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The Central Serotonergic System Mediates the Analgesic Effect of Electroacupuncture on *Zusanli* (ST36) Acupoints

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Key Words
Electroacupuncture · Analgesia · Serotonin

Abstract
Evidence in the past decade indicates that the mechanisms of anti-nociception of electroacupuncture (EAc) involve actions of neuropeptides (i.e., enkephalin and endorphin) and monoamines (i.e., serotonin and norepinephrine) in the central nervous system. Our present results using a subcutaneous injection of formalin to test pain sensation in mice provide further understanding of the involvement of serotonin in the actions of EAc-induced analgesia. Our observations show that (1) EAc at three different frequencies (2, 10 and 100 Hz) elicited an anti-nociceptive effect as determined by behavioral observations of reduced hindpaw licking; (2) exogenously intracerebroventricular administration of 5-hydroxytryptamine (5-HT) exhibited an analgesic effect, which partially mimicked the analgesic actions of EAc; (3) the anti-nociception of EAc at different frequencies was attenuated after reduced biosynthesis of serotonin by the administration of the tryptophan hydroxylase inhibitor, *p*-chlorophenylalanine, and (4) the 5-HT_{1A} and 5-HT_3 receptor antagonists, pindobind-5-HT_{1A} and LY-278584, respectively, blocked three different frequencies of EAc-induced analgesic effects, but the anti-nociceptive effect of 100 Hz EAc was potentiated by the 5-HT_2 receptor antagonist, ketanserin. These observations suggest that 5-HT_{1A} and 5-HT_3 receptors partially mediate the analgesic effects of EAc, but that the 5-HT_2 receptor is conversely involved in the nociceptive response.

Electroacupuncture (EAc) is a traditional Chinese medicinal practice which consists of passing an electrical current through needles inserted into either acupoints or trigger points to alleviate pain. Although EAc has been widely used to relieve pain, the analgesic mechanism of EAc remains unclear. Cheng and Pomeranz [6] previously hypothesized that two distinct pain-relieving mechanisms are involved in the effects of EAc, including endogenous endorphin and non-endorphin systems. They showed that the effect of low-frequency EAc is mediated by endogenous endorphin and the effect of high-frequency EAc is non-endorphinergic, because of observations that the non-selective opioid receptor antagonist, naloxone, com-

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pletely blocked the analgesic effects induced by low-frequency EAc stimuli (i.e., 4 or 6 Hz) but not the analgesic actions induced by high-frequency EAc (200 Hz). The non-endorphinergic actions may be mediated by monoaminergic neurons [5, 30, 32]. Evidence from animal experiments implies that EAc is capable of influencing the synthesis and release of serotonin (5-HT) and norepinephrine (NE) in the central nervous system (CNS). EAc accelerates the genetic expression of c-fos in serotonergic neurons of the nucleus raphe dorsalis, nucleus raphe centralis, and the superior and rostral ventromedial medulla [10, 17]. In addition, it has been documented that 5-HT_1, but not 5-HT_{1A}, 5-HT_2, but not 5-HT_{2A}, and 5-HT_3 receptors are involved in EAc-induced analgesia, whereas the activation of 5-HT_{1A} and 5-HT_{2A} receptors suppresses EAc-induced analgesia [30]. EAc stimuli-induced alteration of NE concentrations in the CNS is variable. Wang et al. [34] reported that EAc inhibits the release of NE from the nucleus reticularis paragiantocellularis lateralis, which in turn mediates the anti-nociceptive effect. However, Shen et al. [26] conversely observed that NE concentrations in the cortex, hypothalamus and brainstem increase after EAc stimuli.

Herein, we further employed a formalin-induced pain model in mice and pharmacologically elucidate the involvement of 5-HT receptor subtypes in EAc-induced analgesia. An acute nociceptive response, which was induced by a subcutaneous injection of formalin into the dorsal part of the hindpaw and was represented by the elevation, licking, biting, or shaking of the affected paw, was utilized as a measure index to evaluate the degree of 'pain sensation'. Formalin induces biphasic responses; an early phase of the response occurs immediately after formalin injection and lasts for 5 min; the late phase of the response begins approximately 20 min after injection and continues for 20–30 min [13, 23, 31, 36, 37]. We measured the duration in which the mouse licked its hindpaw in response to the formalin injection as the nociceptive index. We herein report that EAc stimulation reduced the duration of licking, indicating that EAc possesses an analgesic effect. Furthermore, our observations that pretreatment with the respective 5-HT_{1A} and 5-HT_3 receptor antagonists, pindobind-5-HT_{1A} and LY-278584, attenuated EAc-induced analgesia suggest that the anti-nociceptive effects of EAc at frequencies of 2, 10 and 100 Hz are partially mediated by 5-HT_{1A} and 5-HT_3 receptors. In contrast, potentiation of the EAc-induced analgesic effect by the 5-HT_2 receptor antagonist, ketanserin, suggests that the 5-HT_2 receptor contributes to the nociceptive response instead of mediating EAc-induced analgesia.

Materials and Methods

Materials

All stock solutions were prepared in pyrogen-free saline (PFS). Aliquots of these stock solutions were stored at 4 °C until use. The exact doses of substances used in different experiments are given in the 'Experimental Protocol' below. The sources of substances were as follows: 5-hydroxytryptamine HCl (Sigma, St. Louis, Mo., USA), *p*-chlorophenylalanine (PCPA; Sigma), pindobind-5-HT_{1A} (RBI, Natick, Mass., USA), ketanserin (RBI), and LY-278584 (RBI).

Animals

Male ICR mice (18–25 g; National Laboratory Animal Breeding and Research Center, Taiwan) were used in these experiments. These mice were housed in individual recording cages, and maintained at 23 ± 1 °C with a 12:12-hour light:dark cycle. Food and water were available ad libitum. The surgical procedure of implantation of a guide cannula directed into the lateral ventricle was performed when these animals were anesthetized with ketamine/xylazine (87/13 mg/kg); they were also injected with an analgesic (butorphanol tartrate) and a broad-spectrum antibiotic (penicillin G benzathine). Animals were allowed to recover for 7 days prior to initiation of the experiments.

Acupoints and EAc Doses

The acupoints used in experiments were the bilateral *Zusanli* (ST36). Before manipulation with EAc, mice were acclimated to 5 min of restraint daily for 1 week. Mice were restrained in a plastic tube, and the hind limbs were fixed as when EAc is performed. Two steel (32-gauge) needles were used for bilateral puncture into the *Zusanli* acupoints to 3–5 mm of depth, and an electrical current at frequencies of 2, 10 and 100 Hz with a gradual increase of stimulation intensity up to 3 mA was generated by a Coulbourn E13-65 Stimulator (Coulbourn Instruments, Allentown, Pa., USA). The current passed through the steel needles into the acupoints. The administration of current lasted 5 min. After receiving EAc, mice were placed in an observation box for 25 min followed by the formalin test.

Formalin Test

The time spent licking the hind limb was counted every 5 min and measured for 40 min after a subcutaneous microinjection of 25 µl of a 1% formalin solution into the dorsal area of the hind limb of the mouse. The early phase of the pain reaction time was defined as 0–5 min after formalin injection, while the late phase was between 15 and 40 min after the injection.

Experimental Protocol

Animals were divided into several experimental groups. The first group of mice (n = 8) was used to test the effects of the 5-min restraint and non-acupoint stimulation on formalin-induced nociception. The non-acupoints were located 2 cm away from the *Zusanli* of the hind limbs. The second group of mice (n = 24) was used to observe the effects of 2, 10 and 100 Hz of EAc manipulations on the formalin test in the early and late phases. The third group of mice (n = 40) was intracerebroventricularly (ICV) administered the vehicle (PSF) or 5-HT (3, 10, 30, 100 and 300 µg) 10 min prior to the formalin injection to determine the analgesic effect. The fourth group of mice (n = 24) was depleted of 5-HT by intraperitoneal (IP) administration of PCPA (200 mg/kg) 10 min prior to the EAc manipulation; the licking

time after formalin injection was determined. The fifth group of mice (n = 24) was ICV administered with pindobind-5HT$_{1A}$ (5-HT$_{1A}$ receptor antagonist, 1 μg), ketanserin (5-HT$_2$ receptor antagonist, 1 μg), or LY-278584 (5-HT$_3$ receptor antagonist, 1 μg) 10 min prior to EAc to elucidate the involvement of serotonergic receptors in the EAc-mediated analgesic effects. The injection volumes were brought to 5 μl for ICV injection and 1 ml for IP injection.

Statistical Analyses

All values are presented as the mean ± SEM. Unpaired Student's t test was used to compare differences between the experimental and control groups. One-way analysis of variance (ANOVA) for the licking time was used in the experimental groups. If statistically significant differences were detected, Dunnett's post-hoc comparisons were made to determine which manipulation during the experimental conditions deviated from values obtained from the same animals during control conditions. An α level of $p \leq 0.05$ was taken as indicating a statistically significant difference between the experimental results and control.

Results

Effects of Restraint and Non-Acupoint Stimulation

Our results indicate that neither the 5-min restraint nor 5-min restraint + non-acupoint stimulation altered the behaviors induced during either the early or late phases after formalin injection. The licking times during the early phase after formalin injection, after pretreatment with 5-min restraint, and after pre-manipulation with 5-min restraint + non-acupoint stimulation were 75.6 ± 3.5, 73.6 ± 4.5 and 74.2 ± 4.1 s, respectively. The durations of licking time in the late phase were 120.5 ± 9.8 s after pretreatment with the 5-min restraint and 123.2 ± 10.2 s after 5-min restraint + non-acupoint stimulation, which did not deviate from the 119.8 ± 6.9 s obtained after formalin injection.

Analgesic Effects of EAc at Three Different Frequencies

The durations of licking time were statistically reduced in both the early and late phases by pre-stimulation with EAc at three different frequencies. Pre-stimulation with EAc at frequencies of 2, 10 and 100 Hz reduced the total duration of licking time from 73.6 ± 2.1 to 43.6 ± 1.4, 51.3 ± 2.9 and 42.6 ± 3.0 s, respectively, during the early phase ($p < 0.05$; fig. 1). During the late phase the licking times were reduced from 118.7 ± 7.9 to 56.1 ± 5.1, 63.2 ± 4.3 and 59.3 ± 7.3 s, respectively, when pretreated with EAc stimuli at frequencies of 2, 10 and 100 Hz ($p < 0.05$; fig. 1).

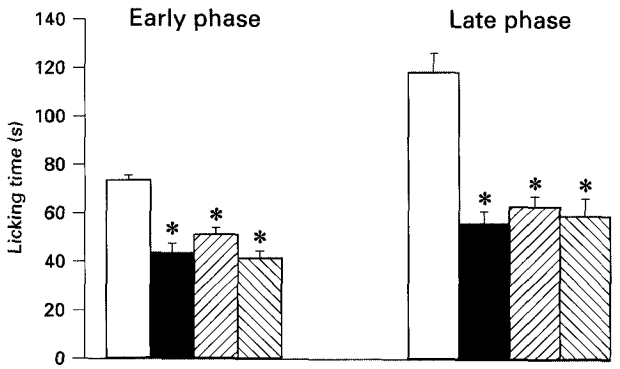

Fig. 1. Analgesic effects exhibited by EAc as reduced licking time on the formalin test. EAc stimuli given at three different frequencies of 2, 10 and 100 Hz statistically reduced the durations of licking time during the early- and late-phase responses. The open bar indicates values obtained from control mice (without EAc treatment; n = 8) after injection with formalin; the closed bar represents values obtained after pretreatment with 2 Hz of EAc prior to the formalin injection (n = 8); the left-hatched bar depicts values obtained after pretreatment with 10 Hz EAc prior to the formalin injection (n = 8), and the right-hatched bar shows values obtained after 100 Hz of EAc pretreatment prior to the formalin injection (n = 8). All values are presented as the mean ± SEM. * Values obtained after pretreatment with EAc statistically differed from those obtained after formalin injection only ($p < 0.05$).

Effects of Central Administration of 5-HT on Analgesia

ICV administration of 5-HT into mice reduced the formalin-induced nociceptive responses in both the early and late phases. Reduction in licking time in the early phase was observed to occur in a dose-dependent manner after ICV administration of 5-HT, although only the highest dose (300 μg) of 5-HT achieved a statistically significant reduction in the late-phase response. Licking time in the early phase was reduced from 39.8 ± 1.9 s after administration of the vehicle (PFS) to 15.7 ± 2.0, 10.3 ± 2.0 and 12.6 ± 1.9 s after ICV administration of 30, 100 and 300 μg of 5-HT, respectively ($p < 0.05$; fig. 2). In addition, the licking time in the late phase was reduced from 50.6 ± 4.3 s after vehicle (PFS) administration to 26.1 ± 4.5 s after ICV administration of 300 μg of 5-HT ($p < 0.05$; fig. 2).

Effects of 5-HT Depletion on EAc-Induced Analgesia

IP administration of 200 mg/kg PCPA into mice blocked the EAc-induced analgesic effects in the early

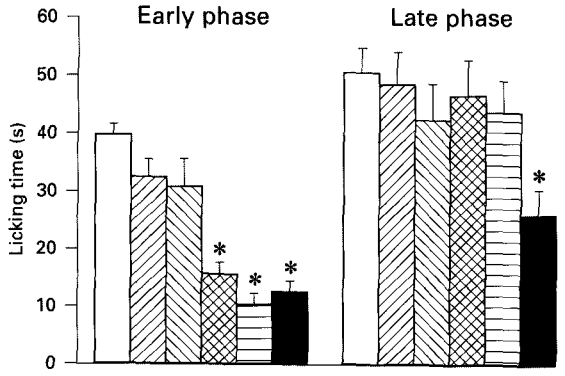

Fig. 2. Reduction in the nociceptive responses induced by formalin in both the early- and late-phase responses after ICV administration of 5-HT in mice. The open bar represents values of licking time obtained after the formalin injection, and the second to the sixth bars depict pre-injection of 5-HT in doses of 3, 10, 30, 100 and 300 μg, respectively (n = 8 for each dose administration). Durations of licking time are represented as the mean ± SEM. * Values obtained after ICV pretreatment of 5-HT differed statistically from those obtained from formalin injection only ($p < 0.05$).

phase of the formalin test, whereas a synergistic effect of anti-nociception was observed during the late phase (fig. 3).

Involvement of Serotonergic Receptors in EAc-Induced Analgesia

The EAc-induced reduction in licking time was attenuated by ICV administration of a 5-HT_{1A} receptor antagonist (pindobind-5-HT_{1A}, fig. 4, upper panel) or by a 5-HT_3 receptor antagonist (LY-278584) during both the early and late phases of the formalin test (fig. 4, lower panel). In contrast, the 5-HT_2 receptor antagonist, ketanserin potentiated the EAc-induced decrease in licking time (fig. 4, middle panel).

Discussion

Acupuncture, an important aspect of traditional Chinese medicine, has been associated with many therapeutic effects. Several theories regarding the mechanisms of acupuncture have recently been proposed. One of the hypotheses of acupuncture's efficacy is mediation of the perceptive 'gate control' in the dorsal horn of the spinal cord. Melzack and Wall [20] introduced the 'gate-control theo-

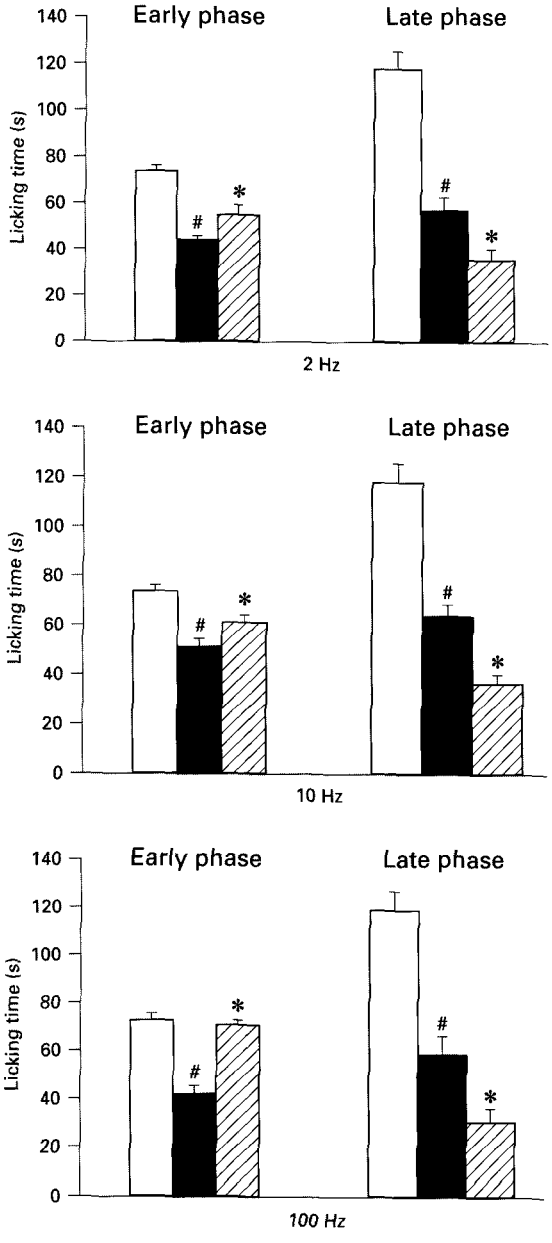

Fig. 3. Effects of pretreatment with PCPA on EAc-induced analgesic actions. Upper, middle and lower panels represent EAc frequencies given at 2, 10 and 100 Hz (n = 8 for each stimulation frequency), respectively. The open bar represents values obtained after formalin injection; the closed bar shows values of licking time obtained after EAc stimuli + formalin injection, and the hatched bar indicates values obtained after pretreatment with PCPA (200 mg/kg, IP) prior to EAc + formalin injection. # Values after EAc statistically differed from those of the formalin injection only ($p < 0.05$). * Values obtained after pretreatment with PCPA (200 mg/kg, IP) prior to EAc + formalin injection differed from those obtained after EAc stimuli + formalin injection ($p < 0.05$).

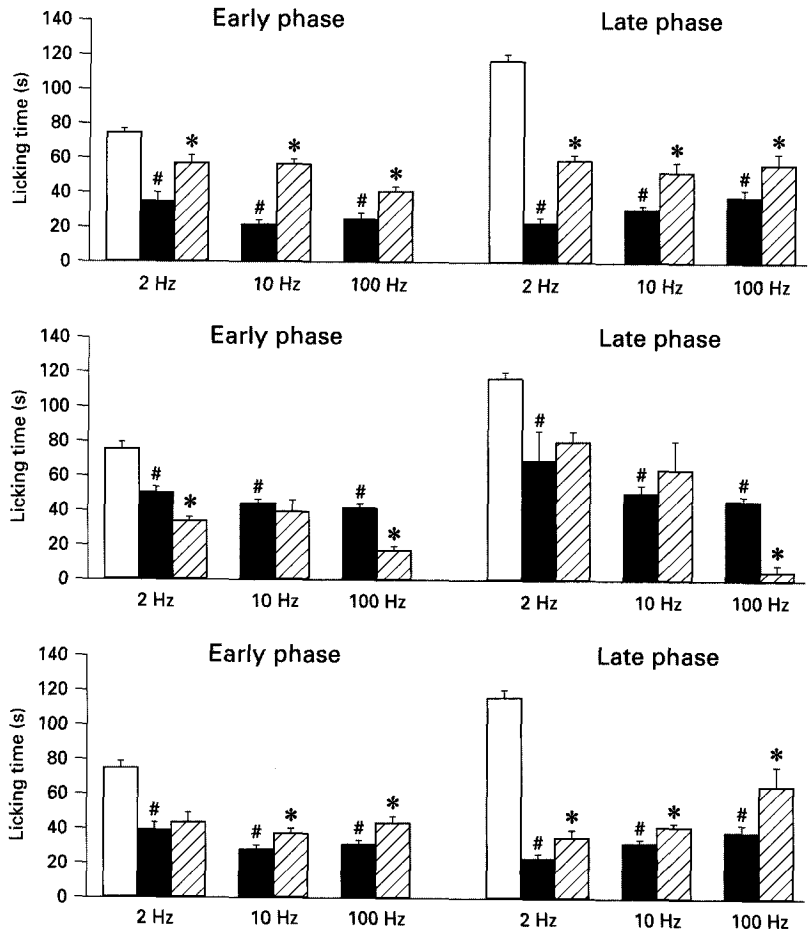

Fig. 4. Effects of 5-HT receptor antagonists on EAc-induced analgesia. Upper panel: effects of the 5-HT_{1A} receptor antagonist, pindobind-5-HT_{1A} (n = 8). Middle panel: effects of the 5-HT_2 receptor antagonist, ketanserin (n = 8). Lower panel: effects of the 5-HT_3 receptor antagonist, LY-278584 (n = 8). The open bar represents control values (formalin injection); the closed bar indicates values obtained after EAc (at frequencies of 2, 10, or 100 Hz) + formalin injection, and the hatched bar depicts values obtained after pretreatment with pindobind-5-HT_{1A} (upper panel), ketanserin (middle panel), or LY-278584 (lower panel) prior to EAc + formalin injection. # Values after EAc statistically differed from those of formalin injection ($p < 0.05$). * Values obtained after pretreatment with 5-HT antagonists prior to EAc + formalin injection statistically differed from those obtained after EAc + formalin injection ($p < 0.05$).

ry' providing evidence that stimulation of low-threshold myelinated primary afferent fibers decreases the response of dorsal horn neurons to unmyelinated nociceptors, whereas blockade of conduction in myelinated fibers enhances the nociceptive response of dorsal horn neurons. Acupuncture and transcutaneous electrical nerve stimulation stimulate Aα or Aβ myelinated afferents via a spinal 'gate-control' circuit, which suppresses the nociception mediated by unmyelinated C fibers [3, 9, 21]. However, if the 'gate-control theory' as applied to the mechanism of acupuncture were valid, then the analgesic effect of acupuncture would only occur when both the myelinated afferents stimulated by acupuncture and the nociceptive afferents feed into the same spinal segments. Most acupuncture applications do not adhere to this situation, even though the spinal 'gate-control' circuit may partially contribute to the mechanism. Another hypothesis related to the synthesis and release of endogenous neurotransmitters after acupuncture states that an endogenous opioid-like substance isolated from mammalian brain tissue and human cerebrospinal fluid acts as an analgesic to opioid receptors [12]. The analgesic effect of acupuncture may be partially due to the release of endogenous endorphins [22]. In addition, Chen and Han [4] elucidated how EAc at a frequency of 2 Hz induces secretion of β-endorphin acting on the μ and δ opioid receptors to produce analgesic effects. These aforementioned results strongly imply that endogenous opioid-like substances may mediate the analgesic effect of acupuncture. Bilateral microinjection of morphine into the posterior hypothalamus, periaqueductal gray matter, or ventral tegmental area elicits powerful suppression of nociceptive behaviors in the formalin test [18], and this anti-nociception may depend upon activation of descending pathways that act as relays in the

mesencephalic periaqueductal gray matter and then in the nucleus raphe magnus and nucleus reticularis paragigantocellularis [7, 15, 25], which respectively implies the involvement of serotonergic and noradrenergic systems. Furthermore, endogenous β-endorphin and enkephalin synthesized from the arcuate nucleus of the hypothalamus elicited the same effects [19, 24]. These observations suggest that at least two systems, endogenous opiate substances and monoaminergic neurons, may mediate the effects of EAc.

Evidence demonstrated that the pain response curve induced by 0.5% formalin was biphasic, consisting of an early phase and a late phase. Neurotransmitters/modulators (i.e., 5-HT, NE, substance P, and bradykinin), which are implicated in central mechanisms, participate in the manifestation of the early-phase response. Histamine, prostaglandin and bradykinin, which are implicated in peripheral inflammatory mechanisms, are involved in the late phase [11, 13, 27, 31, 33]. In the present study, we found that the durations of licking time were reduced in the early and late phases by pre-stimulation with EAc on *Zusanli* (ST36) using 2, 10, or 100 Hz. We further pharmacologically elucidated the involvement of serotonergic systems in EAc-induced analgesia, and found that ICV administration of 5-HT reduced the formalin-induced nociceptive responses in the early and late phases. The result of ICV administration of 5-HT mimics that of EAc, suggesting that the serotonergic system contributes to anti-nociceptive mechanisms. It has been documented that acupuncture analgesia involves the descending serotonergic pathway and the noradrenergic system, although the results in the literature are controversial [5, 16, 28, 29, 35, 39, 40]. Herein we hypothesized that EAc-induced analgesia is partially mediated by 5-HT. Our result that depletion of 5-HT by IP administration of PCPA into mice attenuated EAc-induced analgesic effects in the early phase of the formalin test indicates the involvement of 5-HT in EAc-induced analgesia. As for the late-phase response of the formalin test, PCPA exhibited a synergistic effect of anti-nociception with EAc regardless of the frequencies used with EAc. As mentioned above, the late-phase response of the formalin test was mediated by the peripheral mechanisms of bradykinin or prostaglandin. 5-HT has been reported to enhance the peripheral effect of bradykinin in the generation of muscle pain and muscular hyperalgesia in humans [1, 2]. Therefore, depletion of 5-HT may reduce the sensitization of nociceptive afferents by attenuating the effects of bradykinin or prostaglandin in the peripheral nervous system.

We further determined the involvement of central 5-HT receptor subtypes in EAc-induced analgesia. Our results show that ICV administration of the respective 5-HT_{1A} and 5-HT_3 receptor antagonists, pindobind-5-HT_{1A} and LY-278584, blocked EAc-induced anti-nociceptive effects; whereas the 5-HT_2 receptor antagonist, ketanserin, potentiated high-frequency EAc-induced analgesic actions. These results suggest that 5-HT_{1A} and 5-HT_3 receptors mediate EAc-induced analgesia, whereas the activation of 5-HT_2 receptor suppresses EAc effects. These observations are somewhat consistent with previous results elucidated by Takagi and Yonehara [30]. The explanation for the different results in the late phase between 5-HT depletion and 5-HT antagonism may be primarily due to peripheral versus central mechanisms, because we administered PCPA IP and injected 5-HT antagonists ICV. This hypothesis however needs to be further determined in future studies.

One possible interfering factor with the outcome of EAc arising from our experimental protocol is the effects of restraint. Stress may activate endogenous opioids and induce analgesia [8, 38]. A 30-min period of restraint significantly decreased the duration of paw licking but had no effect on the duration of paw elevation during the early phase; in contrast, this kind of restraint reduced the duration of paw elevation but not paw licking during the late phase [8]. Therefore, it is reasonable to be concerned that stress-induced analgesia may interact or potentiate mechanisms of EAc analgesia. However, previous reports from the literature indicate that stress-induced analgesia is exhibited when the restraint period lasts at least 30 min [8, 14]. Our results delineate that neither 5-min restraint nor 5-min restraint + non-acupoint stimulation altered the nociceptive effects induced by formalin injection. This observation suggests that stressors-induced analgesia, such as restraint or electrical stimulation in our current experimental design, was not additive to the EAc-induced analgesia.

Collectively, our results suggest that 5-HT partially mediates EAc-induced analgesic effects. Both 5-HT_{1A} and 5-HT_3 receptors mediate EAc-induced analgesia, whereas the activation of 5-HT_2 receptors inhibits EAc effects.

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