must be restricted carefully, and the development of effective therapies to control the morphine dependence is required.

Acupuncture, a well-known traditional therapy in eastern Asia, has been gaining interest as a useful therapy in drug addiction [3,4]. A number of studies have shown that acupuncture ameliorate the behavioral disorder [5–9], abnormal Fos expression [10,11], and the abnormalities of neuronal function in the brain [12–14] caused by morphine.

Most of all, acupuncture has shown to suppress morphine self-administration [15]. In particular, our previous studies demonstrated that acupuncture at SI5 reduced morphine-seeking behavior following abstinence [16,17]. However, all of these self-administration studies evaluating acupuncture effects used the fixed ratio (FR) schedule and no study has examined the effect of acupuncture using a progressive ratio (PR) schedule.

In self-administration studies, the schedule can be divided into FR and PR. FR is appropriate for evaluating pharmacological effects, whereas PR is optimal for clinical application because it is usually used to evaluate the severity of the reinforcing powers of abused drugs [18].

Therefore, the present study was designed to further examine whether acupuncture could suppress the reinforcing power of morphine in a PR schedule.

In addition, many studies assessing the effects of acupuncture on morphine revealed a relation with the gamma aminobutyric acid (GABA) system [7,15,19,20].

Therefore, we also investigated the possible neuronal mechanism in the effects of acupuncture, focusing on the GABA pathway.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Daehan Animal, Seoul, Korea) weighing 270–300 g at the beginning of the experiment were used. Rats were housed in an environment of 12 hours light–dark cycle (light on at 7:00 AM), a room temperature of 22 ± 2°C, and a humidity of 60 ± 2% with free access to food and water. After a 3-day adaptation period, animals were used for experiments. Stress was minimized throughout all experiments in accordance with the protocols approved by the Institutional Animal Care and Use Committee at Daegu Haany University, Daegu, Republic of Korea.

2.2. Apparatus

Animals self-administered food and morphine in the operant chambers (Fig. 1) sound-attenuated by double cubicles (inner plastic and outer wood) and ventilated (Med Associates, St. Albans, VT, USA). The operant chamber was equipped with two lights. House lights were mounted on a wall, and cue lights were located above the active lever on the opposite wall. Inactive lever was installed under the house light, which was illuminated when the session started and then turned off for 15 seconds when animals pressed the active lever. Cue lights were turned on for 5 seconds when animals pressed the active lever. Following the disappearance of cue lights, rats spent 10 seconds in darkness of "time-out" (TO). During the TO period, both lever responses were recorded but produced no result. The response of inactive lever was recorded but made no consequence. When an animal pressed the active lever, a signal was delivered to the computer installed with experiment program (Schedule manager, Med Associates). Then, a motor pump located outside the operant chamber pushed the syringe and the morphine solution in the syringe was delivered to the animal's jugular vein via swivel (Med Associates) and Tygon tubing (Saint-Gobain PPL Corp., Aurora, HO, USA) according to the experimental program. The Tygon tubing extended from the swivel and was shielded by a metal spring; it was secured to a screw embedded in a catheter assembly on the back of the animals.

2.3. Food training

Rats that had acclimatized to the experimental environment for 3 days were trained to press the active lever for food pellets (45 mg, Bio-serve, Frenchtown, NJ, USA) under an FR 1 schedule. They were required to achieve 100 food pellets by pressing the active lever spontaneously within 3 hours, except for the 1st day when they were exposed to an overnight schedule to promote learning to use the active lever. The house light was not illuminated, but the cue light turned on. Food training was performed once a day, and rats were given food restriction (70% of the daily intake) during food training to facilitate learning the active lever. When the animal had taken 100 food pellets for 3 consecutive days, it underwent intravenous catheter implantation surgery.

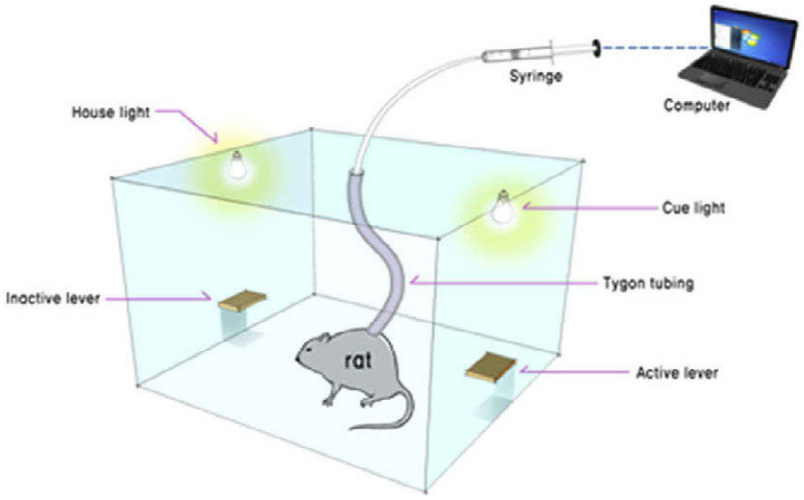

Figure 1 Schematic of the operant chamber.

2.4. Surgery

Rats that had succeeded in food training (achieve 100 food pellets for 3 consecutive days) were allowed free access to food and water for 3 days. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg), and a chronic silastic catheter (Dow Corning, Midland, MI, USA; 0.02″ ID × 0.037″ OD) coated with tridodecylmethyl ammonium chloride heparin (Polysciences Inc., Warrington, PA, USA) was implanted surgically into the right jugular vein and fixed with mersilene surgical mesh (Ethicon Inc., Somerville, NJ, USA). The catheter was exteriorized in the back of rats using 22 gauge guide cannulae (Plastics One, Roanoke, VA, USA) through skin incision. Silastic tubing and guide cannula were embedded in dental cement and secured with Prolene surgical mesh. 0.2 mL of saline was infused into the catheter once a day during recovery period, and heparin (30 U/mL) was contained to prevent clogging and maintain patency.

2.5. Morphine training

2.5.1. FR

After at least 1 week of recovery period, animals were trained to self-administer morphine hydrochloride (JEIL Pharmaceutical Co. Ltd., Daegu, Korea) using daily 2 hours session. 0.2 mL of saline containing heparin (30 U/mL) was flushed into the catheter immediately before and after each daily session to maintain patency of the silastic tubing. If rats pressed the active lever, 0.1 mL of morphine (0.5 mg/kg per infusion) was infused for 5 seconds. The dose of 0.5 mg/kg was based on a previous study [21]. Morphine training was performed initially under an FR 1 schedule for 3 days. Thereafter, the FR was increased to 3 on Days 4—6 and increased to 5 on Days 7—9.

2.5.2. PR

At the end of the FR training, animals that had taken morphine stably proceeded to the PR schedule. The number of active lever presses required for the morphine infusion was escalated as follows: 1, 2, 4, 6, 10, 14, 20, 26, 34, 44, 56, 70, 86, 104, 124, 154, 194, 244, and 304. When animals did not press the active lever for 1 hour, the session was finished, and the number of infusions at the finish was defined as the break point (BP). After 10 days of PR training, animals that had established a stable baseline BP (variation < 20% of the mean of 3 consecutive days) received acupuncture treatment on the next day of baseline (Fig. 2).

2.6. Acupuncture

Acupuncture treatment was performed bilaterally for 1 minute at each acupoint immediately before the start of test session. Stainless steel needles (0.18 mm diameter, 8 mm length; Dongbang Acupuncture Inc., Chingdao, China) were inserted vertically into a depth of 2—3 mm, and stimulations were made by bidirectional twisting of the needle at a frequency of twice per second for a total of 2 seconds as the needle was inserted and withdrawn from the acupoints. Acupuncture treatment was performed in awaken state without anesthetization under a slight movement restriction. Animals received daily handling for 2—3 minutes throughout all experiments to minimize the stress from the movement restriction.

Figure 2 Schematic of the schedule of experiment.

2.7. Experimental design

2.7.1. The first experiment: effects of acupuncture

Rats of SI5 group ($n = 8$) were given acupuncture at the bilateral SI5 points located on the posteromedial aspect of the wrist, in the depression between the triquetrum bone and the ulnar styloid process [22]. Rats of LI5 group ($n = 8$) received acupuncture at bilateral LI5 points located on the posterolateral aspect of the wrist, at the radial side of the dorsal wrist crease, distal to the radial styloid process, in the depression of the anatomical snuffbox [22]. Rats of the control group ($n = 8$) received the same treatment as the acupuncture group without needle stimulation. Acupuncture treatments were performed after establishment of baseline using a counter balance design according to random assignment.

2.7.2. The second experiment: neuronal involvement

To further investigate the possible neuronal mechanism associated with the effects of acupuncture, animals were assigned into following groups. The control group ($n = 6$) and SI5 group ($n = 6$) were given the same treatment as the first experiment. The SI5 + bicuculline (BIC) group ($n = 6$) received acupuncture at SI5 and the selective $GABA_A$ receptor antagonist BIC (Tocris, Ellisville, MO, USA; 1 mg/kg), which was injected intravenously 30 minutes prior to acupuncture. The SI5 + SCH 50911 (SCH) group ($n = 6$) received acupuncture at SI5 and the selective $GABA_B$ receptor antagonist SCH (Tocris; 2 mg/kg), which was injected intravenously 30 minutes prior to acupuncture. The BIC group ($n = 6$) and SCH group ($n = 6$) received intravenous injections of BIC or SCH, respectively, without acupuncture stimulation.

2.8. Immunofluorescence — examination of c-Fos expression

To confirm the suppressive effects of acupuncture on morphine-craving, c-Fos expression was examined. Rats received acupuncture immediately after the self-administration on the day after the last test and were anesthetized with pentobarbital (50 mg/kg, i.p.). Following transcardiac perfusion with 4% paraformaldehyde in 0.1M sodium phosphate buffer, brains were fixed and cryoprotected in 30% sucrose solution. Frozen brains were cut into 30 μm-thick sections using a cryostat at −20°C. Free-floating sections were then incubated sequentially as follows: 1 hour in 0.3% Triton X-100 and 5% normal donkey serum in PBS, 24 hours at 4°C with rabbit polyclonal antic-Fos antibodies (1:500, sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 2 hours at room temperature with Alexa Fluor 488 donkey antirabbit immunoglobulin G (1:200, Life Technologies Ltd.). The c-Fos immunoreactivity positive cells were counted in both hemispheres at ×400 magnification by a blinded researcher.

2.9. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and *post hoc* Tukey test. The statistical significance was regarded with $p < 0.05$.

3. Results

In the first experiment to evaluate the effects of acupuncture on morphine craving, the basal levels of infusion (means of the BP of the last 3 consecutive days of the PR period) before tests were compared among groups to determine whether the amount of morphine taken by animals were significantly different. One-way ANOVA and *post hoc* Tukey test revealed that there was no significant difference among groups ($p > 0.342$; Fig. 3A).

However, acupuncture has suppressed morphine-craving on the test session. The BP on the test session was significantly ($^{**}p < 0.01$) suppressed by SI5 acupuncture compared with the control group, and this effect proved to be different from LI5 ($^{\#}p < 0.05$, Fig. 3B). This suppression was also shown in the number of active lever response ($^{*}p < 0.05$, Fig. 3C). In addition, the number of inactive lever response was reduced by SI5 (1.50 ± 0.98) compared with the control group (6.13 ± 2.40) but not significant (not shown). When BPs were compared with the basal levels, the similar suppression by SI5 was exhibited ($^{**}p < 0.01$, $^{\#}p < 0.05$, Fig. 3D).

In the second experiment performed to investigate the possible neuronal mechanism underlying the effects of acupuncture, basal level (mean of the BP of the last 3 consecutive days of PR period) did not show significant difference ($p > 0.264$, Fig. 4A).

However, on the test session, the BP decreased by SI5 ($^{***}p < 0.001$) was reversed by BIC ($^{\#\#\#}p < 0.001$) and SCH ($^{\$\$\$}p < 0.001$, Fig. 4B). In the same manner with the BP, the number of active lever response and the percentage of BP compared with the basal level were reversed by BIC and SCH (Figs. 4C and 4D).

In the immunohistochemistry performed to confirm the acupuncture effect, acupuncture at SI5 suppressed c-Fos expression in both VTA and NAc (Figs. 5A and 5B). Representative images of c-Fos expression are shown in Figs. 6 and 7.

4. Discussion

In the first experiment designed to investigate whether acupuncture suppresses morphine-craving behavior in PR schedule, animals trained to self-administer morphine took similar amounts of morphine (Fig. 3A). However, acupuncture at the specific acupoint SI5, but not at the control acupoint LI5, suppressed morphine-craving. The BP was decreased by 37.21% of the control group (Figs. 3B and 3D). A similar suppressive effect of acupuncture was observed in the active lever response (Fig. 3C). These observations are consistent with our previous studies demonstrating suppressive effects of acupuncture at SI5 on morphine-seeking behavior after abstinence [16,17].

SI5 is the River point as well as the Fire point of the Small Intestine meridian. This point is generally used to control the balance between the Heart meridian and the Small Intestine meridian, and is often selected for stress-related problems [23]. In Korean medicine, among the five phases, brain functions belong to Fire, and according to the meridian theory, Fire corresponds to the Heart and Small Intestine meridian. Therefore, results of the first experiment

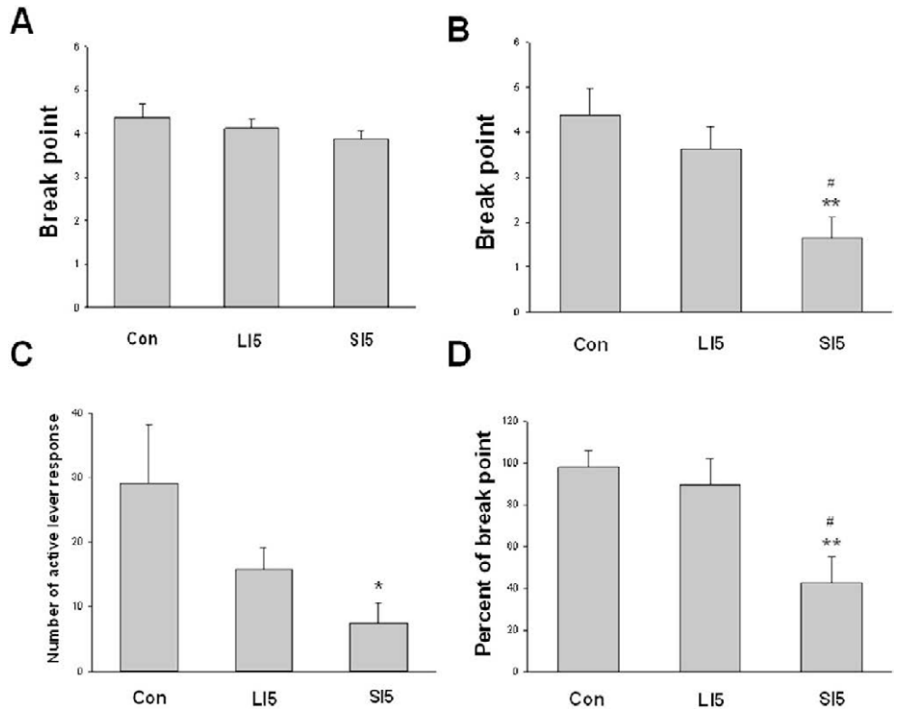

Figure 3 Results of the first experiment. (A) Basal levels (means of the BPs of the last 3 days of PR session before the test); (B) BPs on the test session; (C) active lever responses on the test session; and (D) percentages of BP compared with the basal levels. Results are mean ± SEM, one-way ANOVA, and *post hoc* Tukey test. BP = break point; PR progressive ratio; SEM = standard error of mean; ANOVA = analysis of variance.

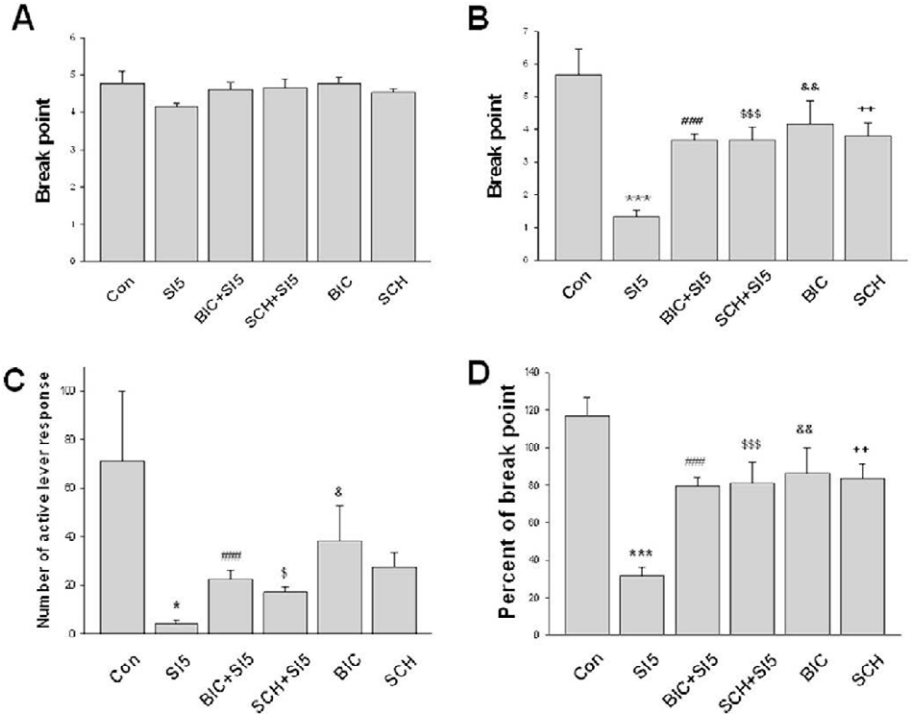

Figure 4 Results in the second experiment. (A) Basal levels (means of the BPs of the last 3 days of PR session before the test); (B) BPs on the test session; (C) active lever responses on the test session; and (D) percentages of BP compared with the basal levels. Results are mean ± SEM, one-way ANOVA, and *post hoc* Tukey test. BP = break point; SEM = standard error of mean; ANOVA = analysis of variance.

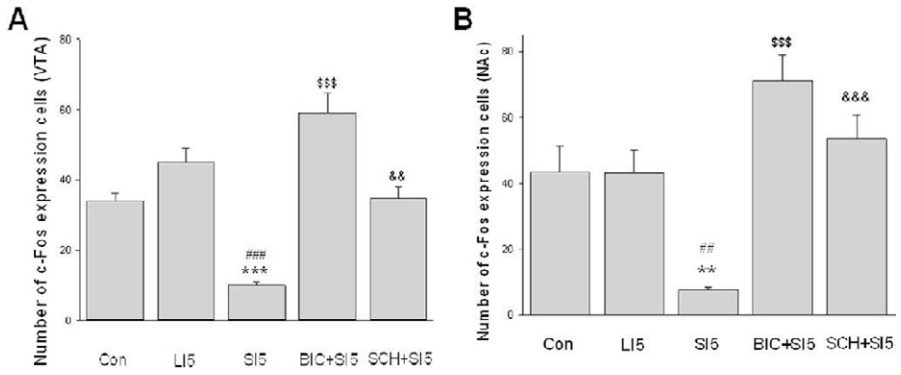

Figure 5 Numbers of c-Fos reactive cells. (A) VTA and (B) NAc. VTA = ventral tegmental area; NAc = nucleus accumbens.

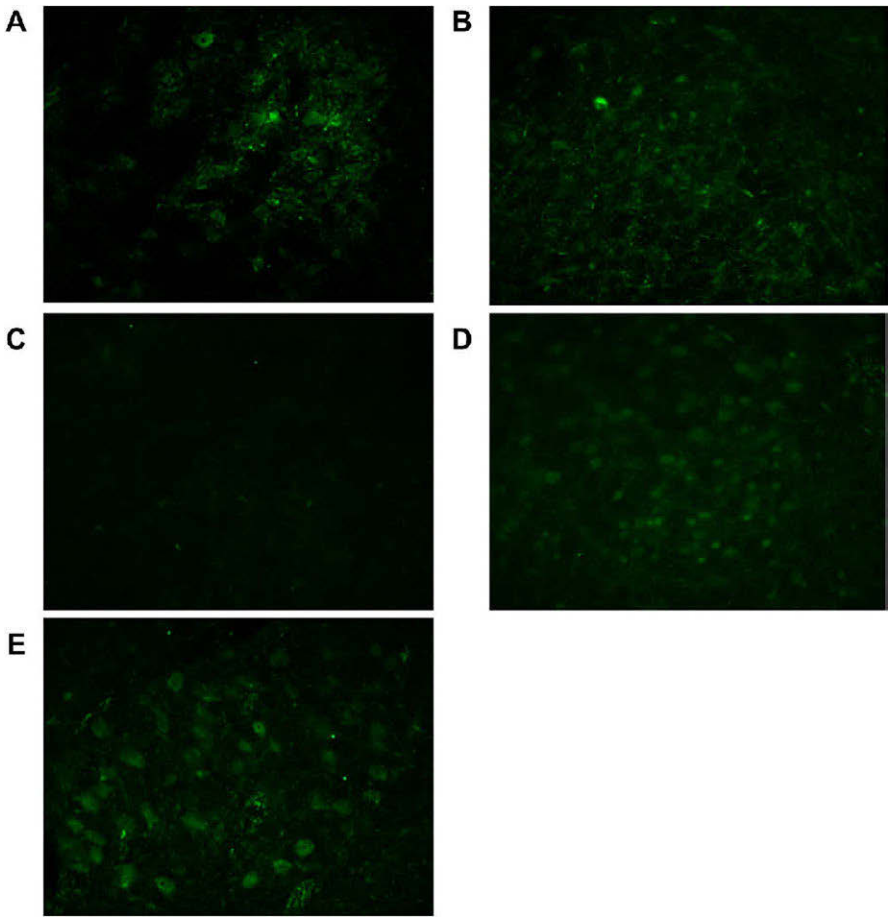

Figure 6 Representative c-Fos expression in VTA. (A) control group; (B) LI5 group; (C) SI5 group; (D) BIC + SI5 group; and (E) SCH + SI5 group. Magnification ×400. VTA = ventral tegmental area.

that SI5 acupuncture suppressed the BP in morphine PR schedule are explained by the meridian theory of Korean medicine.

LI5, the River point and the Fire point of the Large intestine meridian, is located on the wrist joint similarly to SI5. However, the Large Intestine meridian corresponds to Metal among the five phases and is less associated with psychiatric disorders than Small intestine meridian. Therefore, LI5 is thought to be ineffective.

In self-administration studies, an FR schedule is usually used to investigate pharmacological functions, whereas PR is generally used to evaluate the reinforcing powers of abused-drugs [18]. In this study, acupuncture at SI5 reduced the BP suggesting that it has potential to treat morphine-craving.

In the second experiment designed to investigate the neuronal mechanism, acupuncture at SI5 exerted suppressive effects on BP, similarly to the first experiment.

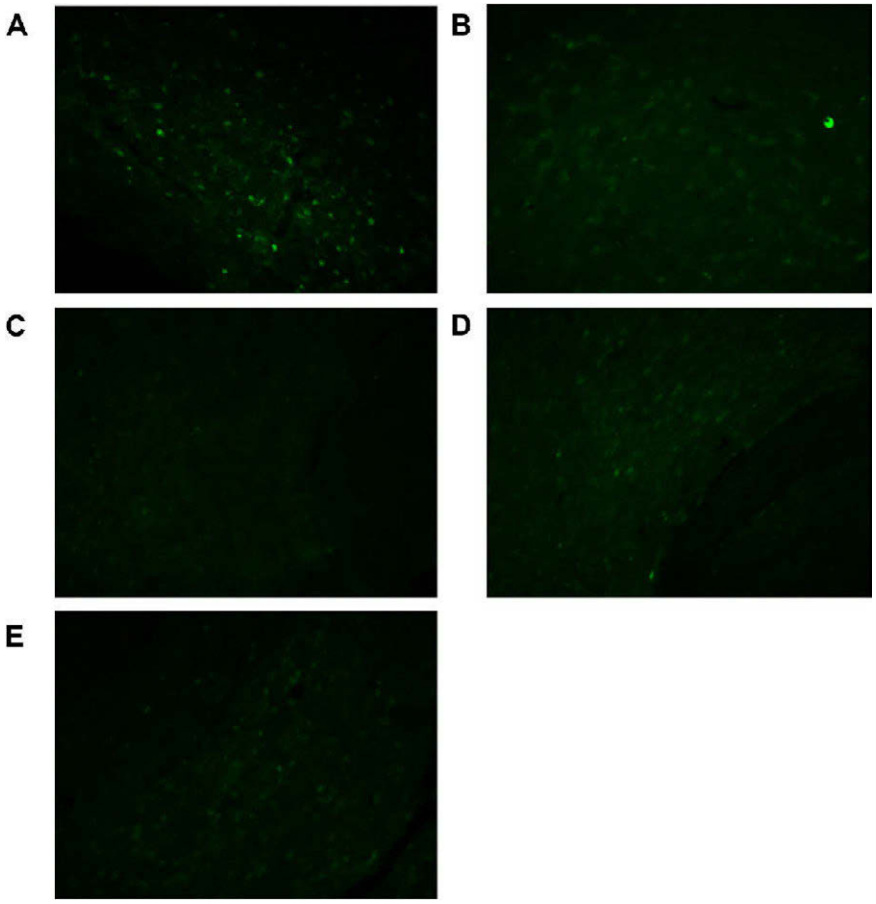

Figure 7 Representative c-Fos expression in NAc. (A) control group; (B) LI5 group; (C) SI5 group; (D) BIC + SI5 group; and (E) SCH + SI5 group. Magnification ×400. NAc = nucleus accumbens.

However, this suppression was reversed by pretreatment with the selective $GABA_A$ receptor antagonist BIC or the selective $GABA_B$ receptor antagonist SCH (Fig. 4B). SI5 reduced BP by 23.46% of that in the control group, which was restored to 64.72% by BIC and SCH. A similar reversal was observed in the active lever response and the percentage of infusion compared with the basal level (Figs. 4C and D). These results suggest that the effects of acupuncture at SI5 on morphine-craving are mediated, at least in part, through GABAergic pathway.

In the mesolimbic system of midbrain, DAergic neurons, which are representative excitatory neurons, project from the VTA to the NAc. Enhancing these neurons reinforces the effects of drugs abused. By contrast, activation of the $GABA_A$ and $GABA_B$ receptors on the DAergic neurons inhibits the DA pathway and attenuates drug abuse. The current results, whereby SI5 acupuncture's inhibition of morphine-craving was blocked by GABA receptor antagonists, may suggest that SI5 exerts suppressive effects via GABA receptors on DA neurons in the VTA.

In this study, the BIC and SCH alone groups were designed to investigate the influences of each GABA receptor antagonist on morphine-craving behavior. The results demonstrated that the antagonists alone had no significant difference from the control group. This suggests that neither GABA receptor antagonist affected morphine-craving, which is consistent with our previous studies [15—17].

Interestingly, the examination of c-Fos expression demonstrated that acupuncture at SI5 suppressed expression markedly (Figs. 5—7). In addition, these acupuncture effects were blocked by BIC and SCH. These neurochemical results are consistent with the behavioral data. Generally, c-Fos expression means neuronal activity. Our data revealed that SI5 acupuncture reduced c-Fos expression in both the VTA and NAc. These results suggest that acupuncture suppressed the DA neuron activity in rats craving for morphine.

Taken together, these data demonstrated that acupuncture at SI5 reduced the BP markedly in a PR schedule of morphine, and these effects were reversed by $GABA_A$ and $GABA_B$ receptor antagonists. In addition, these results were confirmed by examining c-Fos expression in the VTA and NAc, eventually suggesting that SI5 acupuncture suppressed morphine-craving via GABA receptor system in VTA DA neurons.

In addition, according to our previous studies, acupuncture has also shown suppressive effect on the abnormal locomotor activity induced by morphine [12,24], and the suppressive effects proved to be selective to morphine showing not effective on food-taking behavior [17]. Therefore, acupuncture seems to certainly have suppressive effects on morphine and this effect is not likely to be produced due to motor impairment or depressive function.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

Acknowledgments

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2010-0025821) and the Research Fund for Overseas & Returned Scholars from the Heilongjiang Provincial Department of Education, China (No. 1154h22).

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