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Research report

Involvement of opioid receptors in electroacupuncture-produced anti-hyperalgesia in rats with peripheral inflammation

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Abstract

Our previous study showed that electroacupuncture (EA) significantly attenuated inflammatory hyperalgesia. It has also been reported that EA analgesia in uninjured animals is mediated by μ and δ opioid receptors at 2–15 Hz and by κ opioid receptor at 100 Hz. Because persistent pain changes neural response to external stimulation, we hypothesized that (1) the mechanisms of EA anti-hyperalgesia may be different under conditions of persistent pain and that (2) combining EA with a sub-effective dose of morphine could enhance EA anti-hyperalgesia. Hyperalgesia, decreased paw withdrawal latency (PWL) to a noxious thermal stimulus, was induced by subcutaneously injecting complete Freund's adjuvant (CFA) into the hind paws of rats. Selective antagonists against μ (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-ThrNH2, CTOP), δ (naltrinodole, NTI) and κ (nor-binaltorphimine, BNI) opioid receptors were administered intrathecally 10 min before each of two EA treatments at acupoint Huantiao (GB30), one immediately post and the other 2 h post-CFA. Morphine was given (i.p.) 40 min before the second EA treatment. PWL was measured before and 2.5 and 5 h post-CFA. Both 10 and 100 Hz EA-produced anti-hyperalgesia were blocked spinally by μ- and δ- but not κ-receptor antagonists. EA combined with a sub-threshold dose of morphine (2.5 mg/kg) enhanced anti-hyperalgesia additively (10 Hz EA) or synergistically (100 Hz EA) compared to that produced by each component alone. These results suggest selective involvement of μ and δ, but not κ, receptors in EA-produced anti-hyperalgesia in rats. A combined EA and opioid drug protocol may provide an improved treatment strategy for inflammatory pain.

Theme: Sensory systems
Topic: Pain modulation: anatomy and physiology

Keywords: Acupuncture; Spinal cord; Freund's adjuvant; Analgesia; Inflammation; Pain

1. Introduction

It is documented that as many as 42% of patients use complementary and alternative medicine to fulfill their needs in lieu of or as an adjunct to conventional medicine [6], and patients are turning increasingly to acupuncture for pain relief. Our previous study with an animal model of inflammatory pain showed that electroacupuncture (EA) of 10 Hz/3 mA or 100Hz/3 mA at acupoint Huantiao (GB30) significantly attenuated hyperalgesia [16,17].

It has been reported that EA-produced analgesia is mediated by endogenous opioid systems in uninjured animal models [9]. However, there are distinct differences between transient and persistent pain. In contrast to the transient pain studied using uninjured animal models, persistent pain is associated with long lasting alterations of the nervous system [24,34]. Persistent pain sensitizes peripheral nociceptive receptors and increases the hyperexcitability of the central nervous system (e.g. spinal dorsal horn neurons) in relation to the transmission and modulation of noxious messages. Moreover, it is more clinically relevant: as acupuncture treatment is often used to relieve ongoing, chronic pain, it is worthwhile to investigate the mechanisms

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underlying the anti-hyperalgesic effect of EA under pathophysiological conditions.

Opioids are the mainstay of pharmacological therapy in the management of chronic pain [20]. However, adverse effects such as sedation, nausea, vomiting, constipation and respiratory depression limit their utility, especially at the higher doses often needed by patients with chronic pain [1,5]. Clearly, alternatives or adjunctives to opioid therapy would be clinically useful in the treatment of persistent pain. This study was designed to study one such adjunct, EA, to (1) investigate the mechanisms of its anti-hyperalgesic effects and to (2) examine the effect of combinations of EA and low-dose morphine on inflammatory hyperalgesia in a rat model of inflammatory pain.

2. Materials and methods

2.1. Intrathecal cannulation

Male Sprague–Dawley rats weighing 280–320 g (Harlan) were kept under controlled conditions (22 ± 0.5 °C, relative humidity 40–60%, 7 a.m. to 7 p.m. alternate light–dark cycles, food and water ad libitum). The animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine.

Rats were prepared for intrathecal injection and allowed to recover for 7 days after the operation prior to experimentation. Under pentobarbital sodium anesthesia (50 mg/kg i.p.), the atlanto-occipital membrane at the level between the head and neck (i.e., approximately the obex level) was exposed and a 7.5-cm length of PE-10 tubing was inserted into the subarachnoid space through a slit made in the membrane. The catheter was advanced to the level of the lumbar spinal cord and filled with saline (approximately 7–10 μl), and the outer end was plugged. Animals with gross signs of motor impairment were excluded from the study. At the end of the experiments, Evans blue was injected via the catheter and the location of the distal end of the catheter was verified.

2.2. Induction of hyperalgesia

Inflammation and hyperalgesia were induced by injecting complete Freund's adjuvant (CFA; Sigma, St. Louis, MO; suspended in an 1:1 oil/saline emulsion, 0.08 ml, 40 μg *Mycobacterium tuberculosis*) subcutaneously into the plantar surface of one hind paw of the rat using a 25-gauge hypodermic needle [26]. The inflammation, manifesting as redness, edema and hyper-responsiveness to noxious stimuli, was limited to the injected paw, appeared shortly after the injection, and lasted about 2 weeks. Hyperalgesia was determined by a decrease in paw withdrawal latency (PWL) to a noxious thermal stimulus.

2.3. Experimental procedures

Two sets of experiments were conducted: (1) EA plus opioid sub-receptor antagonists and (2) EA plus the opioid receptor agonist morphine.

In Experiment 1, rats were randomly divided into the following groups ($n = 7–9$ per group): (1) μ opioid receptor antagonist CTOP [D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-ThrNH2] (Sigma) plus 10 Hz (2 and 5 nmol in 10 μl, i.t.) or 100 Hz (5 nmol) EA; (2) δ opioid receptor antagonist, naltrindole hydrochloride (NTI; Sigma) plus 10 Hz (4 and 10 nmol in 10 μl, i.t.) or 100 Hz (10 nmol) EA; (3) κ opioid receptor antagonist, nor-Binaltorphimine dihydrochloride (BNI; Sigma) plus 10 Hz (5, 10 and 20 nmol in 10 μl, i.t.) or 100 Hz (20 nmol) EA; and (4) vehicle control (10 μl saline, i.t.). All antagonists were dissolved in saline and administered 10 min before each of the two EA treatments.

In Experiment 2, rats were divided into the following groups ($n = 7–9$ per group): (1) morphine sulphate (Sigma), 2.5, 5 and 7.5 mg/kg (0.5 ml, i.p.); (2) vehicle control (0.5 ml saline, i.p.); (3) morphine (2.5 mg/kg) plus 10 Hz EA; (4) saline plus 10 Hz EA; (5) morphine (2.5 mg/kg) plus 100 Hz EA; and (6) saline plus 100 Hz EA. Morphine was dissolved in saline and administered (i.p.) 40 min before the second of two EA treatments.

2.4. Acupuncture treatment

Previously determined EA parameters of low frequency (10 Hz) and high frequency (100 Hz) at 3 mA and 0.1 ms pulse width, which showed significant anti-hyperalgesic effects in the rat inflammation model [16,17], were chosen for the present study. The equivalent of human acupoint GB30 [19] on the rat's hind limbs was treated bilaterally. GB30 was chosen based on traditional Chinese medicine (TCM) meridian theory [19], its successful use in our previous studies, and its use in studies by others [16,17,29]. Our previous study [17] showed that EA at acupoint GB30, but not at Waiguan (also known as the fifth acupoint on the Triple Energizer Meridian), or at sham points, including the opposite aspect of GB30 and an abdominal point, showed significant anti-hyperalgesia. In humans, GB30 is located at the junction of the lateral 1/3 and medial 2/3 of the distance between the greater trochanter and the hiatus of the sacrum; underneath are the sciatic nerve, inferior gluteal nerve and gluteal muscles [4]. Waiguan is located 2 units (based on the standard acupuncture measurement of 12 units between the transverse cubital crease and the transverse wrist crease) above the dorsal crease of the wrist, between the radius and ulna. Underneath are the posterior interosseous nerve and the anterior interosseous nerve [4]. The sham point at the aspect opposite to GB 30 is located at the center of the medial aspect of the thigh, and the sham abdominal point is located 3-mm lateral to the umbilicus, bilaterally. The comparable landmarks were used to locate GB30, Waiguan, and the sham points in the rats.

The animals were gently handled for 30 min each day for 2–3 days and habituated to the acupuncture treatment before the experiment. After cleaning the skin with alcohol swabs, disposable acupuncture needles (gauge # 32, 0.5 in. in length) with electrodes soldered to their handles were swiftly inserted bilaterally, approximately one half inch deep, into GB30 by one investigator while another gently held the animal. The needles and the electrodes were stabilized with adhesive tape [17]. The procedure typically lasted less than 20 s and caused little distress to the animal. EA stimulation was delivered by an electrical stimulator (A300 Pulsemaster, World Precision Instruments) via an isolator (A360D Stimulus Isolator, World Precision Instruments), which converts electrical voltage into electrical current. While EA frequency was held constant, intensity was adjusted slowly (over the period of approximately 2 min) to the designated level of 3 mA, which is the maximum EA current intensity that a conscious animal could tolerate. Mild muscle twitching was observed. To maximize the anti-hyperalgesic effect and treat animals prophylactically, two 20 min EA treatments were given, once immediately after CFA administration and again 2 h post-CFA. During EA treatment, each rat was placed under an inverted clear plastic chamber (approximately $5 \times 8 \times 11$ in.) but was neither restrained nor given any anesthetic. The animals remained awake and still during treatment and gave no observable signs of distress. For sham control, acupuncture needles were inserted bilaterally into GB30 without electrical stimulation or manual needle manipulation. Sham EA showed little anti-hyperalgesia in our previous study [17] and seems to be an appropriate control for non-specific needling effects. The sham treatment and the EA-treated animals were handled identically.

2.5. Behavioral test

Rats were tested for hind paw thermal hyperalgesia by a method previously developed [10,16]. The rats were placed under a clear plastic chamber on the glass surface of the Paw Thermal Stimulator System (UCSD, San Diego) and allowed to acclimatize for 30 min. A radiant heat stimulus was applied from underneath the glass floor with a high intensity projector lamp bulb (CXL/CXR, 8 V, 50 W). The heat stimulus was directed onto the plantar surface of each hind paw, and the PWL to the nearest 0.1 s was automatically determined. The intensity of the thermal stimulus was adjusted to derive an average baseline PWL of approximately 10.0 s in naive animals. A 20-s cut-off was used to prevent tissue damage [10,16].

The mean PWL was established by averaging the latency of four tests with a 5-min interval between each test. PWL measurements were made pre-CFA and at two designated intervals post-CFA injection: 2.5 and 5 h. The investigator who performed the behavioral tests was blind to the treatment assignments.

Table 1
Effects of opioid antagonists CTOP, NTI and BNI on 10 Hz EA anti-hyperalgesia in rats with CFA-induced hind paw inflammation ($n = 7–9$)

Groups	Baselines	After 10 Hz EA or sham EA
Sham EA	10.37 ± 0.25	5.23 ± 0.31
EA + saline	10.13 ± 0.58	7.06 ± 0.71*
EA + 2 nmol CTOP	10.41 ± 0.27	6.79 ± 0.56*
EA + 5 nmol CTOP	10.01 ± 0.49	5.39 ± 0.42
EA + 4 nmol NTI	10.03 ± 0.77	7.29 ± 0.74*
EA + 10 nmol NTI	10.19 ± 0.41	5.59 ± 0.28
EA + 5 nmol BNI	10.29 ± 0.23	7.28 ± 0.50*
EA + 10 nmol BNI	10.62 ± 0.47	6.81 ± 0.34*
EA + 20 nmol BNI	10.49 ± 0.47	7.38 ± 0.45*

*$p < 0.05$ compared to sham EA group.

2.6. Data analysis

The results are presented as mean ± S.E.M. (Tables 1 and 2) or % change ± S.E.M. (Figs. 1 and 2). The % changes in Fig. 1 were calculated as (post-CFA PWL-baseline PWL)/baseline PWL × 100%. The % changes in Fig. 2 are presented as: (PWLs of experimental groups minus PWLs of control group)/(PWLs of control group) × 100%. The actual PWL data were used for two-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc test. $P < 0.05$ was considered significant in all cases. The effect of the combination of EA and morphine was compared to the sum of the effects produced by drug and EA alone. An additive effect is obtained if the effect is equal to the sum of the effects of each individual treatment given alone, while a synergistic effect is obtained if the effect of the EA-drug combination is significantly greater than the sum of the effects of each individual treatment given alone.

3. Results

3.1. EA anti-hyperalgesia

Before CFA injection, the overall mean baseline PWL to noxious heat stimuli was similar in all groups of rats, and there was no significant difference in PWL between left (10.13 ± 0.48 s) and right hind (10.43 ± 0.38 s) paws. Following a 0.08-ml injection of CFA into the left hind paw, its latency significantly decreased compared to that of the contralateral hind paw, which remained at the pre-CFA level. EA at both 10 Hz and 100 Hz (EA + saline groups) significantly increased the PWL of the CFA-injected hind paw, an anti-hyperalgesic effect, at 2.5 h post-CFA injection compared to sham control (Tables 1 and 2).

3.2. Effects of CTOP, NTI and BNI on EA anti-hyperalgesia

As shown in Table 1, CTOP dose-dependently prevented the anti-hyperalgesic effects of EA. CTOP at 5 nmol, but not

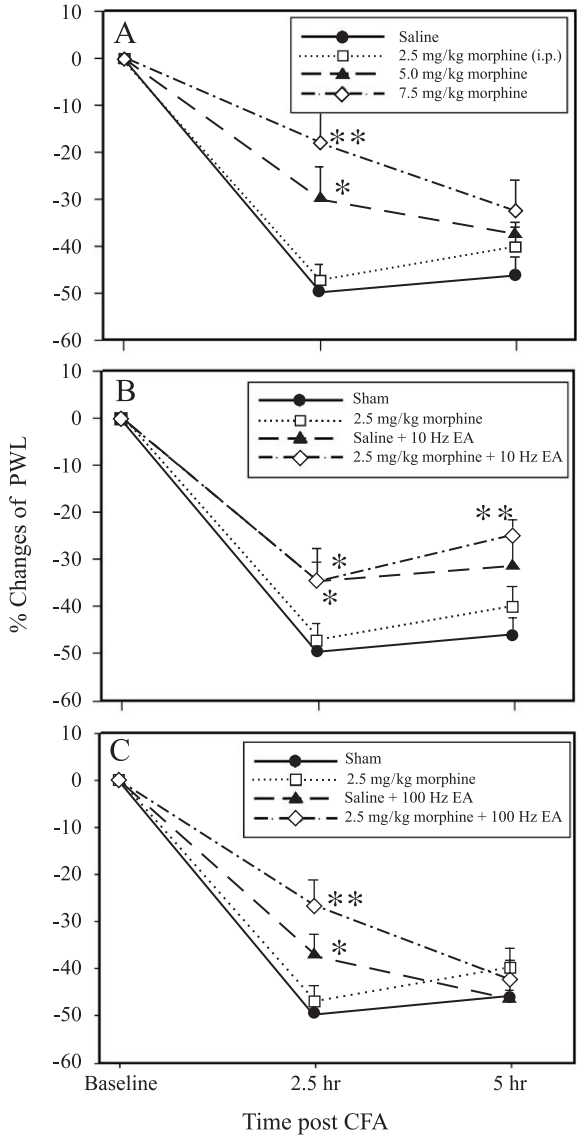

Fig. 1. Effects of EA and morphine on CFA-induced hyperalgesia. Morphine was dissolved in saline and administered (i.p.) 40 min before the second of two EA treatments. (A) Morphine at 2.5–7.5 mg/kg (i.p.) dose-dependently inhibited hyperalgesia. Both 5 and 7.5 mg/kg showed significant anti-hyperalgesia, while 2.5 mg/kg showed no anti-hyperalgesia. (B) A combination of 10 Hz EA and 2.5 mg/kg morphine more effectively inhibited hyperalgesia than either did individually at 5 h post-CFA, which suggests an additive effect. (C) The combination of 100 Hz EA and 2.5 mg/kg morphine inhibited the hyperalgesia to a significantly greater extent than 100 Hz EA did alone at 2.5 h post-CFA, which suggests a synergistic effect. *$P<0.05$ and **$P<0.01$ compared to saline (A) or sham control (B and C).

at 2 nmol, blocked 10 Hz EA-produced anti-hyperalgesia. The higher 5 nmol dose also blocked the anti-hyperalgesia produced by 100 Hz (Table 2). Similarly, NTI dose-dependently prevented EA anti-hyperalgesia: 10 nmol but not 4 nmol of NTI blocked the effect of the 10 Hz treatment (Table 1). NTI at 10 nmol also blocked the anti-hyperalgesic effect of 100 Hz (Table 2). In contrast, no dose of BNI (5, 10, 20 nmol) impeded the anti-hyperalgesic effects of either 10 (Table 1) or 100 Hz EA (Table 2).

Table 2
Effects of opioid antagonists CTOP, NTI and BNI on 100 Hz EA anti-hyperalgesia in rats with CFA-induced hind paw inflammation ($n=7–9$)

Groups	Baselines	After 100 Hz EA or sham EA
Sham EA	10.37 ± 0.25	5.23 ± 0.31
EA + saline	10.40 ± 0.39	6.71 ± 0.40*
EA + 5 nmol CTOP	9.88 ± 0.50	5.35 ± 0.52
EA + 10 nmol NTI	9.89 ± 0.41	4.68 ± 0.62
EA + 20 nmol BNI	10.44 ± 0.32	6.72 ± 0.55*

*$p<0.05$ compared to sham EA group.

3.3. Effects of combinations of EA and morphine on hyperalgesia

As shown in Fig. 1A, morphine dose-dependently inhibited CFA-induced hyperalgesia: 7.5 mg/kg showed the most significant anti-hyperalgesia ($P<0.01$); 5 mg/kg produced moderately significant anti-hyperalgesia ($P<0.05$);

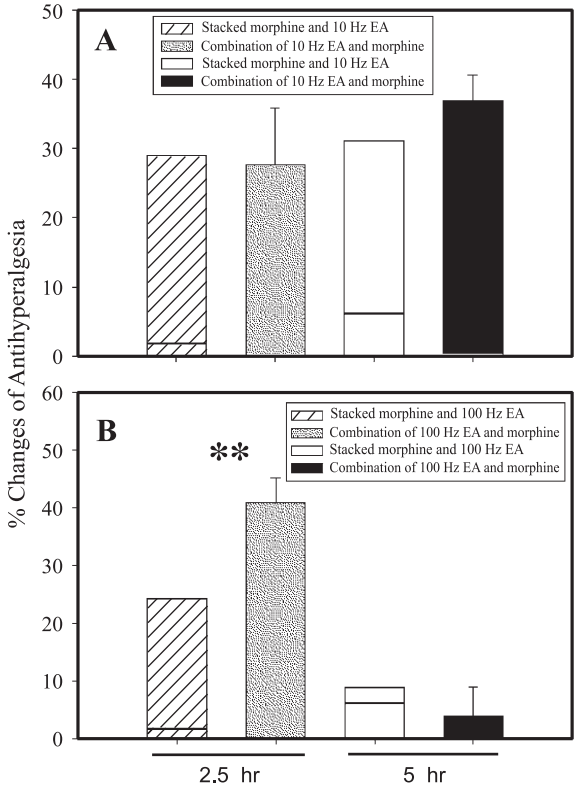

Fig. 2. Additive and synergistic effects of EA and morphine (2.5 mg/kg) on CFA-induced hyperalgesia. Anti-hyperalgesia is presented as (PWLs of experimental groups minus PWLs of saline control group)/(PWLs of control group) × 100%. (A) Note that the anti-hyperalgesic effect of the combination (column 4) of 10 Hz EA and morphine is greater, although not statistically significant, than the sum (column 3) of the anti-hyperalgesic effects produced by morphine or 10 EA alone at 5 h post-CFA, an additive effect. (B) Note that the anti-hyperalgesic effect of the combination (column 2) of 100 Hz EA and morphine was significantly greater than the added effect (column 1) of 100 Hz and morphine alone at 2.5 h post-CFA, an synergistic effect. **$P<0.01$ compared to stacked effects of 100 Hz EA and morphine alone at 2.5 h post-CFA.

2.5 mg/kg showed no anti-hyperalgesia. A combination of 10 Hz EA and a sub-effective dose of 2.5 mg/kg morphine produced significant anti-hyperalgesia at 2.5 and 5 h post-CFA injection, while 10 Hz EA alone showed significant anti-hyperalgesia only at 2.5 h post-CFA (Fig. 1B). A combination of 100 Hz EA and 2.5 mg/kg morphine significantly enhanced anti-hyperalgesia compared to 100 Hz EA alone at 2.5 h post-CFA (Fig. 1C). In order to discern the additive or synergistic effects of EA combined with morphine, anti-hyperalgesia was expressed as (PWLs of experimental groups minus PWLs of control group)/(PWLs of control group) × 100% and summarized in Fig. 2. Although not statistically significant, the anti-hyperalgesic effect of the combination of 10 Hz EA and morphine is greater than the sum of the anti-hyperalgesic effects produced by morphine or 10 EA alone at 5 h post-CFA, which suggests an additive effect. The anti-hyperalgesic effect of the combination of 100 Hz EA with morphine at 2.5 h post-CFA is significantly greater than the sum of the anti-hyperalgesia of either of these alone, which suggests a synergism of this combination at that time point (Fig. 2).

4. Discussion

4.1. μ and δ, but not κ, opioid receptors mediated EA anti-hyperalgesia

The present study demonstrated that both μ and δ opioid receptor antagonists, but not κ opioid receptor antagonists, blocked the anti-hyperalgesia of 10 and 100 Hz EA in a CFA-induced peripheral inflammatory pain rat model. These data indicate that both lower and higher frequency EA-produced anti-hyperalgesia were mediated by the μ and δ, but not κ, receptors. This suggests that the significant anti-hyperalgesia produced by high and low frequency EA is the result of the activation of endorphin/endomorphin (for μ receptors) and enkephalin (for δ receptors) systems but not dynorphin (for κ receptors) systems at the spinal level during persistent pain. Consistent with our study, previous studies reported that the analgesic effects of μ and δ opioid receptor agonists are potentiated during persistent inflammation [12,14,21]. For example, μ or δ receptor, but not κ receptor, agonists dose-dependently reduced mechanical hyperalgesia following repeated intramuscular injections of acid [25]. Dose–response curves for intrathecally administered μ- and/or δ-opioid agonists, determined by hind paw withdrawal latencies to noxious thermal stimuli, were shifted to the left for carrageenan-inflamed hind paws compared to contralateral non-inflamed paws. The intrathecally administered selective κ-receptor agonist showed no activity in this analgesic assay on either inflamed or non-inflamed paws [14]. In contrast to our studies using a persistent pain animal model, previous studies on uninjured rats demonstrated that lower frequency (2–15 Hz) EA activates endorphin/endomorphin and enkephalin, while high frequency (100 Hz) EA activates dynorphin [9]. It is known that persistent pain induces long-term neuroplasticity, which will change neural response to external stimulation [15,23]. Therefore, EA intervention activates the nervous system differently during persistent pain than in health.

Previous studies have demonstrated that endomorphin is localized in the superficial laminae of the spinal cord dorsal horn [18] and that it dose-dependently inhibits the evoked C-fiber response of spinal neurons [3]. Behavioral studies have shown that endomorphin produced antinociception in the tail flick test [22,27] and inhibited the behavior elicited by intrathecally injected substance P [27]. It has also been reported that acupuncture reduces electrophysiological spinal neuron response to noxious stimuli [32]. We hypothesize that EA treatment induces release of endomorphin in the spinal cord, which inhibits the transmission of noxious messages by acting on μ receptors in the spinal cord and results in suppression of hyperalgesia in rats with persistent peripheral inflammation.

Similarly, enkephalin is localized in the spinal cord neurons [2,11], and δ-opioid agonists inhibit excitatory transmission in spinal dorsal horn neurons [7] and substance P-elicited behavior [13]. It is also plausible that EA induces the release of enkephalin, thus inhibiting hyperalgesia in rats with persistent peripheral inflammation.

4.2. Combination of EA with morphine enhanced anti-hyperalgesia

Another significant finding is that EA treatment combined with a sub-effective dose of morphine enhanced EA-produced anti-hyperalgesia. Opioids are the main pharmacological therapy for alleviating moderate to severe acute and chronic pain [8,20]. However, adverse effects become a management problem and require additional intervention [1,5]. Our data showed that 10 Hz EA and morphine, which act at the same opioid receptors, interact additively to inhibit hyperalgesia, and that 100 Hz EA and morphine interacted synergistically. A previous study on rats demonstrated that a combination of EA treatment and fentanyl, an opioid receptor agonist, significantly enhanced and prolonged analgesia compared to EA alone [28]. A study on humans demonstrated that transcutaneous electrical acupoint stimulation (TEAS) combined with 0.05 mg/kg morphine induced significantly greater analgesia than TEAS alone [33]. Other clinical trials [30,31] have also shown that combined acupuncture-drug anesthesia caused fewer postoperative complications and better outcomes. Therefore, our study and these others suggest that acupuncture combined with low-dose morphine can improve pain control under pathophysiological conditions with few or reduced side effects.

In summary, spinal μ and δ, but not κ, opioid receptors mediated 10 and 100 Hz EA-produced anti-hyperalgesia, and the EA combined with morphine intensified anti-hyperalgesia. These findings suggest that EA may be clinically

effective as an adjunct to opioids in the treatment of persistent pain.

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