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C-Fos proteins are not involved in the activation of preproenkephalin gene expression in rat brain by peripheral electric stimulation (electroacupuncture)

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Abstract

The present work was designed to study the role of the oncogene product c-Fos in activating the transcription of preproenkephalin (PPE) gene following a kind of peripheral electric stimulation known as electroacupuncture (EA) stimulation. The temporal patterns of rat brain *c-fos* and PPE mRNA expression were evaluated using the method of Northern blotting, showing that *c-fos* mRNA expression, which peaked at 2 h after the termination of EA, was always ahead of the PPE mRNA expression which began at 4 h and peaked at 48 h after EA. The methods of immunocytochemistry (ICC) and in situ hybridization (ISH) techniques were combined to identify the co-existence of c-Fos protein and PPE mRNA at the cellular level. The results showed that only a small percentage of PPE mRNA-containing neurons depicts Fos-like immunoreactive nuclei. These findings suggest that c-Fos protein may not be involved in the activation of brain PPE gene transcription induced by peripheral electric stimulation.

Keywords: *c-fos* mRNA; Fos protein; Preproenkephalin; Electroacupuncture; Transcription factor; Brain; Oncogene

The transcription of preproenkephalin (PPE) gene in the neurons was proposed to be mediated by c-Fos protein [17]. This hypothesis was based on the following findings: (1) *c-fos* gene expression always precedes PPE expression in the neuron where *c-fos* and PPE products co-exist, as in the case of seizure [21] and peripheral tissue inflammation [6]. (2) There exists AP-1 consensus sequence in the promoter region of PPE gene, which has high affinity for AP-1 complexes containing *c-fos* component [17,21]. (3) c-Fos and c-Jun act synergistically in stimulating transcription from the 5′-control region containing AP-1 consensus sequence of PPE in transactivation assays [17]. This hypothesis, however, is not universally applicable and has met with much opposing evidence. A dissociation of the *c-fos* and PPE expression has been observed in many occasions [5,13,15,22,23], and it has been shown that in extracts from unstimulated and stimulated rat striatum [15] and mouse hypothalamus [1], the PPE cAMP response element 2 (CRE-2) site interacts with CRE-binding protein (CREB)-like, rather than AP-1 proteins. Therefore, whether the relation between the induction of c-Fos/c-Jun and the expression of PPE is a cause-and-effect one is still unclear and worth further investigation. In the present study, the role of c-Fos protein in electroacupuncture (EA)-induced PPE gene expression was investigated using the methods of Northern blotting as well as double-labeling of c-Fos protein and PPE mRNA.

Female Wistar rats weighting 180-250 g were provided by the Animal Center of the Beijing Medical University and were randomly divided into three groups. In group 1, rats received electrical stimulation via stainless-steel needles inserted into hind leg points, one near the knee joint (ST36, 5 mm lateral to the anterior tubercle of tibia) and the other near ankle joint (SP6, at the level of the superior border of the medial melleolus, between the posterior border of the tibia and the anterior border of the Achilles tendon), respectively. The details of the procedure have been described elsewhere [2]. Briefly, stainless steel needles inserted into ST36 and SP6 points of 5 mm depth in both hind legs were fixed in situ and connected to the output leads of an electronic stimulator (Han's

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Acupoint Nerve Stimulator, HANS, made in Beijing, China) delivering square wave pulses of 0.3 ms pulse width, with constant current output adjustable in the range of 0–3 mA. The intensity was set at 1 mA and then increased stepwise to 2 mA and then 3 mA, each lasting for 10 min. The total stimulation period was 30 min. The frequency of EA stimulation was shifting automatically between 2 Hz and 15 Hz, each lasting for 3 s. This was to mimic the manual twisting of the acupuncture needle, and was considered as the most effective mode of stimulation for inducing analgesia [2]. In group 2, needles were inserted into the acupoints without connecting to the stimulator. In group 3, rats were restrained in the holder with no special treatment, serving as the blank control group.

For Northern blotting, the rats were decapitated 1, 2, 4, 8, 24, 48 and 72 h after the termination of electrical stimulation, respectively. The brain tissues (whole brain less cerebral cortex and cerebellum) were rapidly removed and were immediately frozen in liquid nitrogen. The total RNA fraction was extracted according to Chomozynski [3]. Aliquots of 30 μg of the RNA were denatured with formaldehyde, run on a 1.2% agarose gel and transferred to nylon sheets (Nytran, Schleicher-Schuell). After baking for 2 h at 80°C, the nylon filters were prehybridized and hybridized sequentially to denatured *c-fos* (donated by Dr. Curran), PPE (donated by Dr. Fuller) and β-actin cDNA probe. The probes were labeled by [^{32}P]dATP using the methods of random primer to a specificity of 6×10^8–9×10^8 cpm/μg. After hybridization, the nylon filters were washed according to the instructions of the supplier (Schleicher-Schuell), dried and exposed to X-ray film (Kodak) at −80°C. The autoradiograms were scanned by a densitometer (IBAS). The filters were then washed in 0.1 M phosphate buffer (pH 7.0) at 100°C for 10 min to remove the probes and were then hybridized with the next probe. The amount of *c-fos* and PPE mRNA were normalized with that of β-actin mRNA in the same sample and then analyzed by ANOVA and *t*-test.

For double-staining of immunocytochemistry (ICC) and in situ hybridization (ISH), 2 h after the termination of EA stimulation or the control manipulation, the rats were deeply anesthetized with 10% chloral hydrate (1.5 ml per rat) and perfused transcardially with 100 ml saline followed by 300 ml 4% paraformaldehyde within 40 min. The brain tissues were removed and postfixed in the same fixative overnight, and placed in a 30% sucrose solution for 36–48 h.

Tissue sections were cut coronally in a cryostat at 30 μm and collected at an interval of five sections. Immunostaining of Fos-like immunoreactivity (FLI) was conducted according to Hsu's ABC method [10] with some modifications which have been described elsewhere [11]. Rabbit antiserum against Fos (1:1000, Oncogene Science, Inc.) was used in these experiments.

For ISH, 30 μm sections were collected in 4% PFA/PBS and fixed for 3–5 h. After pretreated by proteinase K (1 μg/ml), the sections were transferred into hybridization buffer containing 500 ng/ml digoxin-labeled antisense cRNA probe of PPE, and hybridized for 12–16 h at 42°C. Afterward, the sections were washed at high stringency and were digested by RNase to remove excess probe. For immunodetection and color-staining, the procedures were the same as recommended in the detection kit from Boehringer Mannheim Corp. The sections were mounted onto the slides in PBS, dehydrated and coverslipped. Some of the sections were lightly counterstained with Cresyl violet.

For the combined protocol, ICC was performed prior to ISH. Following DAB staining, the sections were transferred to 4% PFA/PBS and postfixed for 3–5 h, followed by the same ISH procedures described above.

Tissue sections were examined at 65, 100 and 200× under lightfield microscopy. Labeled nuclei of FLI or labeled neurons of PPE were identified using lightfield microscopy at 100×. FLI nuclei and PPE neurons were counted only when structures of the appropriate size and shape demonstrated clear increases when compared to the background level. Some of the ISH sections were analyzed by IBAS 2000 image system.

As shown in Fig 1, Northern blotting results revealed that EA induced a rapid *c-fos* mRNA expression which peaked at 2 h and disappeared 8 h after the termination of EA. The expression of PPE gene, however, responded slowly, and the level of PPE mRNA began to increase 4 h after EA, and peaked at 48 h after EA. These findings suggested that there is a good temporal relation between *c-fos* and PPE gene expression.

However, double labeling of FLI and PPE mRNA revealed that although there was some Fos expression in PPE mRNA-containing neurons, the co-existence rates

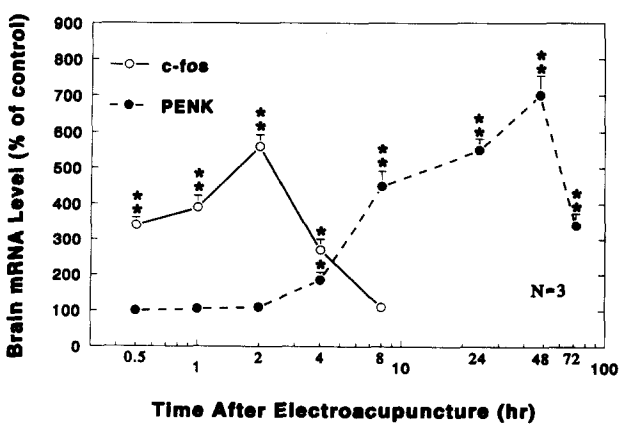

Fig 1. Densitometric quantitation of changes in *c-fos* and proenkephalin mRNAs. The curves for *c-fos* and proenkephalin mRNA are the average of three separate RNA blot time course experiments. The β-actin is used to precisely standardize each lane for the amount of RNA applied. The ordinate is the ratio of *c-fos* or proenkephalin to β-actin.

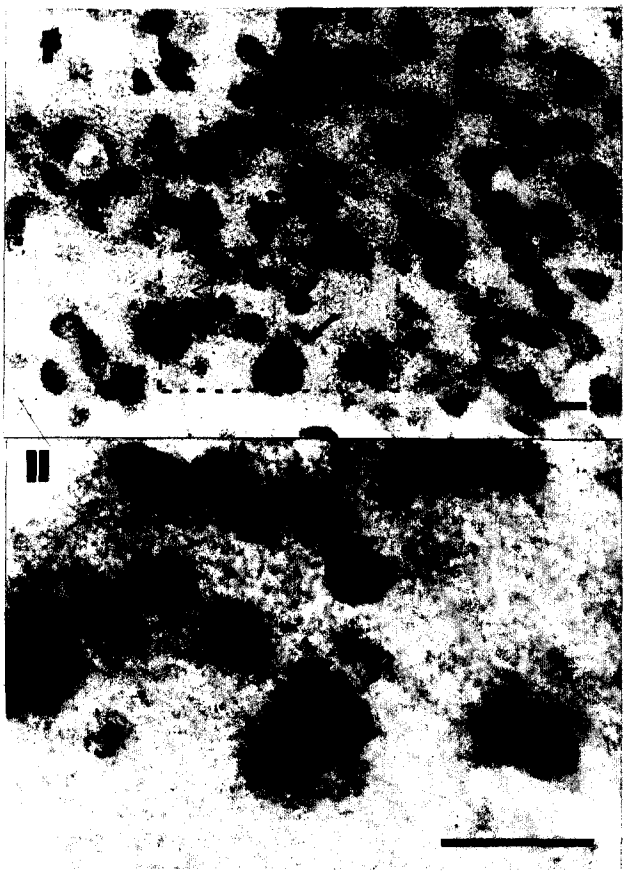

Fig 2. Photomicrographs showing the expression of Fos-like immunoreactivity in neurons containing preproenkephalin mRNA in supramammillary hypothalamic nucleus 2 h after the termination of EA stimulation. (II) is the local magnification from (I). Arrows point to double-labeled neurons, blank or silked arrowheads point to the individually labeled PPE or FLI neurons, respectively. Bars, 25 μm.

were low (Fig. 2, Table 1), ranging from 6.0% to 12.6% of the total labeled PPE neurons.

A battery of studies has been performed to explore the molecular mechanisms underlying acupuncture-induced analgesia [7–9,12]. While increased release and gene expression of endogenous opioid peptides in the CNS have been shown to be the most plausible mechanisms, the question of how the genes encoding opioid peptides are activated by EA stimulation is still pending for investigation. Several studies seem to indicate c-Fos and c-Jun as possible 'third messenger' in this extent [6,17,18,21]. The data shown above indicate that a sequential expression of *fos* mRNA and PPE mRNA in the brain as a consequence of peripheral electrical stimulation does not necessarily imply a cause-and-effect relationship. In fact, only a small percentage of neurons showed a coexpression of Fos-like proteins and PPE mRNA. In another study it was shown that even in the brain areas where there is a coexpression of Fos and PPE, the expression of PPE was not affected by the successful blockade of *c-fos* gene expression (to be published). It is therefore not likely that Fos proteins would be the candidates of transcription factor for PPE expression. Further studies are on the way to explore whether other factors, such as cAMP-responsive element binding protein (CREB), are involved in this response.

Concerning the role of Fos and Jun in the regulation of PPE expression in animal, several reports have provided evidence that Fos and Jun are not involved in the PPE transcription in some paradigms, such as haloperidol-induced PPE expression [16,20], amphetamine-induced PPE transcription [4,14] and hippocampal-stimulation-induced PPE transcription [13], etc. However, results from one paradigm could not be easily extrapolated to another. For example, the role of Fos and Jun in seizure-induced PPE expression is still under investigation and was predicted to be of large possibility [19]. In a recent study we have shown that the distribution patterns of EA-induced Fos and PPE mRNA expression in rat brain were greatly different from those produced by other stimuli such as noxious stimuli, stress , cocaine, haloperidol, amphetamine and seizure, etc. (to be published). Even in the category of EA stimulation, the distribution patterns of Fos expression in rat brain induced by EA of different frequencies (e.g., 2 Hz versus 100 Hz) are also different from each other. It has been characterized that the analgesic effect induced by peripheral stimulation of different frequencies (within the range 2–100 Hz) is mediated by different neural pathways and different kinds of neu-

Table 1

Induction of FLI in PPE neurons as visualized by combined ICC and ISH

Brain areas	FLI	PPE	CoE	CoE/PPE (%)	CoE/FLI (%)
Arcuate (Arc) nucleus	58.3 ± 7.4	74 ± 3.5	6.7 ± 2.3	9.1	11.5
Paraventricular (PAH) nucleus	99.3 ± 12.8	116 ± 5.2	7 ± 2.6	6.0	7.0
Ventromedial (VMH) nucleus	122 ± 14.3	97 ± 4.1	9.6 ± 2.1	9.9	8.1
Supramammillary (SuM) nucleus	85.3 ± 10.4	88 ± 3.9	6.3 ± 1.2	8.4	9.8
Periaqueductal gray (PAG)	69.3 ± 12.8	58 ± 2.7	7.3 ± 1.5	12.6	10.5

Shown are mean ± SEM of the number of FLI nuclei (FLI), PPE neurons (PPE), neurons with co-expression of FLI and PPE (CoE), the ratio of co-expressed neurons to the number of FLI (CoE/FLI) and to the number of PPE neurons (CoE/PPE). The values were based on four animals and each site in each animal was counted on three sections. The number of FLI neurons was counted under lightfield microscopy with the naked eye, whereas the number of PPE neurons was counted by the image system mentioned above. The frequency of EA used in this study was 2 Hz, since this frequency was shown to selectively activate the enkephalinergic rather than the dynorphinergic neurons [7].

ropeptides [7]. So electroacupuncture may be used as a tool for further analysis of the interaction between proto-oncogenes and certain central neuropeptides.

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