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# **Intensity-dependent analgesic effect of electroacupuncture: behavioral response and *c-fos* expression in rat spinal cord dorsal horn**

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## Abstract

Although induction of deep aching sensation, De-Qi, is generally accepted as a standard acupunctural practice, whether an intense nociceptive stimulation is required for optimal analgesic effect remains controversial. In freely moving rats we first demonstrated that intramuscular injection of 100  $\mu$ l 5% formalin, a noxious irritant, in the Zusanli acupoint (ST-36) induced intense nociceptive behavior. Tail-flick latency and *c-fos* expression were then compared among halothane-anesthetized rats receiving either low-intensity electroacupuncture (10 $\times$  threshold of local muscle contraction), high-intensity (20 $\times$  threshold) electroacupuncture (30 min, 4Hz), intramuscular formalin, or normal saline injection at the right Zusanli acupoint. We found that formalin injection at the acupoint markedly increased tail-flick latency and induced greater *c-fos* expression at the superficial and deep laminae of spinal cord dorsal horn compared to saline injection. Electroacupuncture prolonged the cumulative tail-flick latency in an intensity-dependent manner but, in contrast to formalin, did not provide intensity sufficient to induce *c-fos* expression throughout the 2nd to 5th lumbar segments. Our data indicated that although noxious chemical stimulation at the acupoint can elicit comparable analgesic effect, non-noxious electrical stimulations produce even greater analgesic responses in an intensity-dependent manner. These findings suggest that generation of intense aching sensation, De-Qi, is not required for the analgesic effect provided by electroacupuncture.

Classification Terms: Theme F (SENSORY SYSTEMS)

Topic: Pain modulation: anatomy and physiology

Key Words: electroacupuncture, tail flick, formalin, *c-fos* expression, stimulation intensity, anesthesia

## 1. Introduction

Traditional acupuncturists often increase the needling strength to “maximal tolerable level” or “beyond the pain threshold” by rapidly stirring or thrusting the needles as this is considered to provide the optimal therapeutic effect. The intense aching, soreness and occasional pain, also known as “De-Qi”, during strong acupuncture stimuli could sometimes lead to fainting, or even neurogenic shock [1]. Strong stimulation also combines with causes emotional distress like nervousness, anxiety or fear, and patients may refuse to receive the subsequent needling because of the distress. which may further frustrate patients from subsequent needling.

Although clinical experience has observed that acupuncture analgesia depends largely on the presence of “De-Qi”, it remains unclear whether a stimulation-evoked nociceptive response is essential for this effect during electroacupuncture (EA), an electrical stimulation through needles inserted at acupoints. Kawakita and Funakoshi [2] demonstrated that high intensity, but not low intensity, EA stimulation at rat hindlimb acupoints activated A $\delta$ -fibers and persistently depressed the jaw-opening reflex in anesthetized rats. In primates, electrical stimulation of the A $\alpha$  and A $\beta$  fibers produced a minor and short-lived inhibition on the spinothalamic tract neurons, while recruitment of A $\delta$  and C fibers produced long-lasting inhibition up to 30 min [3,4]. In a transverse slice of spinal cord, transient A $\delta$ -afferent stimulation could induce long-term depression for 2 h [5]. Based on their parametric studies of EA, Romita et al. suggested that recruitment of high-threshold primary afferents, both A $\delta$  and C fibers, was essential to yield a persistent extrasegmental inhibition [6,7]. In contrast, Toda and Ichioka [8] demonstrated that long-lasting suppression of digastric electromyogram amplitudes could be obtained using an intensity of electrical stimulation just sufficient to activate

A $\delta$ , whereas further augmenting the strength could not enhance the suppression. In awake mice, electric activation of large-diameter A-fibers was sufficient to initiate behavioral hypoalgesia lasting for 20 min after low frequency EA [9]. Human studies revealed that tooth pain threshold could be elevated for 30 min after termination of an unpleasant but not painful electrical stimulation [10,11,12]. Critical defects among the above studies were the lack of nociceptive control for behavioral comparison and the potential bias from cognitive and emotional interaction. It is generally believed that psychological distress by restraint or manipulation could profoundly modify the behavioral responses in conscious animals [13,14,15].

Under a stable light anesthesia with halothane, we were able to eliminate the potential confounding influence of psychological distress from investigation of the intensity-dependent and nociceptive-related effects on EA-produced analgesia. We first determined the adequate depth, volume and concentration of intramuscular injection of formalin, a noxious chemical irritant, to induce characteristic painful responses in awake rats. These rats were used as the nociceptive control to investigate whether a noxious stimulation to the acupoint is required for maximal analgesic response. Tail flick latency and *c-fos* expression were measured under halothane anesthesia. Experiment was performed with low-intensity EA, high-intensity EA, formalin and normal saline injection at the acupoint for comparison.

## 2. Materials and Methods

### 1. Preparation and EA stimulation

Studies were performed under the approval of the Animal Care and Use Committee and strictly followed the Guidelines for the Care and Use of Experimental Animals of Shin-Kong Memorial Hospital and National Taiwan University Hospital. Male Sprague-Dawley rats (250-350 g) were housed in groups of two to three at  $22 \pm 2^\circ\text{C}$  with a 12-hr dark-light cycle. For experiments, a rat was placed in an induction chamber pre-filled with 5% of halothane till loss of spontaneous movement, then transferred to a transparent restrainer with a rodent mask connected to an anesthetic circuit with 1% halothane in 1 L/min pure oxygen. Anesthetic concentration was continuously monitored by gas analyser (Capnomac, Datex Instrumentarium Corp., Helsinki, Finland) at the exhaled end of the circuit. In a previous study, we have shown that “induction period” of 30 min was sufficient to achieve constant tail-flick latencies and could keep rats at a stable light anesthetic plane [16].

One pair of stainless steel needles (30 G) was inserted to a depth of 5 mm at the right Zusanli (ST-36) acupoint approximately 5 mm inferior-lateral to the anterior tubercle of the tibia. Two needles were separated vertically by a distance of 5 mm and were connected to a Grass model S88 electrostimulator (Astro-Med Inc., Rhode Island, USA) with an electric current of square pulses at 4 Hz, 5 ms continuously for 30 min. The stimulation voltage was gradually increased after the first detection of local muscle twitch, defined as basal intensity and the strength is usually about 0.5-0.8 V. ~~(basal intensity), to~~ either of two selected strengths: low (10~~times~~ basal intensity) or high intensity (20~~times~~ basal intensity) was our study target. The target voltage was reached within the first 5 min from the start of electrical stimulation, and ~~was was~~

maintained ~~at the final voltage~~ for ~~the rest remaining~~ 25 min. The characteristics of rhythmic ankle dorsiflexion during EA ~~maintenance stimulation~~ were ~~recorded~~ observed.

## 2. Tail flick latency under low, high intensities EA or intramuscular injection of formalin

All rats were anesthetized in a transparent holder which placed on an electric heat mattress to maintain a constant rectal temperature at 38 °C and ambient temperature was strictly maintained at 25-27 °C as suggested by Sawamura et al [17]. The tail was naturally extended out ~~of a transparent holder~~ and a 5-cm segment within the distal half of the tail, except the tailtip, was blackened to facilitate radiant heat absorption. A photodetector was set to automatically stop a timer with reaction time measured to 0.01s after tail movement being detected. Heat of the focused projection bulb was set to a basal reaction time of 3-4s and the tail was passively removed if withdrawal did not occur within a 10s cutoff limit. Basal latency was the average of two successive latencies, separated by 2 min, measured immediately before EA stimulation or intramuscular formalin injection, and test latencies at 10-min intervals were ~~recorded~~ obtained from the average of three measurements separated by 2 min, at 10-min intervals for the subsequent 90 min after treatments. ~~All rats were placed on an electric heat mattress to maintain a constant rectal temperature at 38 °C and ambient temperature was strictly maintained at 25-27 °C as suggested by Sawamura et al (2002). Test latency was the average of three measurements, separated by 2 min.~~ To avoid excessive thermal injury to the tail, irradiation was performed in a clockwise manner within the blackened segment. Maximal possible effect (MPE) was calculated as:

$$\text{MPE \%} = (\text{test latency} - \text{basal latency}) / (10 - \text{basal latency}) \times 100 \%$$

## 3. Experimental Protocol

In first part of the study, we sought to determine the appropriate depth,

concentration and volume of formalin intramuscular injection required to induce nociceptive behaviors. In conscious rats, 2% or 5% formalin in either 50 or 100  $\mu$ l was injected 5 mm into a point at the upper one third of right anterior tibial muscle, the same locus of Zusanli acupoint (Fm). Rats receiving either normal saline or 2%, 50  $\mu$ l formalin subcutaneous injection into the plantar surface of right hindpaw served as the negative or positive control (Fp). All rats were transferred into an open cage immediately after injection and observed for 1 h using weighted pain scores [18,19]. Behavioral scores following the intramuscular injection in the hindlimb were compared with those in the rats subjective to intraplantar injections. Twenty  $\mu$ l methylene blue was added to evaluate the spreading of blue injectate in muscles after sacrifice.

In the second part of study, rats were anesthetized under halothane inhalation and categorized into four groups: Control group (C): normal saline of 100  $\mu$ l injected at the right ST36 acupoint; Low intensity EA (E1): EA with 10 $\times$  basal intensity at the right ST36 acupoint; High intensity EA (E2): EA with 20 $\times$  basal intensity at the right ST36 acupoint; and formalin group (Fm): injection with 5%, 100  $\mu$ l formalin at the right ST36 acupoint. Tail flick latencies of the rats in the four groups were measured and compared. All rats were euthanized for immunohistochemical study 90 min after start of EA stimulation or injection.

#### *4. Immunohistochemistry and counting of Fos-labeled neurons*

Under deep anesthesia with an intraperitoneal injection of overdose sodium pentobarbitone, animals were perfused transcardially with 250 ml saline followed by 300 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH=7.4). The spinal cord at the L<sub>2</sub>-L<sub>5</sub> lumbar segments was removed, post-fixed at 4°C for 4 h, and cryoprotected in 30% sucrose at 4°C for 48-72 h. Frozen sections were cut in a cryostat

(30  $\mu\text{m}$ ) and collected as free-floating sections, then incubated with primary rabbit polyclonal anti-Fos antiserum (Santa Cruz Biotechnology, Inc., CA, USA), diluted 1:1500 with PBS 0.1 M, containing 3 % normal goat serum (NGS) and 0.3 % Triton X-100 at 4 °C for 48 h. After washing in PBS, sections were incubated with biotinylated second antibody (goat anti-rabbit antiserum; Vector Laboratories, Burlingame, CA, USA) diluted 1: 200 in PBS for 1 h at room temperature, reacted with elite avidin-biotin-peroxidase complex (Vectastain<sup>®</sup>, Vector Laboratories, Inc., CA, USA) diluted 1:50 for 1 h, and then 0.1 % diaminobenzidine (DAB) solution containing 30%  $\text{H}_2\text{O}_2$  as substrate was added. All sections were mounted on gelatin-subbed slides, air-dried and protected with a coverslip for light microscopic inspection. For each animal at least ten slices of the Fos-labeled neurons in each segment of the 2nd to 5th lumbar spinal dorsal horns were counted. Sections were examined using a dark-field microscope (Axioscope, Zeiss, German) to determine the segmental level according to the gray matter landmarks, as previously described [20]. 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 and VI). Fos-labeled neurons, which showed deep staining distinguishable from background in bright-field illumination, were counted by laminae of layers.

##### *5. Data Analysis*

All data are presented as mean  $\pm$  SEM. Tail-flick latency and weighted pain scores of formalin test were transformed into area under curve (AUC) and were compared with one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. A value of  $p < 0.05$  was considered statistically significant.

### **3. Results**

#### *1. Formalin acupoint injection and nociceptive behaviors*

Formalin injection in either the plantar surface (Fp) and acupoint (Fm and Fm2) exhibited biphasic behavioral hyperalgesia which were distinct from intraplantar normal saline injection (Fig 1). The peak weighted pain scores were  $2.1 \pm 0.1$  at 40 min in Fp group and  $1.5 \pm 0.2$  at 35 min in Fm group. In freely moving rats, intramuscular injection of formalin into upper one third of right anterior tibial muscle exhibited joined toes, clenched claw, elevated hindlimb, crippled walking, and occasional licking of hindfoot for approximately 1 h and gradually disappeared within 2 h. Among the 4 different combinations of various concentrations and volumes, intramuscular injection of 5%, 100  $\mu$ l formalin produced a similar scoring pattern as intraplantar injection of 2%, 50  $\mu$ l formalin.

Spreading of methylene blue was examined. The densely deep blue staining was confined to the deep layer of anterior tibial muscle and scarcely dispersed through interosseous membrane. No appearance of blue staining was found in the posterior calf muscle groups.

#### *2. Intensity-dependent analgesia on tail-flick reflex*

Anesthetic level was maintained at 0.8-1% halothane to preserve corneal and ear pinna reflex while agitation or body movement was markedly abolished during the most intense electrical stimulation. Rats receiving normal saline maintained constant tail-flick latency throughout 2-hr anesthesia (Fig 2A). Tail-flick latencies gradually increased after 30 min stimulation with low and high intensity EA and remained constantly elevated thereafter. Maximal MPE in the E1 group developed at 70-min and was

significantly higher than the saline control (36.6% vs. 4.5%,  $p < 0.05$ ). E2 group showed maximal analgesic effect (77.0%) at 90-min and was significantly different from control (7.2%,  $p < 0.01$ ), E1 (30.0%,  $p < 0.05$ ), or Fm group (38.4%,  $p < 0.01$ ). Cumulative latencies in the low and high intensity EA groups were 7.2- and 11.0-fold greater than the saline control while muscular formalin produced an 8.2-fold increases compared to the saline control ( $p < 0.01$ , Fig. 2B).

### *3. Expression of Fos-like immunoreactive (Fos-LI) neurons in the spinal cord dorsal horn*

The somatotopical distribution of nociceptive neurons in lumbar spinal cord dorsal horn was different between rats receiving intramuscular formalin injection and EA treatment (Fig. 3). Noxious input from formalin irritating at the acupoint markedly increased the number of Fos-labeled neurons at the superficial and deep laminae from L2 to L5 compared to the saline control and EA groups. In contrast, high intensity electrical stimulation *per se* only mildly increased the number of Fos-labeled neurons in ipsilateral corresponding L2 and L3 segments without significance (Fig. 3 and 4). High intensity EA or saline injection showed no difference in Fos-labeled neurons between the right and left sides among various regions of dorsal horn throughout the L2 to L5 segment. These results demonstrated that very few nociceptive neurons at the receptive dorsal horns could be excited by intense EA stimulation.

#### 4. Discussion

This study demonstrated that noxiously-evoked stimulation, i.e. formalin injection, to the Zusanli acupoint resulted in biphasic behavioral hyperalgesia, simultaneously prolonged the tail-flick latency, and markedly evoked *c-fos* expression throughout the lumbar segments. In contrast, 4 Hz electrical stimulation at the Zusanli acupoint produced an intensity-dependent prolongation of tailflick latency but did not induce nociceptive *c-fos* responsiveness in the corresponding spinal dorsal horn. The behavioral and *c-fos* immunohistochemical evidence indicate that the extent of EA analgesia could be generated in proportion to its stimulation intensity within strengths not exceeding the threshold of recruiting nociceptive afferent pathway.

In our study, the intramuscular formalin induced nociceptive behaviors and significant *c-fos* expression at the corresponding spinal dorsal horn. This finding indicates that acupoint formalin injection was noxious in nature. The prolongation in tailflick latency is consistent with a well-known mechanism of “diffuse noxious inhibitory controls” (DNIC), i.e. noxious stimulation in one region can suppress painful response at another region [21,22]. In the practice of acupuncture, deep aching sensation during stimulation was regarded as strong evidence supporting the involvement of DNIC mechanism [7,23,24,25,26]. However, we were able to produce an even greater analgesic response by intense, but not noxious, EA stimulation. This result is consistent with clinical observations that acupuncture could be exercised at an intensity that is “strong but not painful” or “not hurt” [27,28]. Although formalin clearly activates a different neural pathway other than electrical stimulation, both chemical and physical modalities generate potent analgesic effect despite the involvement of two distinct mechanisms. Thus, we suggest that formalin acupoint injection can serve as a useful

positive nociceptive control to gauge the extent of EA stimulation.

The discrepancy between our methods and previous study design could explain the distinct finding in the present report. First, we measured rat's nociceptive behaviors and its biochemical responses under an intact neural axis. This is a clear contrast to previous studies that acute decerebration, or spinalization was required for electrophysiological recordings [2,3,4]. As midbrain and brainstem structures are prerequisite for descending inhibitory control, interrupting the supraspinal connectivity could profoundly alter the manifestation of EA-induced analgesia. Secondly, we stimulated the muscle with electrical needling rather than applying stimulation over the peripheral nerve. The Zusanli acupoint is particularly rich in large A $\beta$  sensory afferents [29]. Electrical acupoint stimulation permits a high intensity to elicit group I or II nerves, while direct peripheral nerve stimulation selectively activates A $\beta$ , A $\delta$ , or C fibers at a much lower intensity [2,3,4,5,30,31]. The simultaneous activation of compound afferents by intramuscular electrostimulation could exert a complex modification to transmission circuit and would thereby inhibit the excitation of nociceptive neurons in the homosegmental substantia gelatinosa [32,33].

As psychological distress clearly plays a critical role in awake rat model, we administered inhalation anesthesia to eliminate the underground bias and used biochemical marker, *c-fos* expression, for detection of nociception. Fos expression in central nervous system has been widely accepted as a reliable marker of neuronal activity in animal nociceptive models [34,35,36]. EA has been shown to reduce total Fos-LI neurons in spinal dorsal horn induced by noxious pinches [37], or by bladder instillation with 1% acetic acid [38]. However, these reports either did not delineate the distribution of EA-evoked Fos expression in dorsal horn or did not differentiate the

impact of EA with that of a nociceptive stimulation in the same receptive field. In this study, we demonstrate that EA at ST36 acupoint evoked a scarce number of Fos-LI neurons in the superficial laminae, and even fewer in the neck and deep laminae from L2 to L5 segments. Our result is in agreement with Lee et al. that *c-fos* expression in laminae III to V was not different between the both sides of dorsal horn [39]. Although the physiological implication of *c-fos* activation remains to be specified, it is still justified correlating behavioral responses with histochemical reactivities to obtain an evidence of relation between the intensity of peripheral stimulation and the magnitude of nociceptive responses. Intramuscular formalin injection, which has been shown to be both behaviorally and histochemically noxious, can be regarded as an ideal nociceptive control. Accordingly, the scant *c-fos* activation indicates the lack of a nociceptive response following low frequency, high intensity EA stimulation.

The use of halothane anesthesia throughout the observation period is critical to allow a maximal EA intensity as well as to abolish psychological distress and cognitive processes. In the pilot study, we found that all rats were hiding, shrinking, and tremulous when the electrical stimulation exceeded 5 to 10 times of the twitch threshold. Under these conditions, application of a higher EA intensity (i.e. 10-20 times basal intensity) was almost impossible. But with the aid of 0.8-1% halothane anesthesia, needle insertion and titration of the intensity were smooth without causing any stressful phenomena to the animals. Stress-induced analgesia [40] has been accepted to play a pivotal role in acupuncture analgesia in awake animal models [14,15]. Moreover, stimulation conditioning at the conscious status can also lead to the well-known placebo analgesic effect in acupuncture practice [41]. In this anesthetized model, EA could still exert a profound analgesic effect in rats that were devoid of emotional distress and

conditioned effects.

Halothane anesthesia, however, is a potential risk influencing tail-flick latency and *c-fos* expression. In this study, serial measurement of rats receiving normal saline injection revealed stable tail flick latencies within 2 hours under 0.8-1% halothane, implying that the carefully gauged anesthesia did not modify the thermal noxious threshold within this period of time. In the meanwhile, EA-treated rats demonstrated a prolonged post-stimulation analgesia that continued for more than 60-min after the stimulation period. As we had previously demonstrated that 2% halothane did not suppress behavioral hyperalgesia and Fos expression after subcutaneous formalin injection [19], the subanesthetic concentration of inhalation anesthesia, 0.8-1% halothane, may provide reliable sedation and preserve EA or chemical irritation on peripheral sensory afferents.

In conclusion, this study has demonstrated that EA prolonged cumulative tail-flick latency in an intensity-dependent manner but, in contrast to formalin injection, did not provide intensities sufficient to induce *c-fos* expression throughout the 2nd to 5th lumbar segments. Although noxious chemical stimulation to the acupoint can elicit comparable analgesic effect, non-noxious electrical stimulations produce even greater analgesic responses in an intensity-dependent manner. Our data suggest that the analgesic efficacy of EA may not rely on induction of an intense painful sensation, an idea which is distinct from the belief of the **traditional** acupuncturists.

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## 6. Figure Legends

Fig. 1. Weighted pain scores in rats receiving 2% 50  $\mu$ l formalin in the plantar surface (Fp, n = 9), 5% 100  $\mu$ l formalin injection at the ST36 acupoint (Fm, n = 9) or more diluted (2% 50  $\mu$ l) formalin (Fm2, n = 4) in the acupoint. (A): Both acupoint injection of 5% or 2% formalin resulted in a similar biphasic pain pattern to intraplantar injection. Fp group had a significantly higher pain scores than Fm group at the last 20 min of the late phase, and Fm2 showed lower pain intensity.

Fig. 2. (A) Tail flick latency in halothane-anesthetized rats receiving low- (E1), high- (E2) intensity EA, formalin (Fm, 100  $\mu$ l, 5%) or normal saline (C, 100  $\mu$ l) injection over the right ST36 acupoint. Change of tail flick latency is expressed as MPE with respect to time 0. Horizontal thick bar indicates EA stimulation period. Control rats exhibited consistent tail flick latency throughout the period of administration of anesthetic. An intensity-dependent analgesia effect of EA was shown. \*  $p < 0.05$  \*\*  $p < 0.01$  for E1, E2 or Fm groups vs. C group; +  $p < 0.05$  ‡  $p < 0.01$  for E2 group vs. E1 group, §  $p < 0.05$  §§  $p < 0.01$  for E2 group vs. Fm. (B) Cumulative MPE (shown by area under curve) for 90 min demonstrated that high-intensity EA produced a greater analgesic effect than low-intensity EA. Formalin acupoint injection had a similar analgesic effect to low-intensity EA. \*\*  $p < 0.01$  .

Fig. 3. Distribution of Fos-LI neurons in lumbar dorsal horns was distinct between high intensity EA treatment (A, B) and intramuscular formalin injection (C, D) groups. Very few Fos expression could be found throughout segments from L2 to L5, but more FLI neurons were induced at superficial laminae by intramuscular injection of formalin. Low intensity EA evoked weaker Fos expression than high intensity EA (Photos not shown). A and C at L2; C, D at L5. Scale bar = 100 mm

Fig. 4. Somatotopical distribution and quantification of Fos-labeled neurons along the lumbar spinal cord dorsal horn (L2-L5). Numbers of Fos-labeled neurons among rats treated with high intensity EA stimulation (E2, n=5) or normal saline (NS, n=5) shows significant difference from those treated with acupoint injection of formalin (Fm, n=8) at the superficial laminae and deep laminae of all segments. No difference is found between E2 group and NS group. S: superficial lamina; NP: nucleus propius; D: deep laminae; T: total number at dorsal horn. \*  $p < 0.05$ , \*\* $p < 0.01$ .