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A minimal stress model for the assessment of electroacupuncture analgesia in rats under halothane

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Abstract

The use of anesthetics in acupuncture analgesia is controversial. We evaluate a steady-state light anesthesia model to test whether minimal stress manipulation and reliable measurement of analgesia could be simultaneously achieved during electroacupuncture (EA) in animals.

A series of experiments were performed. Firstly, EA compliance and tail-flick latencies (TFL) were compared in rats under 0.1%, 0.3%, 0.5%, 0.7%, or 1.1% halothane for 120 min. Under 0.5% halothane, TFL were then measured in groups receiving EA at intensity of 3, 10 or 20 volt (V), 1 or 2 mg/kg morphine, 20 V EA plus naloxone, or control. Subsequently, the effect of EA on formalin-induced hyperalgesia was tested and c-*fos* expression in the spinal dorsal horn was analyzed.

Rats exhibited profound irritable behaviors and highly variable TFL under 0.1% or 0.3% halothane, as well as a time-dependent increase of TFL under 0.7% or 1.1% halothane. TFL remained constant at 0.5% halothane, and needle insertion and electrical stimulation were well tolerated. Under 0.5% halothane, EA increased TFL and suppressed formalin-induced hyperalgesia in an intensity-dependent and naloxone-reversible manner. EA of 20 V prolonged TFL by 74%, suppressed formalin-induced hyperalgesia by 32.6% and decreased c-*fos* expression by 29.7% at the superficial and deep dorsal horn with statistically significant difference.

In conclusion, 0.5% halothane provides a steady-state anesthetic level which enables the humane application of EA stimulus with the least interference on analgesic assessment. This condition serves as a minimal stress EA model in animals devoid of stress-induced analgesia while maintaining physiological and biochemical response in the experiment.
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Keywords: Electroacupuncture; Model; Halothane; c-Fos; Formalin

1. Introduction

Although acupuncture is commonly accepted as a treatment of choice for many pain conditions, the mechanism underlying acupuncture analgesia is still under debate. Many outcome studies in human are classified as "less convincing scientific data" for not including sham needling to exclude the potential placebo effect (NIH Consensus Statement, 1997; Linde et al., 2005). Application of acupuncture to conscious animal is even more complicated because needle insertion *per se* is

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actually a painful stimulus and needle manipulation, either manual or electrical, could further irritate the animal and confound the assessment. Furthermore, tight restraint, strong stimulus, psychological stress or fear are well known as potent activators to produce stress-induced analgesia (Lewis et al., 1980; Vaccarinoa and Kastina, 2001) in awake animal models of electroacupuncture analgesia (EA) (Pomeranz, 1986; Takeshige et al., 1992; de Medeiros et al., 2003). Thus, the scientific merits of the conventional acupuncture models could be severely reduced when the concomitant stressors are not adequately controlled.

Many techniques had been proposed to reduce stressors in acupuncture, including environmental habituation, freely-moving design, spinal transaction, decerebration, or anesthesia. Habituation attenuates the stress in the conscious and restrained rats but it is ineffective in suppressing the stress from needle stimulation (Yang et al., 2002). One study using consciously free-moving rats showed that while distress was markedly reduced, the stimulating intensity of EA, however, was limited to under 3 mA or 3 V and the acupoint should be deep points, like GB30 (Lao et al., 2001, 2004). Spinal transaction or decerebration preserves segmental spinal reflex but sacrifices supraspinal descending modulation (Chung et al., 1984a,b; Romita et al., 1997b). Similarly, anesthesia also has its problem. Various anesthetics had been used in acupuncture studies, such as halothane (Bing et al., 1991; Lee and Beitz, 1992, 1993), urethane (Murase and Kawakita, 2000), ether (Sheng et al., 2001), pentobarbital (Pan et al., 1994), or combination of pentobarbital and chloral hydrate (Romita and Henry, 1996; Romita et al., 1997a; Romita et al., 1997b). These agents, however, theoretically would suppress the neural and behavioral responses thus interfering with the analgesic assessment. Therefore there remains the demand for a better acceptable stress-free model for EA studies.

In this study, we proposed an anesthetic model under low concentration of volatile anesthetics as an alternative choice. The activation of endogenous opioid releases by EA has been established by numerous studies (reviewed by Han (2003, 2004)), hence, we performed a series of experiments to validate this model. First, a dose-finding study was conducted to examine suppressing effect of halothane at different concentrations, ranging from 0.1% to 1.1%, on the tail-flick threshold in rats. Then, we tested EA analgesia under 0.5% halothane with tail-flick test and formalin-induced hyperalgesia. Moreover, formalin-induced c-*fos* expression was analyzed to indicate neuronal responsiveness in this model. Our study indicated that the model proposed here could be a reliable EA model in animals which is minimally biased by stress-induced analgesia and anesthesia.

2. Methods

2.1. Animal preparation and anesthetic technique

The present study was performed with the approval of the National Taiwan University Hospital Animal Care and Use Committee and strictly followed the guidelines of The Care and Use of Experimental Animals. Male Sprague–Dawley rats (250–350 g, CD® (SD) IGS, National Laboratory Animal Center, Taiwan) were housed in groups of three to four in animal room with temperature control at 23 ± 1 °C, 50% relative humidity, with food and water ad libitum, and 12/12 h light/dark cycle. Experiments were performed between 9 am to 4 pm.

The rat was first placed in a $30 \times 20 \times 15$ cm clear acryl chamber pre-filled with a halothane-soaked gauze (average chamber concentration of halothane was over 5%) until the appearance of lying down, semi-closed or fast blinking eyes, or slow breathing. The rat was then relocated to a transparent cylinder holder circulated with desired concentration of halothane in pure O_2 delivered by an anesthetic machine at a flow rate of 2 L/min for at least 5 min. Two holes just bigger than the rat's thigh were made in the holder for hind limb extension, and its rostral end was connected to the breathing circuit (Fig. 1). Anesthetic concentration was monitored by gas analyzer (Capnomac, Datex Instrumentarium Corp., Helsinki, Finland) by sampling from a three-way adaptor connected to the circuit and was corrected by changing the vaporizer setting.

2.2. Study 1: Effect of halothane anesthesia on the tail flick reflex (Fig. 2a)

2.2.1. Tail flick latency (TFL)

The rat's tail was extended out of the holder and tested by an algesic test machine (MK-330B, Muro-

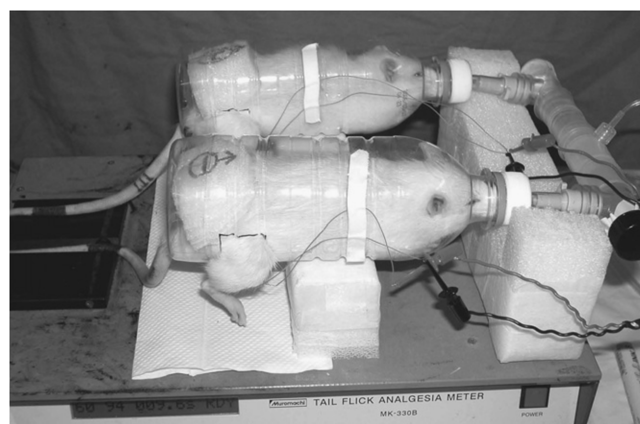

Fig. 1. Photograph of the volatile anesthesia model for tail-flick test and EA stimulation.

machi, Japan). A 2-cm segment within the distal one third of the tail was blackened to facilitate heat absorption. A photodetector would automatically stop a timer with a reaction time measured at 0.01 s after detecting the tail movement. Heat from a heat projector was set so that the baseline tail-flick time was 3–5 s and the tail was passively removed if a withdrawal did not occur within the "cutoff limit" of 10 s. Each "test latency" was an average of three serial withdrawal latencies, separated by 2 min, at two different spots within the blackened segment of the tail.

2.2.2. TFL under different halothane concentrations

In order to select an optimal anesthetic concentration with the least suppression on the withdrawal reflex, five different halothane concentrations: 0.1%, 0.3%, 0.5%, 0.7%, and 1.1% were tested. TFL started immediately after the rat was placed in the holder. The first three measurements were averaged and defined as "basal latency" and the successive averaged latencies at the 10-min intervals were defined as "test latency", individually denoting one time point. We calculated the averages by "% change" of threshold as follows:

$$\%\ \text{Change} = \frac{\text{test latency} - \text{basal latency}}{\text{basal latency}} \times 100\%$$

2.2.3. Behavioral indexes following halothane anesthesia and needle stimulation

Four behavioral indexes were used to evaluate the anesthetic depth and the feasibility of needle manipulation: "Agitation" = strong body withdrawal or vigorous rotation in the holder; "Nervous posture" = body curling or back curving; "Leg withdrawal" = leg withdrew inside the holder when investigator manipulated the hind leg; "Difficult needling" = need to re-insert the dislodged needles more than 2 attempts during the first 30 min. In addition, eye opening/closing, cornea and ear pinna reflexes to soft cotton thread at the end of anesthesia were assessed. "Oversedation" is defined as any two of the three tail flick latencies within each 10-min interval exceeding the cut-off limit. Duration of "Recovery" was measured from stopping anesthesia to the time of being able to walk normally. Over-anesthetized rats were removed from holder to avoid mortality. Rats showing the "positive" behaviors were counted and the result was presented as "(+) rat number/group number". Halothane concentration was adjusted according to the measurement of gas analyzer. Each rat could be subjected to the concentration response study at most twice, usually first at low then at high concentration which were separated by 2–3 days. Electrical stimulus through needles was briefly tested to ensure the correct needle positions. The voltage was limited to within 5 times of the current causing local muscle twitch and a duration less than 1 min at each trial to avoid EA effect.

2.3. Study 2: Effect of EA on tail flick reflex in halothane anesthesia model (Fig. 3a)

2.3.1. Induction period and maintenance period of anesthesia

From result of the concentration response study, 0.5% halothane was selected for the following Study 2 and 3. Anesthesia was divided into 2 periods: induction period (from consciousness to steady-state anesthesia) and maintenance period (treatment period). During the induction period, oxygen flow was set at 2 L/min to facilitate the anesthetic uptake, and then decreased to 1 L/min at the maintenance period. Tail-flick tests started at 5 min after the rat was transferred from the anesthetic chamber to the holder, and were repeated at an interval of 2–3 min. When the values of two successive TFL were very close with a difference within 0.5 s, anesthesia was regarded as stable and the average from the two was defined as "basal latency". Usually, 15–20 min was enough for reaching a stable measurement from our experience and those reported by others (Bing et al., 1991; Cuellar et al., 2004; Kawamata et al., 2005). The "test latency" was the same as that described in Section 2.2. Maximal possible effect (MPE) was calculated by this equation:

$$\text{MPE}\% = \frac{\text{test latency} - \text{basal latency}}{\text{cutoff limit} - \text{basal latency}} \times 100\%$$

2.3.2. EA stimulation

After exposure of the lateral aspect of the right hind limb in the rat, one pair of stainless steel needles (30G) were applied during the first 5 min of the induction period. Needles were inserted to a depth of 5 mm, one in the meridian point Zusanli (ST-36), located between the tibia and fibula approximately 5 mm lateral, 5 mm lower to the anterior tubercle of the tibia, and the other located 5 mm below the Zusanli point. This acupuncture point is traditionally used for pain control (Pomeranz et al., 1977). The paired needles were then connected to a stimulator (Square Wave Stimulator 611, Phipps & Bird Inc., Virginia, USA) that generated stimuli of 4 Hz, 0.5 ms square pulses, for a total of 30 min. The intensity was slowly increased from local muscle twitch at the acupoint, usually 0.7–1.0 V, to the target intensity in 5–10 min, and then maintained at that level for another 25 min.

The rats were allocated into 8 groups which were sham EA group: only needle insertion at acupoint without electrical current; E3 group: low EA (3 V); E10 group: moderate EA (10 V); E20 group: high EA (20 V); M1 group: intraperitoneal (i.p.) morphine 1 mg/kg; M2 group: i.p. morphine 2 mg/kg; NAL group: E20 stimulation plus three doses of i.p. naloxone, respectively, at 10 min before EA (2 mg/kg), 30 min (1 mg/kg) and 60 min (1 mg/kg) after EA; and C group: no EA nor injection as control. Morphine and naloxone

doses were chosen according to previous animal studies as the active comparative control (Lee and Beitz, 1992; Takeshige et al., 1992; Han, 2003, 2004). The anesthetic circuit was designed so that each control or sham rat was always paired with one experimental rat treated with either EA or morphine. Thus, this design made each pair of rats sharing the identical anesthetic concentration, exposure time, ambient temperature, and radiant heat condition on the tail. Electrical stimulation or morphine injection started at the beginning of the maintenance period (time 0).

2.4. Study 3: EA effect on formalin-induced hyperalgesia in halothane anesthesia model (Fig. 4a)

Four groups were included: 10 V EA, 20 V EA, subcutaneous (s.c.) injection of 2 mg/kg morphine, or normal saline at the maintenance period. All rats received intraplantar injection of 50 μl diluted formalin (5%) at the left paws via a 26G needle at10 min after stop of anesthesia. The rats were then transferred at an iron-grid cage for 1-h observation. The weighted pain score, as described by Dubuisson and Dennis (1997), was assessed by computing the weighted average of the time spent for each category as described in our previous study (Sun et al., 1996). To quantify the temporal pattern of pain behavior, two phases were identified: the early phase (initial 10 min) and the late phase (20–60 min), and the cumulative pain scores at the late phase were compared among groups. All rats were euthanized for subsequent immunohistochemical staining study.

2.4.1. Immunohistochemistry and Fos-like immunoreactive (Fos-LI) neurons counting

The rats were euthanized with an overdose of sodium pentobarbital at 60 min after formalin injection, and then perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer 0.1 mol/L (pH = 7.4). The spinal cord at the lumbar enlargement was removed, post-fixed for 4 h, and cryoprotected overnight in 30% sucrose. Frozen sections were cut in a cryostat (30 μm) and collected as free-floating sections. Sections were incubated with primary rabbit polyclonal anti-Fos antiserum (Ab-2, Oncogene), diluted 1:750 with PBS 0.1 mol/L, containing 3% normal goat serum and 0.3% Triton X-100 for 48 h at 4 °C. After being washed in PBS, they were incubated with biotinylated second antibody (goat anti-rabbit antiserum; Vector) diluted 1: 200 in PBS for 1 h at room temperature. The sections were reacted with elite ABC (Vectastain) diluted 1:50 for 1 h and then 0.1% diaminobenzidine solution containing 0.6% nickel ammonium sulfphate and 0.2% H_2O_2 as substrate was added. All sections were mounted on gelatin-subbed slides, air-dried and protected with a coverslip for light microscopic examination. For each animal at least ten slices of the Fos-labeled neurons in the L4-5 spinal dorsal horns were counted. Sections were examined using a dark-field microscope (Axioscope, Zeiss) to determine the segmental level according to the grey matter landmarks, as described by Molander et al. (1984). The dorsal horn of each section was divided into three regions: (1) the superficial laminae (lamina I/II), (2) the nucleus proprius (lamina III/IV) and (3) the deep laminae (lamina V/VI). Fos-LI neurons, which showed a deep staining distinguishable from background in bright-field illumination, were counted with respect to each lamina.

2.4.2. Data analysis

All data were presented as mean ± SEM. Formalin behavioral scores were transformed into area under curve (AUC). Repeated measures analysis of variance (ANOVA) or one-way ANOVA when appropriate was conducted to compare the time effect and group differences in the study of concentration survey, EA analgesic on tail flick test, weighted formalin pain score, and the numbers of Fos-LI neurons. Post-hoc comparison was performed by the least significant difference (LSD) test when indicated. $p < 0.05$ was taken as statistically significant.

3. Results

3.1. Study 1: Rats anesthetized with 0.5% halothane had stable tail-flick latencies

All rats became anesthetized shortly after being placed in the high halothane-prefilled chamber and were transferred to the transparent holder at once. We found that needle insertion should be done as soon as the rat was placed into the holder when the rat was still deeply anesthetized. The timing of needle insertion is especially critical for rats under light anesthesia (e.g. 0.1% and 0.3% halothane).

Many rats under 0.1% halothane woke up from anesthesia, moved vigorously and withdrew their tails into the holder which made the subsequent testing difficult (Table 1). For the 0.3% group, needles insertion was still difficult because of leg withdrawal hiding under the body, or vigorous shaking at electrical stimulation. Comparatively, the rats under 0.5% and 0.7% halothane not only showed tolerance to the electric stimulation, but also preserved tail reflexes and had fast recovery from anesthesia. All rats subjected to 0.5% and 0.7% halothane could walk normally within 5 min after stop of anesthesia, but more rats in the 0.7% halothane group were classified as oversedated. The results demonstrated that anesthesia at 0.5% halothane provided an optimal condition for EA with minimal impact on nociceptive reflex. Therefore, 0.5% halothane was selected for the subsequent studies.

Table 1
Behavioral indexes observed during the 120-min halothane anesthesia

	Halothane concentration				
	0.1%	0.3%	0.5%	0.7%	1.1%
Induction period (0–30 min)					
Agitation	3/5	2/8	0/8	0/8	0/5
Nervous posture	4/5	3/8	0/8	0/8	0/5
Leg withdrawal	4/5	5/8	1/8	0/8	0/5
Difficult needling	5/5	4/8	1/8	0/8	0/5
Maintenance period (30–120 min)					
Eye open	5/5	8/8	6/8	4/8	1/5
Conea reflex	5/5	8/8	6/8	3/8	1/5
Ear pinna reflex	5/5	8/8	8/8	3/8	2/5
Recovery (min)	<1	<3	1–5	1–5	6-10
Over-sedation	0/5	0/8	1/8	3/8	5/5
Death	0	0	0	0	1[a]

The data are expressed as "(+) rat number/group rat number". The definition of each behavioral sign is described in the text.

[a] This rat appeared dyspnea 30 min after the start of anesthesia, probably due to airway obstruction.

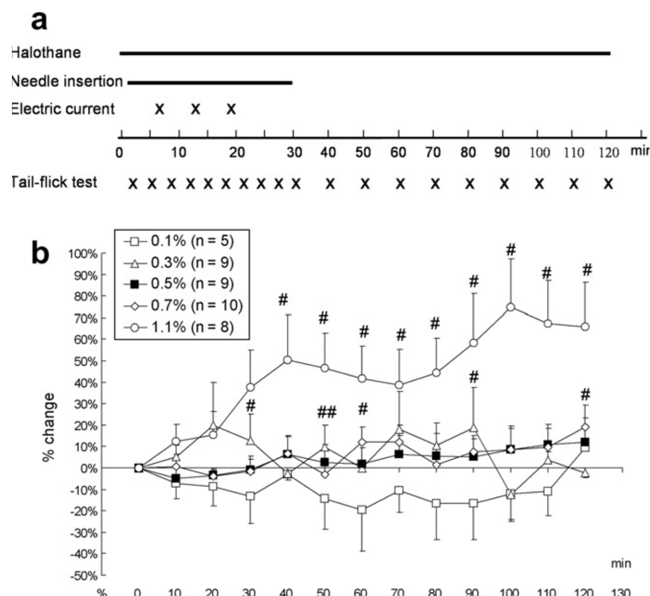

Fig. 2. Effect of different halothane concentrations on the tail-flick latency (TFL). (a) Study design for concentration survey. Time points for tail-flick test, electrical stimulation were marked by ×. (b) TFL under various concentrations of halothane in $O_2$for 120 min showed concentration-dependent effects on nociceptive threshold. TFL is expressed as "% change" with respective to the "basal latency" at time 0 (see text). Statistical analysis was conducted by repeated-measures analysis of variance (ANOVA) to compare the time factor, followed by the Least Significant Difference (LSD) test for post-hoc comparison. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for groups vs. basal latency.

The TFL with different concentrations of halothane are presented in Fig. 2. We chose the % change rather than the MPE for nociceptive threshold changes because the cutoff limit in MPE could obliterate the anesthetic effect. Significant difference was found among various concentration groups ($F_{4,27} = 5.347$, $p = 0.003$). As shown in Fig. 2, halothane at low concentrations (0.1% and 0.3%) caused highly variable thresholds, while high concentration (1.1%) resulted in a time-dependent prolongation of TFL ($p < 0.05$). The latencies under 0.1% or 0.3% halothane ranged from −19.4% to 9.2% and from −12.1% to 19.9%, respectively. Rats in the 0.5% and 0.7% groups had a narrow range of values. The maximal difference of % change of threshold (i.e. maximal value minus minimal value) through the 120-min duration was only 15% in the 0.5% group and 23% in the 0.7% group. Halothane-induced analgesia was shown in the 0.7% group at the end of the study. The TFL under 0.5% halothane was not prolonged until 120-min anesthesia.

3.2. Study 2: EA intensity-dependently prolonged tail-flick latency in the halothane anesthesia model

In the 0.5% halothane groups, sham EA did not produce any analgesia compared with the control ($p = 0.603$) or with the pre-EA baseline (Fig. 3b). Low intensity EA (3 V) mildly prolonged the withdrawal thresholds compared with baseline level ($p < 0.05$), but had no significant difference from that of the control or the sham EA at any time point ($p = 0.052$, Fig. 3b). The MPE maintained at about 20% elevated for a long period after stop of EA. Increasing the stimulating intensity showed a significantly dose-dependent difference in tail-flick threshold between E10 and control as well as between E20 and control from 10 and 30 min after EA, respectively ($F_{7,66} = 12.233$, $p = 0.00$) (Fig. 3c). EA of 20 V had higher analgesic effect than 10 V EA at 40 min after EA stimulation ($p < 0.01$). In the 10 V EA group, two peak MPEs appeared at 50 min (32.0 ± 6.2%) and 70 min (34.6 ± 5.1%), whereas maximal MPEs of the 20 V group were at 60 min (67.2 ± 9.2%) and 90 min (74.3 ± 9.9%), all of which far outlasted the stimulation period. Analgesic effect of the 20 V EA is similar to the effect of i.p. 2 mg/kg morphine ($p = 0.538$, Fig. 3d), but peak effect of morphine appeared earlier than EA stimulation. Naloxone partially reversed the 20 V EA analgesia (Fig. 4b), which was significantly attenuated from 74.3 ± 5.1% to 13.6 ± 4.0% at time 90, with a 82% decrease ($p < 0.01$). There was no hematoma, local tissue swelling or inflammatory changes at needle insertion sites in any rat after the stimulation.

3.3. Study 3: EA suppressed formalin-induced hyperalgesia and decreased c-fos expression in halothane anesthesia model

The typical biphasic nocifensive response was present after intraplantar injection of 5%, 50 μl formalin in all groups (Fig. 4b). The 10 V and 20 V EA obviously

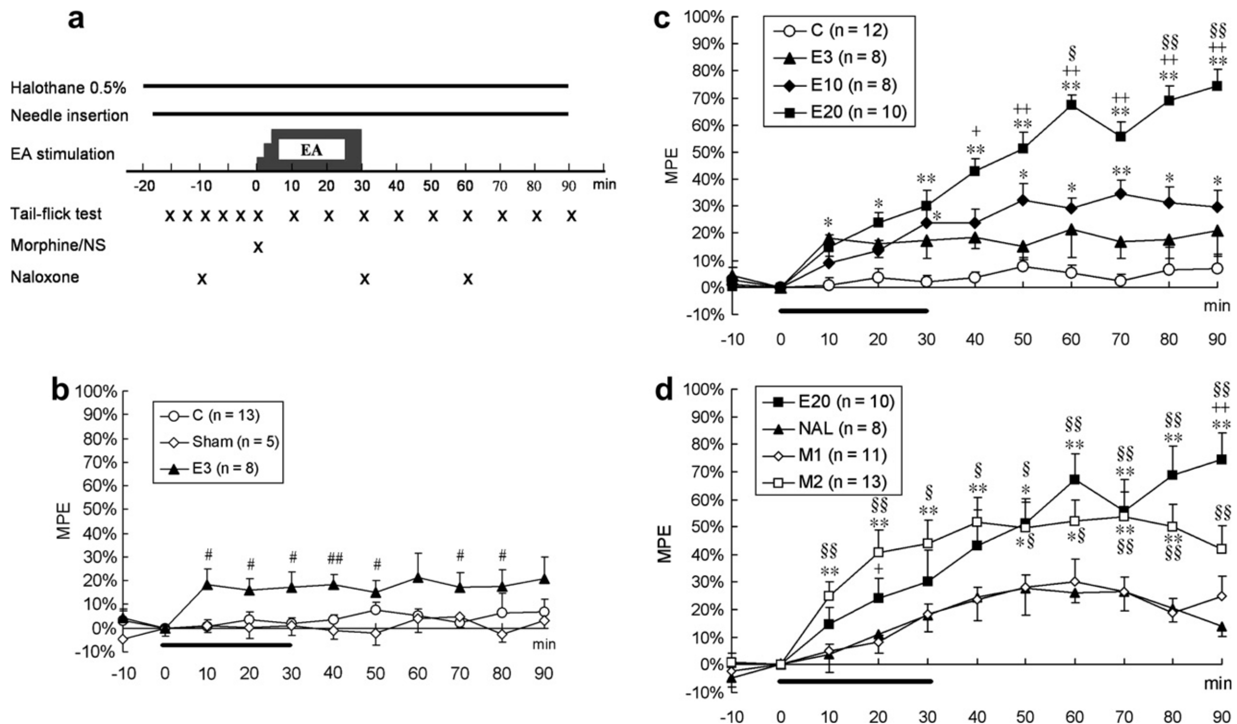

Fig. 3. EA effect on TFL in 0.5% halothane model. (a) Study design for procedures of EA stimulation, tail-flick test, morphine, saline, or naloxone administration. (b–d) Maximal possible effect (MPE) illustrates the effect of sham EA, EA of 3 V (E3), 10 V (E10), and 20 V (E20), morphine at the doses of 1 mg/kg (M1) or 2 mg/kg (M2), and E20 plus naloxone (NAL) on TFL under 0.5% halothane. Horizontal thick bar indicates the EA stimulating period. Repeated measures analysis of variance (ANOVA) and one-way ANOVA were conducted to compare the time factor and group differences. Post-hoc comparison was performed by the Least Significant Difference (LSD) test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for E3 group vs. its pre-EA basal latency. $^{*}P < 0.05$, $^{**}p < 0.01$ vs. C (c) or vs. NAL (d); $^{+}p < 0.05$, $^{++}p < 0.01$ vs. E3 (c) or vs. M2 (d). $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ vs. E10 (c) or vs. M1 (d) (b) M1: i.p. morphine, 1 mg/kg; M2: i.p. morphine, 2 mg/kg ; NAL: E20 plus i.p. naloxone 2 mg/kg at time −10, 1 mg/kg at time 30 and 60, respectively.

inhibited the behavioral hyperalgesia induced by formalin, and this suppression mainly appeared at the late phase. They significantly decreased cumulative pain score of the late phase by over 30% (form 15.27 ± 0.75 in control group to 11.56 ± 0.76 in the E10 and 10.30 ± 1.20 in the E20 group, $p < 0.01$) (Fig. 5d). The 20 V EA is approximately equipotent to s.c. 2 mg/kg morphine given 40 min before formalin injection. EA of 20 V had stronger inhibitory effect than 10 V EA at the latter-half of the late phase, though without statistical significant difference. Unlike EA, morphine had a significant suppressive effect at both the early and late phase of the formalin test (Fig. 4c).

Induction of Fos-LI neurons by 5% formalin injection was predominantly located in L4-5 dorsal horn ipsilateral to the side of formalin injection, and mostly distributed at the superficial and deep laminae (Fig 5a), which clearly indicated hyper-excitation of the post-synaptic secondary neurons or interneurons by formalin. This neuronal excitation could be significantly inhibited by either pretreatment with 20 V EA or 2 mg/kg morphine to the extent of 29.7% and 42.6% decrease, respectively ($p < 0.05$, Fig. 5d). More specifically, morphine and 20 V EA suppressed the Fos expression at the superficial and deep laminae of dorsal horns (Fig. 5b and c), whereas 10 V EA only decreased Fos-LI neurons at the deep laminae (not shown).

4. Discussion

The aim of this study was to search for a low concentration of halothane to enable EA manipulation and reproduce analgesia in rats with minimal stress. Our data showed that rats tolerated needle insertion and EA stimulation well under 0.5% or higher concentration of halothane. At 0.5% halothane, TFL was constantly maintained for 120 min, and EA stimulation could prolonged TFL, suppressed formalin-induced hyperalgesia and inhibited c-*fos* expression in an intensity-dependent manner. The analgesic efficacy was naloxone reversible. These findings correlate well with the generally accepted phenomena in available EA studies and showed appropriate analgesic property in different pain models.

Stimulating the animals without detectable stress is the most favorable feature in this model. It is generally accepted that administration of EA to conscious animal involves three kinds of stressors: immobilization, needle insertion, and electric current stimulation. Immobiliza-

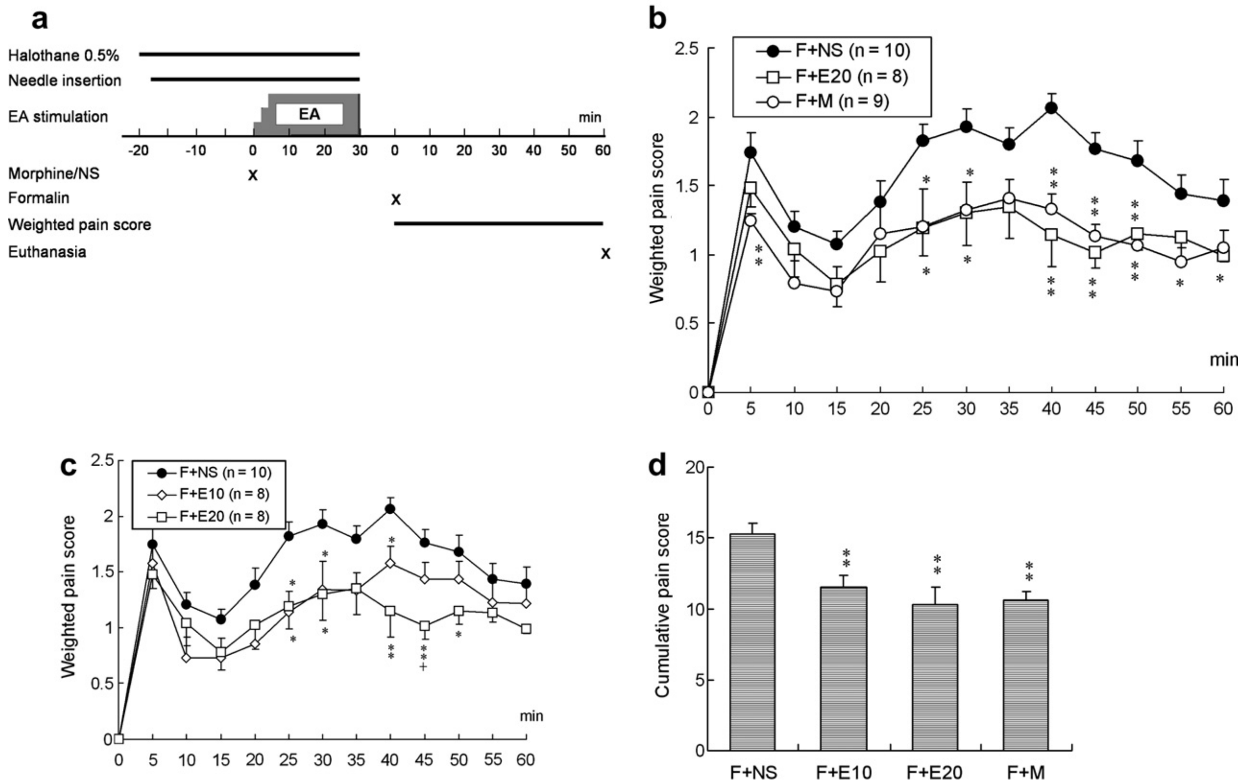

Fig. 4. EA effect on formalin-induced hyperalgesia in 0.5% halothane model. (a) Study design for procedures of formalin injection, EA stimulation, morphine, or saline administration. (b–c) Formalin plantar injection in all groups showed typical biphase of hyperalgesia. Both the 10 V and 20 V EA significantly reversed behavioral hyperalgesia, mainly at the late phase (20–60 min). Morphine had a significant suppressive effect at both the early and late phase. (d) The cumulative pain score at the late phase demonstrated E10, E20, or morphine produced significant analgesia. $^{*}p < 0.05$, $^{**}p < 0.01$ for groups vs. NS; $^{+}p < 0.05$ for E20 vs. E10.

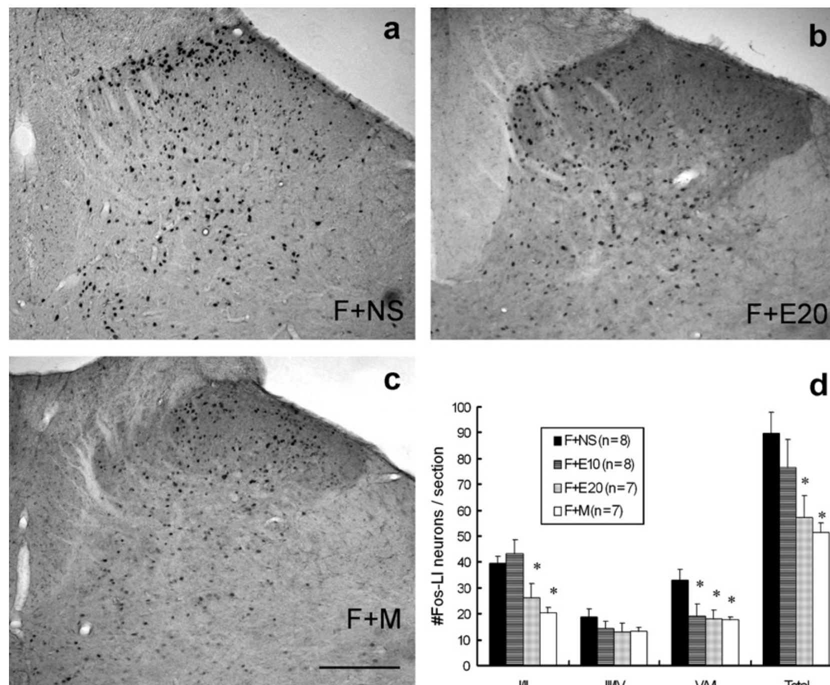

Fig. 5. Differential c-*fos* expression at the L4-5 dorsal horns after the 10 V, 20 V EA, or morphine treatment in 0.5% halothane model. The rats subjected to the formalin injection at hind limb plantar showed strong c-*fos* expression at the superficial and deep laminae in L4-5 dorsal horn (a). Subcutaneous 2 mg/kg morphine (c) and 20 V EA (b) suppressed the Fos expression at the laminae I/II and V/VI of dorsal horns, while 10 V EA only decreased Fos-LI neurons at the laminae V/VI (d).

tion is a well-documented stressor producing potent analgesic effect through the release of endogenous opioid peptides (Vaccarinoa and Kastina, 2001), and is probably the most severe stressor among those having a predominant emotional reaction (Marquez et al., 2002; Armario et al., 2004). Rats in restraint exhibited longer TFL in our pilot study and other study (Mitchell et al., 1998), and had less formalin-induced hyperalgesia than those been habituated (Aloisi et al., 1998). Furthermore, repeated restraint training was reported to markedly reduce immobilization-induced analgesia and associated c-*fos* expression (Umemoto et al., 1994). Acupuncture *per se* is another stressor to animals due to physiological and psychological effect. Our unpublished data showed that the conscious, acclimated rats did not tolerate needle insertions or low electric stimulation without restraint. In immobilized rats, EA increased heart rate and blood pressure, and released three- to four-fold of norepinephrine and epinephrine than rats without EA stimulation (Yang et al., 2002). A recent study indicated that, in un-habituated rats, non-acupoint EA produced comparable analgesic effect as acupoint EA stimulation with respect to behavioral index and c-*fos* expression in the periaqueductal gray (de Medeiros et al., 2003). In this anesthetic study, three volt EA had very mild depression on tail-flick withdrawal, whereas this intensity was advocated to be strongly effective in conscious animals (Ulett et al., 1998). It is presumed that the stress-induced analgesia might participate in EA analgesia in the conscious rats, though supporting evidence is insufficient. Taken together, the reduction in animal distress not only avoid the confounding mechanisms associated with stress-induced analgesia but also conform to the ethical policy at intense EA stimulation.

Volatile anesthetic agent possesses a more favorable pharmacokinetic profile than the conventional non-volatile anesthetics, i.e. shorter wash-in and wash-out period. Animals in this study preserved constant tail-flick reflexes under 0.5% halothane and resumed spontaneous activities within 5 min after anesthesia. The unique advantage of better control of anesthetic depth had never been clearly described despite similar concentration of halothane was used in other studies (Bing et al., 1991; Lee and Beitz, 1992). Faster onset of anesthesia enables a short titration period, and rapid recovery allows to evaluate many nocifensive behaviors, which can only be accomplished in the conscious state, e.g. formalin-induced hyperalgesia. In comparison, parenteral anesthesia by single or repeated injection technique would cause fluctuating plasma concentration, and continuous infusion requires an indwelling catheter and is even harder to achieve a desirable anesthetic level. Therefore, this light halothane model for EA shows superiority over the conventional injectable anesthetic models.

Another advantage of light halothane is that the effect on sensory processing and nociceptive reflex could be minimized. We showed that the constant withdrawal threshold under 0.5% halothane, which is approximately 0.5 minimum alveolar concentration (MAC) (O'Connor and Abram, 1995), serves as a satisfactory negative control to exclude the potential confounding effect by itself. Hence, we need not calculate the "actual EA effect" by subtracting the control latencies in rats receiving anesthesia but no EA from latencies in rats receiving both anesthesia and EA in other studies (Romita and Henry, 1996; Romita et al., 1997a; Romita et al., 1997b). Volatile anesthetics, however, were believed to have modifying effects on nociceptive processing and Fos protein expression (Wall, 1988; Yaksh, 1993; Noviova et al., 2004). Several lines of evidence showed the effect on sensory integrities and neural reactions is concentration-dependent and agent-specific, particularly at concentration over 1 MAC. In rat formalin model O'Connor et al. demonstrated spinal sensitization could be blocked by halothane or isoflurane at 1 MAC, 1.0% and 1.4%, respectively (O'Connor and Abram, 1995). "Windup phenomenon" of wide dynamic range (WDR) neurons at the spinal dorsal horns in mice was suppressed by halothane at 0.9–1.1 individual MAC ($0.9 \pm 0.1\%$) but was not significant at below 0.9 MAC (Cuellar et al., 2004). Exposure duration is another contributing factor because the rats under 0.7% halothane showed significant higher TFL than the control rats in the end of the experiment. Accordingly, we suggest that light anesthesia with 0.5% halothane (0.5 MAC) may not be sufficient to alter nociceptive sensitization and EA effect within an 120-min period.

In formalin-induced hyperalgesic model, EA at 10 V or 20 V manifested an inhibition exclusively at the late phase, but not at the early phase. The differential suppression was reported earlier (Hao et al., 2000) and was suggested to be an evidence that EA analgesic efficacy is insufficient to inhibit the early intense nociceptive barrage. The suppression of Fos expression distributed in the superficial and deep dorsal horns by EA treatment provides adequate explication (Harris, 1998). Superficial laminae I/II serve as the first relayed station from initial peripheral noxious barrage, and in lamina V, WDR neurons polysynaptically receive both noxious and innocuous peripheral inputs and participate in generating a long-lasting after-discharges (Besson and Chaouch, 1987; Price et al., 1994). In this study, formalin induced densely Fos distribution over laminae I, II, and V of the L4-5 dorsal horns as shown by other studies (Hunt et al., 1987; Bullitt, 1990; Presley et al., 1990). Our staining results suggested that EA mainly affected neuron activation in the deep layers and only the strong stimulation (20 V) could suppress Fos expression at both the superficial and deep laminae. Comparatively, Presley and colleagues (Presley et al., 1990) reported that systemic

injection with 10 mg/kg morphine suppressed c-*fos* expression in the deep laminae to a greater extent than that in the superficial laminae. The c-*fos* expression between EA and morphine effect suggests that they share, at least partly, the antinociceptive effect through a complex inhibitory system at the multiple synaptic components.

We proposed a steady-state and easily titratable anesthetic model for studying acupuncture-induced analgesia in rats. In this dose-finding study we found that 0.5% halothane induced constant tail-flick latency for 120 min and permitted easy animal manipulation, strong needle stimulation and immediate recovery. The intensity-dependent EA analgesia was reproduced as gauged by spinal reflex, behavioral hyperalgesia or Fos expression in the spinal cord dorsal horn neurons. Most importantly, this model created a condition with minimal stress, which had been considered as a confounding and confusing factor in conscious model.

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