ELSEVIER

Molecular Brain Research 43 (1996) 157–166

MOLECULAR BRAIN RESEARCH

Research report

# Brain substrates activated by electroacupuncture of different frequencies (I): comparative study on the expression of oncogene c-*fos* and genes coding for three opioid peptides

Hui-Fu Guo [a,1], Jinhua Tian [a], Xiaomin Wang [a], Yuan Fang [b], Yueping Hou [a], Jisheng Han [a,*]

[a] *Neuroscience Research Center, Beijing Medical University, Beijing 100083, People's Republic of China*
[b] *Dept. of Physiology, Oregon Health Science University, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97201-3908, USA*

Accepted 18 June 1996

**Abstract**

Low and high frequency electroacupuncture (EA)-produced analgesia have been shown to be mediated by different brain substrates and different opioid peptides. In this study, Fos-like immunoreactivity (FLI) and in situ hybridization of the three opioid mRNAs were used to examine the effect of low (2 Hz) and high (100 Hz) frequency EA on neuronal activities and the expression of opioid genes. 2 Hz and 100 Hz EA induced a markedly different spatial patterns of Fos expression in the rat brain, suggesting there are distinct neuronal pathways underlying EA of different frequencies. Likewise, 2 Hz and 100 Hz EA exert differential effects on opioid gene expression: while 2 Hz EA induced a more extensive and intensive preproenkephalin (PPE) mRNA expression than 100 Hz EA, it had no effect on preprodynorphin (PPD) mRNA expression which was significantly increased by 100 Hz EA stimulation. In contrast, EA of both frequencies did not affect POMC mRNA expression.

*Keywords:* Electroacupuncture; Stimulation frequency; c-*fos*; c-*jun*; Preproenkephalin; Preprodynorphin; Proopiomelanocortin

## 1. Introduction

Study on the mystery of the ancient healing of 'acupuncture' has been greatly facilitated by the use of advanced technology of neuroscience. On the other hand, the study of the mechanism of acupuncture-induced analgesia has also contributed to the progress of neuroscience. One of the issues worthwhile of mention is the claim that distinct neurochemical mechanisms are involved in mediating analgesia induced by electroacupuncture (EA) of different frequencies [4,14,18–21].

Before the discovery of dynorphin [16], the most important criterion of an 'opioid-mediated effect' was the reversibility of the effect by a small dose of naloxone (1–2 mg/kg). Since naloxone (2 mg/kg) was found to block analgesia induced by EA of low frequency (4 Hz) but not high frequency (200 Hz), the later was considered 'non-opioid' in nature [5]. The discovery of dynorphin A and its relative resistance to naloxone blockade triggered a re-evaluation of the conclusion. Using electrical stimulation of the same pulse width (0.3 ms) and same intensity (3 mA) placed on the same 'acupoints' at the hind leg of the rat, Han and coworkers [19] were able to show that the $IC_{50}$ of naloxone for blocking EA-induced analgesia (EAA) can be as low as 0.5 mg/kg for 2 Hz EA, and as high as 10 mg/kg for 100 Hz EA, suggesting the involvement of dynorphin and the κ-opioid receptor in mediating the high frequency EAA. This was substantiated by a series of evidence using classical physiological approaches of stimulation and abolition techniques as well as pharmacological and neurochemical approaches [4,14,18–21,23,36–39]. These studies may be summarized as: (1) specific brain areas are involved in the mediation of pain by EA of different frequencies, i.e., arcuate versus parabrachial nucleus is the critical area mediating low versus high frequency EAA, respectively [36–39]; (2) low frequency EAA is mediated by beta-endorphin and enkephalin interacting with μ- and δ-opioid receptors, while high fre-

* Corresponding author. Fax: +86 (1) 202-9252.
[1] Present address: Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724, USA. Fax: +1 (516) 367-8880; E-mail: huifu@cshl.org


*PII* S0169-328X(96)00170-2

quency EA analgesia is the result of the activation of κ-receptor by dynorphin [4,14,18–21]. These findings suggested the importance of the stimulation frequency in determining the effect of EAA, which is in agreement with the clinical experience of acupuncture practice that proper maneuver of acupuncture (speed of twisting as well as the frequency of up-and-down movement of the needle) is a cardinal factor in determining the curative effects for different diseases. A rational question is: what are the central pathways responsible for these qualitatively different physiological effects?

Based on the above mentioned discoveries and hypothesizes, as well as the successful use of Fos immunoreactivity as a marker of neuronal activities [3,11], we in the present study: (1) use immunocytochemistry to monitor the effects of low (2 Hz) and high (100 Hz) frequency EA on c-Fos expression in an attempt to identify the brain areas activated by EA of low and high frequency; (2) use in situ hybridization (ISH) to show effects of 2 Hz and 100 Hz EA on the expression of the three opioid genes in the rat brain.

## 2. Material and methods

### *2.1. Animals and electroacupuncture procedures*

Female Wistar rats weighing 180–250 g were obtained from the Scientific Animal Center of Beijing Medical University. For the immunocytochemistry (ICC) detection of Fos expression, the rats were divided into four groups of four rats receiving: (1) 2 Hz EA; (2) 100 Hz EA; (3) needle inserting into the acupoint without electrical stimulation (needle control); (4) no special treatment, serving as naive control. For electroacupuncture, the animal was loosely placed in a plastic holder with hind legs protruding. Two pairs of stainless steel pins of 0.2 mm diameter were inserted into the acupoint Zusanli (S36, 5 mm lower and lateral to the anterior tubercle of the tibia) and Sanyingjiao (SP5, 3 mm proximal to the medial malleolus, posterior to the tibia) of the hindlegs. Rectangular pulses of 2 Hz or 100 Hz at 0.3 ms pulse width were administered with the intensity increasing stepwise from 1 mA to 2 mA up to 3 mA, each lasting for 10 min. In group 3, rats were kept in holders with the needles placed in situ without connecting to electrical stimulator. Two hours after the termination of EA stimulation of the control manipulation, the rats were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and were perfused transcardially with 100 ml saline followed by 200 ml 4% paraformaldehyde for 40 min. The brain tissues were removed and postfixed in the same fixative overnight, and placed in a 30% sucrose solution for 35–48 h.

To observe the effects of 2 Hz or 100 Hz EA on the three opioid mRNAs expression by in situ hybridization (ISH), the animal groups and the electroacupuncture procedure were the same as that for the detection of Fos expression, except it was 24 h after the termination of EA stimulation (or needle) that the animals were anesthetized and perfused. The tissues were postfixed and sucrose protected in the same way as for ICC.

### *2.2. Immunocytochemistry*

Tissue sections were cut coronally in a cryostat at 30 μm. One to three sections were collected every five sections (varied according to the sagital length of the different nucleus, to ensure that at least three sections were collected for each nucleus or brain area) in phosphate buffer (PBS). Free floating sections were processed according to the Hsu's ABC method [24] with some modifications. Briefly, the sections were incubated with the first primary antibody (rabbit antiserum against Fos 1:1,000, Oncogene Science) for 48 h at 4°C. Subsequently the sections were incubated for 1 h at 37°C with the biotin-labeled secondary antibody and ABC reagent (products of Vector Laboratories). The sections were then incubated n 0.05%DAB/0.01% hydrogen peroxide/0.1 N acetate buffer (pH 6.0) containing 2% ammonium nickel sulfate for 2–5 min. After staining, the sections were rinsed in acetate buffer and mounted onto the gelatin coated slides, air dried and neutral palsam coverslipped.

### *2.3. In situ hybridization*

Free-floating method of ISH was used. Briefly, continuous 30 μm coronal sections were collected in three sets for the detection of PPE, PPD and POMC mRNA respectively. After rinses in phosphate buffer and pretreatment with proteinase K (1 μg/ml), the sections were transferred into paraformaldehyde (PFA)/PBS and were then acetylated. After washes in SSC buffer, the sections were incubated in hybridization buffer containing 500 ng/ml digoxin-labeled antisense cRNA probe of PPE, PPD or POMC respectively, and hybridized for 12–16 h at 42°C. The sections then were washed at high stringency and digested with RNase to remove excessive probes. For immunodetection and color-staining, the procedures were the same as recommended in the detection kit from Boehringer Mannheim. The sections were mounted onto the slides in PBS, dehydrated and coverslipped. Some of the sections were lightly counterstained with cresyl violet.

### *2.4. Methodological controls of ISH*

(1) Sections were pretreated with RNase (20 μg/ml); (2) sections were incubated in prehybridization buffer containing an excess of unlabeled PPE or PPD or POMC cRNA probe prior to hybridization; (3) no probe was added to the hybridization buffer; (4) labeled sense instead of antisense cRNA probe was added to the hybridization buffer; (5) standard controls for immunodetection.

### 2.5. Counting and statistics

Tissue sections were examined at 65, 100 and 200 × under lightfield microscopy. Labeled nuclei of FLI or labeled neurons of PPE, PPD or POMC mRNA were identified using lightfield microscopy at 100 × . FLI nuclei and PPE, PPD or POMC-positive neurons were counted only when structures of the appropriate size and shape demonstrated clear increases when compared to the background level. Some of ISH sections were analyzed by IBAS 2000 image system. Numerical data were expressed as mean ± S.E.M., and the statistical significance was determined by Student's *t*-test.

## 3. Results

### 3.1. Effects of 2 Hz and 100 Hz EA on Fos induction in the rat brain

With the whole brain, only a few FLI cells were detected in the pontine nucleus of the untreated rats. In the

Table 1
Effects of 2 Hz and 100 Hz electroacupuncture on c-*fos* expression in rat brain

| Structure | Naive | Needle | 2Hz EA | 100 Hz EA |
|---|---|---|---|---|
| *Telencephalon* | | | | |
| Cerebral cortex | | | 18.8 ± 3.2 | 17.6 ± 4.5 |
| Motor | | | | |
| Sensory | | | 15.6 ± 5.3 | 28.3 ± 3.9 |
| Auditory | | | 20.6 ± 3.7 | 32.2 ± 5.6 |
| Entorhinal | | | 45.5 ± 8.7 | 40.3 ± 7.1 |
| Habenula (Hab) | | | 0 | 30.2 ± 5.2 * |
| Medial optic area (MPO) | | | 50.5 ± 4.8 | 45.3 ± 7.5 |
| Supraoptic nucleus (SON) | | | 22.2 ± 1.9 | 21.3 ± 2.3 |
| Amygdala, medial | | 3.8 ± 1.9 | 69.3 ± 4.8 | 58.5 ± 9.5 |
| Amygdala, cortical | | | 23.5 ± 3.2 | 16.8 ± 6.3 |
| *Diencephalon* | | | | |
| Paraventricular thalamic nucleus (n.) (PV) | | | 0 | 20.5 ± 2.6 * |
| Hypothalamus | | | | |
| Arcuate n. (Arc) | | | 58.3 ± 7.4 * | 5.3 ± 1.4 |
| Paraventricular n. (PAH) | | | 99.3 ± 12.8 * | 34.5 ± 4.6 |
| periventricular n. (Pe) | | | 29.8 ± 3.1 * | 4.5 ± 0.6 |
| Dorsomedial n. (DMH) | | | 92.3 ± 9.6 * | 20.2 ± 3.0 |
| Ventromedial n. (VMH) | | | 122 ± 14.3 * | 31.3 ± 2.8 |
| Anterior n. (AHy) | | | 77.8 ± 6.9 | 63.3 ± 7.7 |
| Supramammillary n. (SuM) | | | 83.5 ± 10.4 * | 29.5 ± 3.3 |
| Area of tuber cinereum (TC) | | | 57.6 ± 6.6 * | 10.5 ± 3.1 |
| *Brainstem* | | | | |
| Periaqueductal gray (PAG) | | | 69.3 ± 12.8 | 80.3 ± 15.1 |
| Inferior colliculus (IC) | | | 70.4 ± 9.8 | 152 ± 23.4 * |
| Lateral lemniscus, ventral | | 5.3 ± 1.5 | 92.0 ± 5.3 * | 24.5 ± 1.04 |
| Lateral lemniscus, dorsal | | | 98.3 ± 6.2 | 37.3 ± 4.2 |
| Pontine n. (P) | 3.3 ± 0.5 | 8.5 ± 2.3 | 34.8 ± 3.1 | 32.8 ± 4.3 |
| Medial geniculate | | | | |
| Medial part | | | 35.6 ± 4.5 * | 8.3 ± 2.1 |
| Ventral part | | 10.1 ± 2.5 | 12.3 ± 3.2 | 14.5 ± 1.04 |
| Dorsal part | | | 11.7 ± 2.6 * | 0 |
| Lateral parabrachial (LPB) | | | 0 | 62.5 ± 12.1 |
| Locus coruleus (LC) | | | 3.2 ± 1.5 | 83.5 ± 5.4 |
| Raphe magnus n. (RM) | | | 7.5 ± 2.4 | 10.3 ± 2.9 |
| Raphe dorsalis n. (DR) | | | 11.3 ± 1.9 | 15.2 ± 1.5 |
| Gigantocellular n. (Gi) | | | 0 | 7.3 ± 1.8 * |
| Paragigantocellular n. (PGi) | | | 0 | 8.5 ± 1.9 * |
| Lateral reticular n. (LRT) | | | 4.5 ± 0.6 | 16.5 ± 1.7 * |
| Solitary tract n. (Sol) | | | 43.3 ± 5.9 * | 5.3 ± 1.1 |

Note: shown are numbers of FLI positive nuclei ( $X \pm$ (S.E.M.) based on four animals, counted on 3 sections in each substrate (the selection of the sections varied according to the sagittal length of different structures, but as evenly as possible in accordance with the rostral-medial-caudal parts for each substrate, and as identical as possible for every animal. Most of the symmetrical structure were counted unilaterally, as EA was administered bilaterally and symmetrical expression were found. The numbers in SuM, RM and DR were counted bilaterally.

* Indicate significant difference ( $P < 0.01$) between 2 Hz and 100 Hz. Naive: rats receiving no EA or needle treatment. Needle: rats administered with stainless steel needles without being connected to EA stimulator.

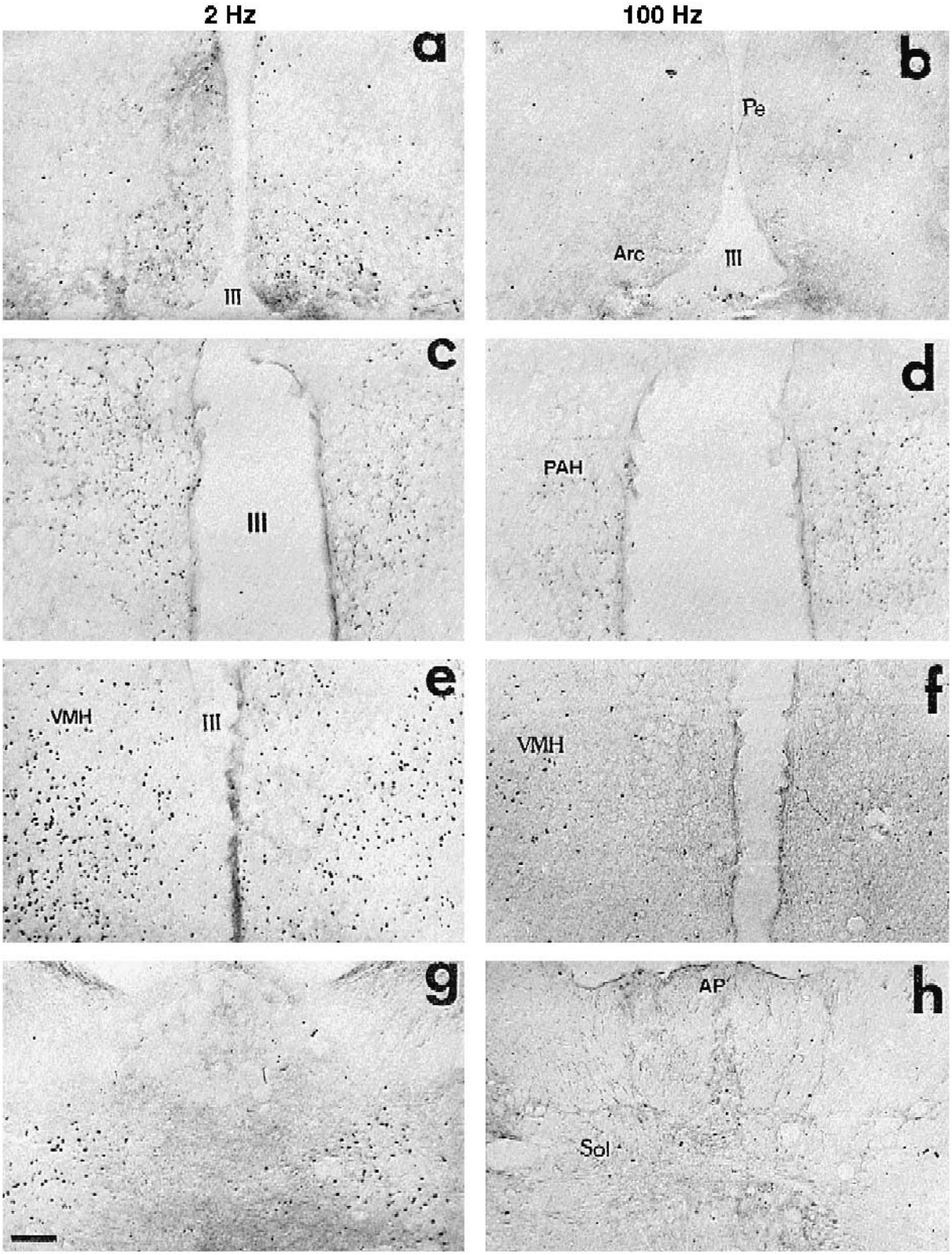


Fig. 1. Photomicrographs showing part of the brain areas where 2 Hz (left column) EA induced more FLI cells than 100 Hz (right column) EA. The areas are arcuate nucleus (Arc, a and b), periventricular nucleus (Pe, a and b), paraventricular hypothalamic nucleus (PAH, c,d), ventromedial nucleus (VMH, e,f) and solitary tract nucleus (Sol, g,h). III, the third ventricle; AP, area postrema. Same magnifications for a–h. Bars = 0.1 mm

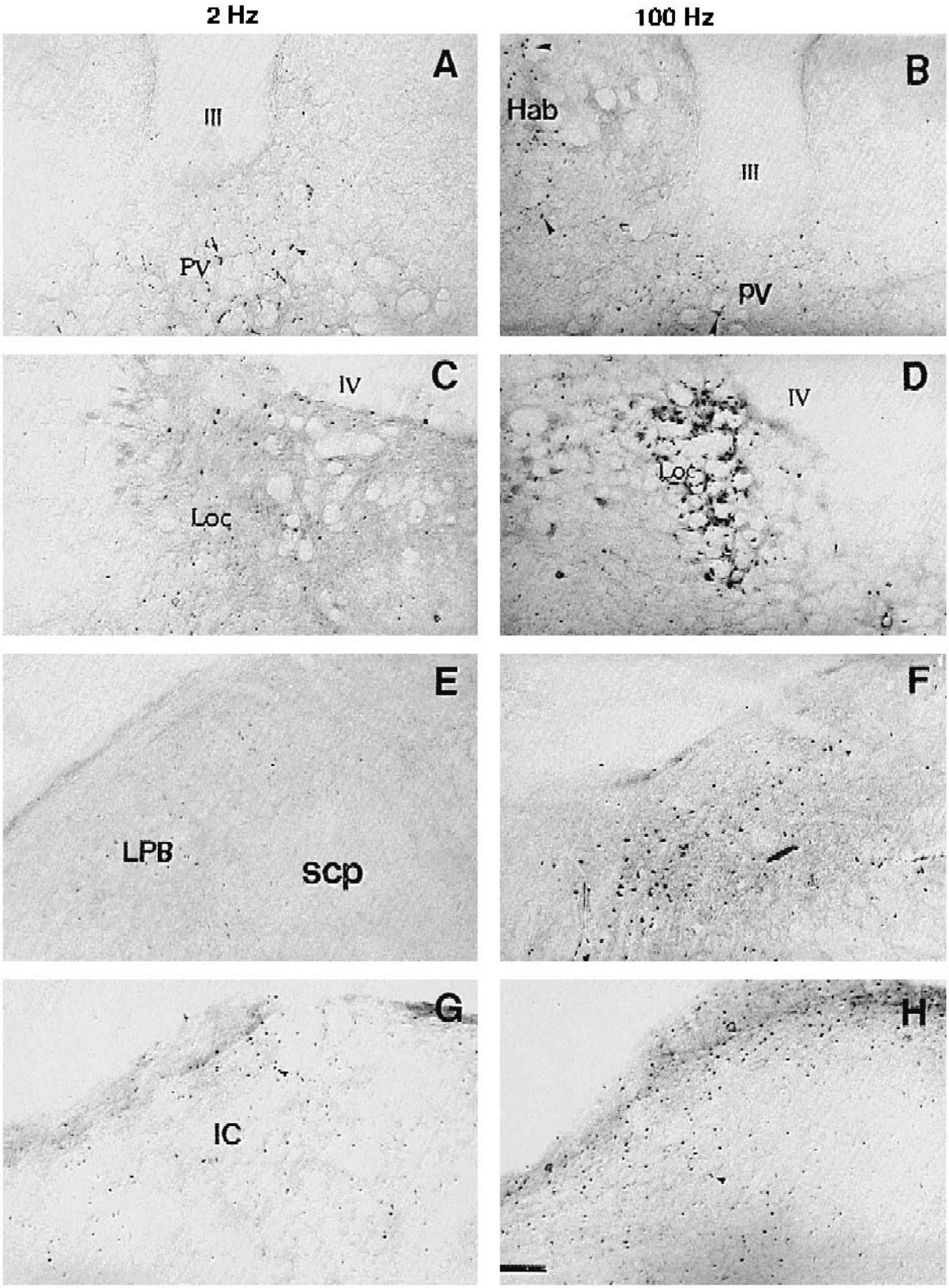


Fig. 2. Photomicrographs showing brain areas were 100 Hz EA (right column) induced more FLI nuclei than 2 Hz EA (left column). These areas are paraventricular thalamic nucleus (PV, a,b), habenula (Hab, a,b), locus coruleus (Loc, c,d), lateral parabrachial nucleus (LPB, e,f) and inferior colliculus (IC, g,h). III, the third ventricle; scp, superior cerebellar peduncle. Arrowheads and arrows show FLI cells and red blood cells respectively. Same magnifications for a–h. Bar = 0.1 mm.

needle group, scattered FLI nuclei appeared in amygdala (cortical part), lateral lemniscus (dorsal part), pontine nucleus and the dorsal part of medial geniculate. Both 2 Hz and 100 Hz EA induced an extensive c-Fos expression. The detailed comparison of the effects of 2 Hz and 100 Hz EA is described as follows (Table 1, Fig. 1 and Fig. 2).

*3.1.1. Telencephalon*

No significant difference was found between 2 Hz and 100 Hz EA in their ability of inducing Fos expression in the telencephalon. The entorhinal cortex displayed a moderate density of FLI cells. followed by the auditory cortex. In entorhinal cortex FLI nuclei distributed mainly in layer

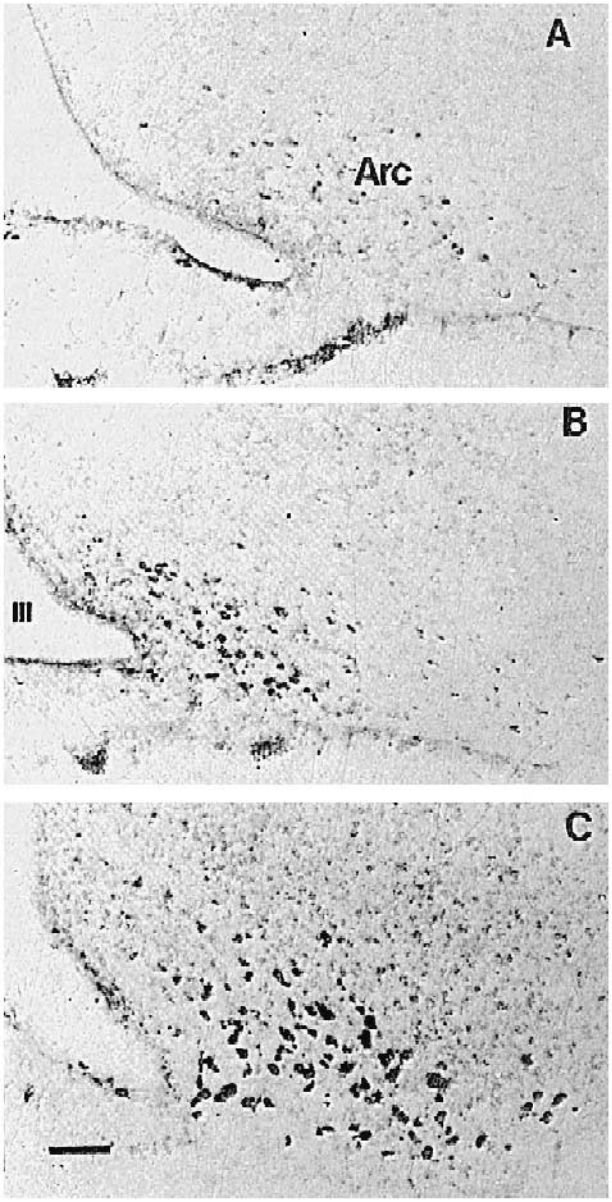


Fig. 3. Photomicrographs showing preproenkephalin mRNA expression in the arcuate nucleus (arc) of naive rats (A) or induced by 100 Hz (B) and 2 Hz (C) EA. III, third ventricle. The same magnifications for A–C. Bar = 0.1 mm.

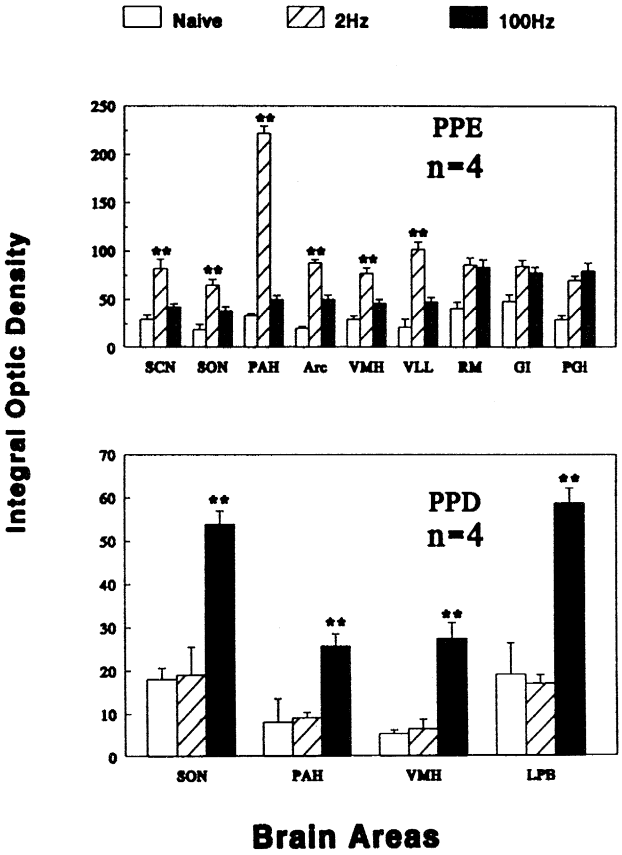


Fig. 4. PPE/PPD mRNA expression induced by 2 Hz or 100 Hz EA. Diagram showing the effect of 2 Hz and 100 Hz EA on PPE/PPD mRNA expression in rat brain. PPE, preproenkephalin; PPD, preprodynorphin; SCN, suprachiasmatic n., SON, supraoptic n.; PAH, paraventricular hypothalamic n.; Arc, arcuate n.; VMH, ventromedial n.; VLL, ventral part of lateral lemniscus n.; RM, raphe magnus n.; Gi, gigantocellular n.; PGi, paragigantocelluar n.; LPB, lateral parabrachial n.

II–III and V–VI, while in auditory cortex FLI cells distributed equally through layer I–IV. Scattered FLI neurons were noticed in motor cortex and the dorsal part of the sensory cortex. No FLI cell was observed in the ventral part of sensory cortex and the dorsal part of auditory cortex. In the medial part of amygdala (ME), a high density of Fos expression was observed, followed by the anterior cortical amygdala nucleus (ACo). Scattered FLI neurons presented in other parts of the amygdala. EA induced a high density of Fos expression in the medial part of the preoptic area and a moderate density of FLI cells in supraoptic nucleus.

*3.1.2. Diencephalon*

In the thalamus, 100 Hz but not 2 Hz EA induced a moderate to low density of FLI expression, especially in lateral habenula (Hab) and paraventricular thalamic nucleus (PV). In the hypothalamus, 2 Hz induced much more FLI cells than 100 Hz EA in arcuate nucleus, paraventricular nucleus, periventricular nucleus, ventromedial nucleus, dorsomedial nucleus, suprammammillary nucleus and the

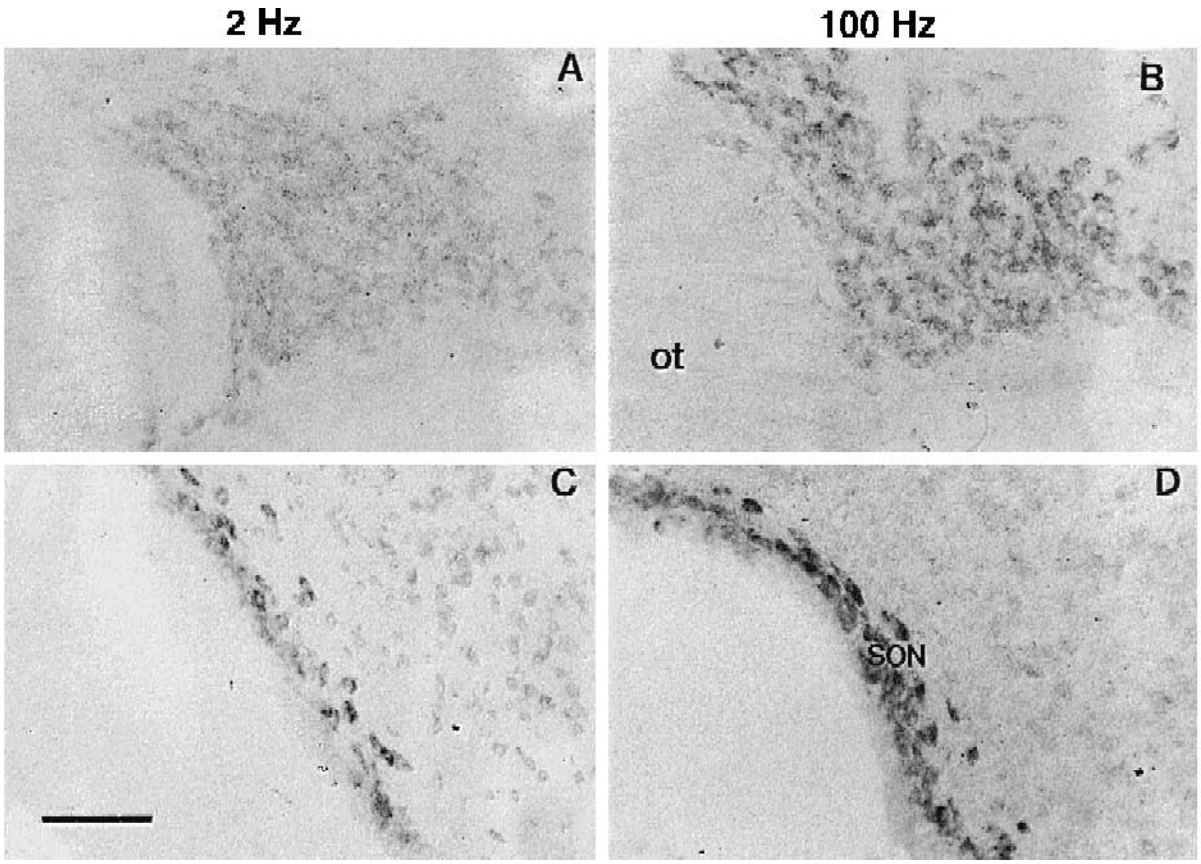


Fig. 5. Photomicrographs showing preprodynorphin mRNA expression induced by 2 Hz EA (A,C) or 100 Hz EA (B,D) in supraoptic nucleus (SON). A,B, chiasmatic part of SON; C,D, post-chiasmatic part of SON. ot, optic tract. The same magnifications for A–D. Bars = 0.1 mm.

area of tuber cinereum. In the anterior hypothalamic nucleus, however, the effects of EA of two frequencies on Fos induction were similar.

*3.1.3. Brainstem*

In mesencephalon, EA of both frequencies induced a moderate density of Fos expression in the caudal part of PAG, in which although the number of 2 Hz and 100 Hz EA-induced FLI cells showed no significant difference, their distributing patterns were different. 100 Hz EA-induced FLI nuclei distributed evenly around the aqueduct, whereas 2 Hz EA-induced FLI cells mostly present in the dorsolateral part of the caudal PAG. In superior colliculus, EA of both frequencies induced only sparse Fos expression, whereas in the inferior colliculus, 100 Hz EA induced significantly more FLI nuclei as compared to that induce by 2 Hz EA. 2 Hz EA induced more Fos expression than 100 Hz EA in the lateral lemniscus (LL) and the medial geniculate nucleus (MG).

In the pons, both 2 Hz and 100 Hz EA induced high density of FLI nuclei in the pontine nucleus and low density of FLI neurons in the dorsal raphe nucleus. In the lateral parabrachial nucleus, however, only 100 Hz EA induced high density of Fos expression, 2 Hz EA exerted no effect.

In the rostroventral medial reticular formation (RVM), 100 Hz EA induced more FLI nuclei that 2 Hz EA in the gigantocellular nucleus, paragigantocellular nucleus and lateral reticular nucleus. On the contrary, 2 Hz EA induced much more FLI neurons in the caudal part of solitary tract nucleus. In the raphe magnus nucleus that 2 Hz and 100 Hz EA were equally effective in induction of Fos expression.

*3.2. Effects of 2 Hz and 100 Hz EA on the mRNAs expression of the three opioid genes*

*3.2.1. Methodological controls*

Treatment with excessive unlabeled probes greatly reduced the signal, indicating that the signal was due to a limited-capacity binding to the probe. Hybridization with sense probes revealed a low background signal but no cellular morphology was observed. No signal was visualized in the sections treated with RNase or in the sections incubated with probe-free hybridization buffer, as well as the control sections for immunodetection.

*3.2.2. Constitutive expression of opioid mRNAs*

ISH with digoxin-labeled antisense cRNA probes revealed a high constitutive expression of opioid mRNAs, of

which PPE mRNA has the most extensive expression, followed by PPD mRNA. The expression of POMC mRNA is limited in the arcuate nucleus and suprachiasmatic nucleus. The distribution of opioid mRNAs were largely in agreement with previous reports resulted from hybridization with radiolabeled probes [2,15,22,27,29,33], with some minor differences, e.g., the high density of labeled neurons of PPE mRNA in hippocampus, and the expression of dynorphin in the post-chiasmatic part of the supraoptic nucleus (SON, Fig. 5) which has not been reported.

*3.2.3. Effects of EA on opioid mRNAs expression*

It was found that the insertion of needles exhibited no significant influence on the opioid mRNA levels. Electroacupuncture produced a frequency dependent expression of PPE mRNAs. 2 Hz EA induced more increase of PPE mRNA than 100 Hz EA (Fig. 3, Fig. 4) in arcuate nucleus, paraventricular hypothalamic nucleus and the ventral nucleus of lateral lemniscus. The increase involved not only the average cellular density, but also the number of labeled cells. Both frequencies, however, were effective in increasing the PPE mRNA level in RVM (Gi, PGi and RM).

Concerning PPD mRNA, it was 100 Hz EA that greatly increased the PPD mRNA levels in supraoptic nucleus, paraventricular hypothalamic nucleus, ventromedial nucleus and lateral parabrachial nucleus (Fig. 4, Fig. 5). 2 Hz EA was totally inactive in this context.

No change in POMC mRNA level was noticed following either 2 Hz or 100 Hz EA stimulation.

## 4. Discussion

*4.1. Differential central patterns of Fos induction by EA of different frequencies and its relation to different frequency EA-produced analgesia*

The present study is the first to provide morphological evidence for activation of different neuronal pathways by EA of different frequencies administered at the same acupoint with the same intensity. The findings that 2 Hz EA induced much more Fos expression than 100 Hz EA in arcuate nucleus, and 100 Hz but not 2 Hz induced Fos expression in the parabrachial nucleus are coincident with the previous discovery that Arc versus PBN is cardinal for mediating 2 Hz versus 100 Hz EA analgesia [20,36–39]. Besides Arc and PBN, however, there are other brain areas displaying frequency-dependent Fos induction (Fig. 1, Fig. 2 and Table 1). Although some of the areas, such as habenula [41,42] and locus coruleus [12], are known to participate in EA analgesia, whether they underlying frequency dependence are still pending for further investigation.

The absence of Fos expression does not necessarily mean the lack of neuronal activity. The following are points for consideration. (1) It was reported by Luckman et al. [26] that the induction of c-*fos* expression in the magnocellular neurons in the supraoptic nucleus requires synaptic activation and not simply increased spike activity. (2) Activation of other immediate early genes (IEGs) such as c-*jun* and zif/268 might compensate for the deficiency of c-*fos* [9]. (3) The positive signal of IEGs can only reflect the activities of the neuronal cell bodies rather than the neuropils. (4) It would be reasonable to postulate that EA may produce some inhibitory effects on neuronal activities, which could not be detected by the Fos method.

*4.2. Comparison of the central patterns of Fos induction by EA, noxious stimuli or stressful stimuli*

It has been suggested that acupuncture might only be certain form of noxious stimulation or stressful stimuli. It would therefore be interesting to compare the magnitude and site specificity of Fos expression elicited by the three types of stimuli. It is well known that thalamus is the center for the relay of pain signals. Stimulation of certain thalamic nucleus may evoked severe pain [10]. While noxious stimulation elicits extensive Fos expression in the thalamus [3], EA induces essentially no Fos expression in thalamus in case of 2 Hz stimulation, or very limited Fos induction in habenula and paraventricular thalamus nucleus in case of 100 Hz EA stimulation (Fig. 1 and Fig. 2).

Compared to stress stimuli, the patterns of acute stressful stimuli (cold water swim, restraint and immobilization, etc.)-induced c-*fos* expression share some similarities with that of EA-induced Fos expression, especially in the hypothalamus and some forebrain areas such as PAH, VMH, DMH, SuM, SON, MPO and amygdala etc. [7,13]. However, detailed comparison of EA-and acute-stress-induced c-*fos* expression seems not to support to this suggestion. Stresses induce much more extensive c-*fos* mRNA expression [9] including many areas of the cerebral cortex, hippocampus, striatum, thalamus and the lower brainstem where EA induced no or relatively low density of FLI neurons. In addition, the patterns of c-*fos* expression induced by various kinds of stressful stimuli are very similar, whereas Fos induction by EA of 2 Hz and 100 Hz are essentially different. A recent report by Pan et al. [31] showed that in the pituitary, EA (4 Hz)-induced fos expression was limited to the anterior lobe where beta-endorphin is secreted, whereas immobilization stress-induced Fos expression located mainly in the intermediate lobe where ACTH is secreted.

*4.3. Differential effects of 2 Hz and 100 Hz EA on the opioid mRNAs expression*

Previous studies in this laboratory [17] revealed that EA was able to accelerate the PPE mRNA expression in rat brain, which began 4 h after the termination of 30 min EA stimulation, reached a high level at 24 h and peaked at 48 h after EA. Ji et al. [25] also noticed the increase in PPE

mRNA at 24 h after EA stimulation. Based on these findings, we chose 24 h as the time point for detecting the effects of EA on opioid gene transcription.

It is interesting to note that 100 Hz EA accelerated both PPE and PPD mRNA expression whereas 2 Hz EA produced a much higher increases in the transcription of PPE gene, but no increase in that of PPD gene. These results are partly in agreement with the previous findings that low frequency EA releases enkephalin in brain and spinal cord and high frequency EA releases dynorphin in the spinal cord [4,14,18–21]. An interesting issue is, whether cerebral dynorphin are involved in high frequency EA analgesia? Dynorphin in brain has been shown to antagonize morphine analgesia [35] and EA analgesia [32]. However, a recent study [1] showed that i.c.v. injected dynorphin or κ-agonist elicited pain relief in cold-water-induced tail flick test but not in heat-induced tail flick test. Thus the analgesic effect of cerebral dynorphin is more particular.

That no change in POMC mRNA level following EA seems not in agreement with the previous findings that 2 Hz EA-induced analgesic effect could be blocked by beta-endorphin antibody into PAG of the rat [23,40], and that beta-endorphin in the Arc nucleus was essential for 2 Hz EA-induced analgesia [20]. This discrepancy may be explained by the high constitutive expression of POMC mRNA which is enough to cope with the EA-induced release of beta-endorphin. In a recent study Yu et al. [43] reported that an EA (3 Hz) stimulation lasting for 60 min accelerated the POMC expression which began at 8 h and peaked at 72 h after the termination of EA. So it is possible that the EA stimulation (1–3 mA, 30 min) used in the present study is not strong enough to increase POMC transcription.

### *4.4. Comparison of the brain areas showing Fos expression and opioid mRNAs expression*

A very interesting question presently concerning Fos and opioid genes is the role of Fos protein in the transcription of opioid genes, since Fos protein-contained AP-1 complexes were supposed to be the transcription factor of many target genes including the three opioid genes [6–8,28,30,34]. To demonstrate the relation of EA-induced Fos and opioid genes expression, it would be helpful to compare the distribution of Fos and opioid mRNAs expression. The analysis revealed that: (1) 2 Hz EA induced an increase of Fos expression in Arc without visible increase of POMC mRNA expression; (2) EA induced a marked increase of PPE mRNA in reticular formation of ventral medulla (RVM) where few FLI neuclei were observed; (3) PPD mRNA expression was accelerated in the post-chiasmatic part of SON where no Fos expression was noticed following EA stimulation. It is obivous that in the above mentioned cases the expression of opioid genes is not the result of Fos induction.

However, there were some areas in which both Fos and opioid mRNAs expression were noticed following EA, these areas were: (1) SON, Arc, VMH, PAH and VLL, where Fos induction and PPE mRNA increase were observed following low freqnency EA; (2) SON (chiasmatic part), PAH, VMH and LPB where 100 Hz EA induced the expression of both Fos protein and PPD mRNA. To clarify whether PPE and PPD transcription in these regions are linked to Fos induction, we need proof from the experiment in which the EA-induced Fos (Jun) expression be specifically blocked or inhibited, and to see if the transcription of PPE/PPD gene could be affected. This will be pursued in our next project.

## Acknowledgements

The authors wish to thank Dr. V. Hollt for the generous donation cRNA probes (carriers) of preproenkephalin, preprodynorphin and proopiomelanocortin, and Dr. John Connolly for improving the writing. This work was supported by a grant from the National Institute of Drug Abuse, the United States of America, DA 03983.

## References

[1] Adams, J.U., Chen, X.H., Kim Deriel, J., Adler, M.W. and Liu-Chen, L.Y., Intracerebraventricular treatment with an antisense oligonucleotide to κ-opioid receptors inhibited κ-agonist induced analgesia in rats, *Brain Res.*, 667 (1994) 129–132.

[2] Bronstein, D.M., Przewlocki, R. and Akil, H., Effects of morphine treatment of proopiomelanocortin systems in rat brain, *Brain Res.*, 519 (1990) 101–111.

[3] Bullitt, E., Expression of c-*fos*-like protein as a marker for neuronal activity following noxious stimulation in the rat, *J. Comp. Neurol.*, 296 (1990) 517–530.

[4] Chen, X.H. and Han, J.S., Analgesia induced by different types of opioid receptors: another cross-tolerance study, *Behav. Brain Res.*, 47 (1992) 143–149.

[5] Cheng, R.S.S. and Pomeranz, B., Electroacpuncture analgesia could be mediated by at least two pain-relieving mechanisms: endorphin and non-endorphin system, *Life Sci.*, 25 (1979) 1957–1962.

[6] Cole, R.L., Konradi, C., Douglass, J. and Hyman, S.E., Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum, *Neuron*, 14 (1995) 813–823.

[7] Comb, M., Mermond, N., Hyman, S.E., Pearlberg, J., Ross, M.R. and Goodman, H.M., Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible expression, *EMBO J.*, 7 (1988) 3793–3805.

[8] Curran, T., Fos and Jun-oncogenic transcription factors, *Tohoku J. Exp. Med.*, 168 (1992) 169–174.

[9] Cullinan, W.E., Herman, J.P., Battagliia, D.F., Akil, H. and Watson, S.J., Pattern and time course of immediate early gene expression in rat brain following acute stress, *Neuroscience*, 64 (1995) 477–505.

[10] Davis, K.D., Tasker, R.R., Kiss, Z.H., Hutchison, W.D. and Sostrovsky, J.O., Visceral pain evoked by thalamic microstimulation in humans, *NeuroReport*, 6 (1995) 369–374.

[11] Dragunow, M. and Faull, R., The use of c-*fos* as a metabolic marker in neuronal pathway tracing, *J. Neurosci. Methods*, 29 (198) 261–265.

[12] Du, H.J. and Zhao, Y.F., Influences of the lesion or stimulation of locus coreleus on the effects of inhibiting visceral-somatic reflex by acupuncture, *Acta Physiol. Sinica*, 31 (1979) 153–162.

[13] Ducan, G.E., Johnson, K.B. and Breese, G.R., Topographic patterns of brain activity in response to swim stress: assessment by 2-deoxyglucose uptake and expression of Fos-like immunoreactivity, *J. Neurosci.*, 13 (1993) 3932–3943.
[14] Fei, H., Xie, G.X. and Han, J.S., Low and high frequency electroacupuncture stimulation release (met5) enkephalin and dynorphin A in rat spinal cord, *Sci. Bull. (Beijing)*, 32(1987) 1496–1501.
[15] Gee, C.E., Chen, C.L.C. and Roberts, J.L., Identification of proopiomelanocortin neurons in rat hypothalamus by in situ hybridization, *Nature*, 306 (1983) 374–376.
[16] Goldstein, A., Tachibana, S., Lowney, L.I., Hunkapiller. M. and Hood, L., Dynorphin (1013), an extraordinary potent opioid peptides, *Proc. Natl. Acad. Sci. USA*, 76 (1979) 666–667.
[17] Guo, H.F., Cui, X., Hou, Y.P., Tian, J.H., Wang, X.M. and Han, J.S., C-Fos proteins are not involved in the activation of preproenkephalin gene expression in rat brain by peripheral electric stimulation (electroacupuncture), *Neurosci. Lett.*, 207 (1996) 163–166.
[18] Han, J.S., Chen, X.H., Sun, S.L., Xu, X.J., Yuan, Y., Yan, S.C., Hao, J.X. and Terenius, L., Effect of Low- and high- frequency TENS on met-enkephalin-Arg-Phe and dynorphin A immunoreactivity in human lumbar CSF, *Pain*, 47 (1991) 295–298.
[19] Han, J.S., Ding, X.Z. and Fan, S.G., The frequency as the cardinal determinant for electroacupuncture analgesia to be reversed by opioid antagonists, *Sci. Bull. (Beijing)*, 38 (1986) 475–482.
[20] Han, J.S. and Wang W., Mobilization of specific neuropeptides by peripheral stimulation of identified frequencies, *News Physiol. Sci.*, 7 (1992) 176–180.
[21] Han, J.S., Xie, G.X., Ding, X.Z. and Fan, S.G., High and low frequency electroacupuncture analgesia are mediated by different opioid peptides, *Pain*, (Suppl.) 2 (1984) 543.
[22] Harlan, R.E., Shivers, B.D., Romano, G.J., Howells, R.D. and Pfaff, D.W., Localization of preproenkephalin mRNA in the rat brain and spinal cord by in situ hybridization, *J. Comp. Neurol.*, 258 (1987) 159–184.
[23] He, C.M. and Han, J.S., Influence of beta-endorphin antibody injected into mesencephalonic periaqueductal gray on analgesia produced by electroacupuncture of different frequencies in rat, *Acupuncture Res. (Beijing)*, 15 (1990) 97–103.
[24] Hsu, S.L. and Fanger, H., Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures, *J. Histochem. Cytochem.*, 29 (1981) 577–580.
[25] Ji, R.R., Zhang Q. and Han, J.S., Electroacupuncture stimulation promotes preproenkephalin mRNA expression in rat spinal cord and medulla, *Acta Physiol. Sinica (Shanghai)*, 45 (1993b) 395–399.
[26] Luckman, S.M., Dyball, R.E.J. and Leng, G., Induction of c-*fos* expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity, *J. Neurosci.*, 14 (1994) 4825–4830.
[27] Mansour, A., Fox, C.A., Meng F., Akil, H. and Watson, S.J., κ-1 Receptor mRNA distribution in the rat central nervous system: comparison to κ-receptor binding and prodynorphin mRNA, *Mol. Cell Neurosci.*, 5 (1994) 124–144.
[28] Messersmith, D.J., Gu, J., Dubner, R., Douglass, J. and Ladarola, M.J., Basal and inducible transcriptional activity of an upstream AP-1/CRE element (DYNCRE3) in the prodynorphin promoter, *Mol. Cell Neurosci.*, 5 (1994) 238–245.
[29] Morris, B.J., Haarmann, I., Kempter, B., Hollt, V. and Hera, A., Localization of prodynorphin mRNA in rat brain by in situ hybridization using a synthetic oligonucleotide probe, *Neurosci. Lett.*, 69 (1986) 104–108.
[30] Naranjo, J.R., Mellstrom, B., Acaval, M. and Sassone-Corsi, P., Molecular pathways of pain: Fos/Jun-mediated activation of a non-canonical AP-1 site in the prodynorphin gene, *Neuron*, 6 (1991) 607–617.
[31] Pan, B.H., Castro-Lopes, J.M. and Coimbra, A., c-*fos* expression in the hypothalamo-pituitary system induced by electroacupuncture or noxious stimulation, *NeuroReport*, 5 (1994) 1649–1652.
[32] Ren, M.F., Lu, C.H. and Han, J.S., Dynorphin-a-(1-13) antagonizes morphine analgesia in the brain and potentiates morphine analgesia in the spinal cord, *Peptides*, 6 (1985) 1015–1020,
[33] Sherman, T.G., Cevelli, O., Douglass, J., Herbert, E., Burke, S. and Watson, S.J., Hypothalamic dynorphin and vasopressin mRNA expression in normal and Brattelbora rats, *Fed. Proc.*, 5 (1986) 1150–1155.
[34] Sonnenberg, J.L., Rauscher III, F.J., Morgan, J.I. and Curran, T., Regulation of proenkephalin by Fos and Jun, *Science*, 246 (1989) 1622–1625.
[35] Walker, J.M., Katz, R.J. and Akil, H., Behavioral effect of dynorphin 1–13 in the mouse and rat: initial observations, *Peptides*, 1 (1980) 341–345.
[36] Wang, Q., Mao, L.M. and Han, J.S., Diencephalon as a cardinal neuronal structure for mediating 2 Hz-, but not 100 Hz-electroacupuncture-induced tail flick reflex suppression, *Behav. Brain Res.*, 37 (1990) 149–156.
[37] Wang, Q., Mao, L.M. and Han, J.S., The arcuate nucleus of hypothalamus mediates low but not high frequency electroacupuncture analgesia in rats, *Brain Res.*, 513 (1990) 60–65.
[38] Wang, Q., Mao, L.M. and Han, J.S., The role of PAG in mediation of analgesia produced by different frequency electroacupuncture stimulation in rats, *Int. J. Neurosci.*, 53 (1990) 167–172.
[39] Wang, Q., Mao, L.M. and Han, J.S., The role of parabrachial nucleus in high frequency electroacupuncture analgesia in rats, *Chin. J. Physiol. Sci.*, 7 (1991) 363–371.
[40] Xie, G.X., Han, J.S. and Hollt, V., Electroacupuncture analgesia blocked by microinjection of anti-beta-endorphin antiserum into periaqueductal gray of the rabbit, *Int. J. Neurosci.*, 18 (1983) 287–292.
[41] Yu, L.C. and Han, J.S., Involvement of arcuate nucleus of hypothalamus in the descending pathway from nucleus accumbens to habenula subservient antinociceptive effect, *Chin. Sci. Bull.*, 33 (1988) 1744.
[42] Yu, L.C., Shi Y.S. and Han, J.S., Study on the descending analgesic pathway from accumbens to habenula, *Sci. Bull. (Beijing)*, 32 (1987) 1344–1346.
[43] Yu, Y.H., Gao, M. and Ke, L.F., Time-course of the change of proopiomelanocortin mRNA in the arcuate hypothalamic nucleus of the rat following electroacupuncture, *Acta Shanghai Med. Univ.*, 21 (Suppl.) (1994) 59–61.