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Research report

Brain substrates activated by electroacupuncture (EA) of different frequencies (II): role of Fos/Jun proteins in EA-induced transcription of preproenkephalin and preprodynorphin genes

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Abstract

Antisense oligodeoxynucleotides (ODNs) of c-*fos* and/or c-*jun* were used in this study to investigate the role of Fos and Jun proteins in electroacupuncture (EA)-induced transcription of the opioid genes preproenkephalin (PPE), preprodynorphin (PPD) and proopiomelanocortin (POMC). As the results showed, EA-induced Fos and Jun expression was blocked efficiently and specifically by c-fos and c-*jun* antisense ODNs, respectively. This treatment significantly prevented EA-induced PPD, but not PPE, mRNA expression. These results suggest that Fos and Jun proteins are involved in PPD rather than PPE gene transcription activated by EA stimulation.

Keywords: Electroacupuncture; Fos; Jun; Preproenkephalin; Preprodynorphin; Antisense oligodeoxynucleotide

1. Introduction

The AP-1 complexes formed by the dimerization of c-Fos and c-Jun protein are thought to be 'third messengers' in the signal transduction cascade, serving as transcription factors for a battery of target genes [6,24,26,32]. A family of possible targets are the three opioid genes: preproenkephalin (PPE), preprodynorphin (PPD) and proopiomelanocortin (POMC) [2,11,23,27,32]. Four lines of evidence suggest that AP-1 complexes modulate opioid expression: (1) The temporal relation of a quick c-*fos* expression followed by a slow opioid gene expression has been revealed in the cases of seizure [32], peripheral inflammation [9], injection of caffeine [33] as well as electroacupuncture (EA) [12]; (2) The co-existence of c-*fos* and opioid mRNA in neurons [27,29]; (3) Existence of the AP-1 consensus sequence in the promoter region of PPE [5,32], and POMC gene [2] or the noncanonical AP-1 like site in the PPD gene promoter [25,27], which have high affinity for c-*fos*-contained AP-1 complexes; (4) Antisense c-*fos* ODN has been showed to be capable of efficiently blocking PPD gene expression in the spinal cord dorsal horn neurons induced by formalin [16] and in the cultured dorsal horn neurons induced by serotonin [23], as well as blocking beta-endorphin secretion in AtT-20 cells [11]. Since EA is known to induce c-*fos* expression which precedes the PPE gene expression [12,18,19], it was suggested that c-Fos may be responsible for EA-activated PPE gene transcription. The sequential expression of c-*fos* and PPE, however, does not necessarily mean the cause and effect of Fos and PPE. In contrast, results from the co-existence experiment are not in favor of this hypothesis [12]. Therefore, the role of Fos/Jun proteins in EA-induced PPE as well as PPD mRNA expression deserves further investigation. To do this, we used c-*fos* and c-*jun* antisense ODNs to specifically block EA-induced c-Fos and c-Jun expression, and to observe their effects on EA-induced PPE and PPD mRNA expression.

2. Material and methods

2.1. Animals and surgery

Female Wistar rats weighing 180–250 g were obtained from the Scientific Animal Center of Beijing Medical

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University. Each animal was implanted with a stainless steel cannula (O.D. = 0.6 mm, I.D. = 0.35 mm) under the anesthesia of hydrate chloride (0.3–0.4 g/kg, i.p.) according to the coordinates of the rat by Paxinos and Watson [30]. The coordinates were as follows: (1) the third ventricle: B 2.3–3.3 mm, H 8.5 mm, L 1.5 mm, with an angle of 12° from the center; (2) ventromedial hypothalamic nucleus: B 2.6 mm, L 1.0 mm, H 8.0 mm; (3) parabrachial nucleus: B 9.2 mm, L 5.0 mm, H 5.0 mm. The animals were allowed to recuperate for 5 days before the injection of oligonucleotides or saline.

2.2. Injection of oligodeoxynucleotides (ODNs)

All ODNs were synthesized by DNA International (USA) and were dissolved in saline at a concentration of 2.5 mol/l. Antisense or sense c-*fos* (antisense: 5′-GAA CAT CAT GGT CGT-3′; sense: 5′-ACG ACC ATG ATG TTC-3′) [3,31] and c-*jun* (antisense: 5′-CGT TTC CAT CTT TGC AGT-3′; sense: 5′-ACT GCA AAG ATG GAA ACG-3′) [11] ODNs were injected through an injection cannula (O.D. = 0.3 mm). The volume of each ODN injection was 2 μl to be finished in more than 3 min. For combined injection, ODNs were injected sequentially instead of simultaneously. EA stimulation was administered 8 h after the injection of ODNs.

2.3. Electroacupuncture procedures

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 (SP6, 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.

Two (for ICC detection of FLI) or 24 h (for ISH detection of opioid mRNAs) after the termination of EA stimulation or 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% para-formaldehyde 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.

2.4. Immunocytochemistry

Tissue sections were cut coronally in a cryostat at 30 μm. Series sections of interesting brain areas (such as LPB or hypothalamus) were collected in phosphate buffer (PBS). Free floating sections were processed according to Hsu's ABC method [15] with some modifications. Briefly, the sections were incubated with the first primary antibody (rabbit antiserum against Fos 1:1000 or Jun 1:1000, 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 in 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.

2.5. In situ hybridization

Free-floating method of ISH was used. Briefly, continuous 30 μm coronal sections from interesting brain areas were collected. 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 were then 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 Boe-

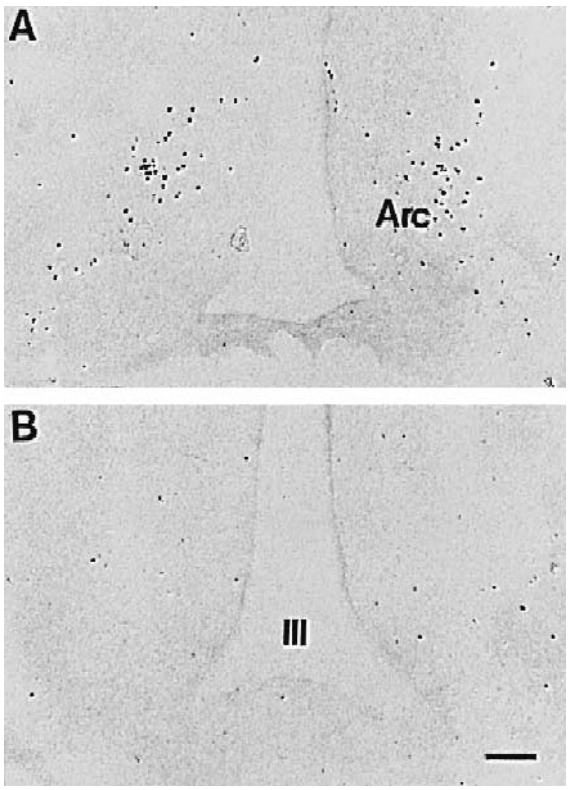

Fig. 1. Photomicrographs showing the effect of c-Fos sense (A) or antisense (B) oligodeoxynucleotides injected into the third ventricle on EA-induced Fos expression in arcuate nucleus (Arc). III: third ventricle. Magnifications: A = B. Bar = 0.1 mm.

Table 1
Influence of sense (S) and antisense (AS) oligodeoxynucleotides (ODNs) of c-Fos on 2 Hz EA-induced Fos expression

Structure	Control (%)	Saline	S-ODN	AS-ODN (%)
Arc	55.8 ± 7.4 (100)	50.3 ± 8.9	51.3 ± 8.9	7.6 ± 2.0 (13.1) *
SON	22.2 ± 1.9 (100)	16.6 ± 1.5	18.3 ± 2.5	5.0 ± 2.0 (25.6) *

Control: without intracerebral injection; Saline: injection of the same volume of saline; * $P < 0.01$ compared with control, saline or S-ODN groups; %: percent remaining as compared with control group.

Table 2
Influence of sense (S) and antisense (AS) oligodeoxynucleotides (ODNs) of c-Jun on 2 Hz EA-induced Jun expression

Structure	Control (%)	Saline	S-ODN	AS-ODN (%)
VMH	158.3 ± 16.3 (100)	152 ± 10.4	167 ± 15.6	24.6 ± 3.2 (15.6) *

Control: without intracerebral injection; Saline: injection of the same volume of saline; * $P < 0.01$ compared with control, saline or S-ODN groups; %: percent remaining as compared with control group.

hirnger Mannheim. The sections were mounted onto the slides in PBS, dehydrated and coverslipped. Some of the sections were lightly counterstained with Cresyl violet.

2.6. 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.

2.7. Experimental design

(1) *To test the efficiency and specificity of the antisense ODNs*, the animal groups were: (a) sense ($n = 4$) or antisense ($n = 4$) c-*fos* ODNs injected into the third ventricle (IIIv); (b) sense ($n = 4$) or antisense ($n = 4$) c-*jun* ODNs injected into VMH; (c) saline injection into IIIv ($n = 4$) or VMH ($n = 4$). All the animals were administered with 2 Hz EA, and perfused 2 h after the termination of EA for the immunodetection of FLI and JLI.

(2) *To investigate the effect of antisense ODNs on the EA-induced opioid genes expression*, the animal groups were: (a) injection of sense ($n = 4$) or antisense ($n = 4$)

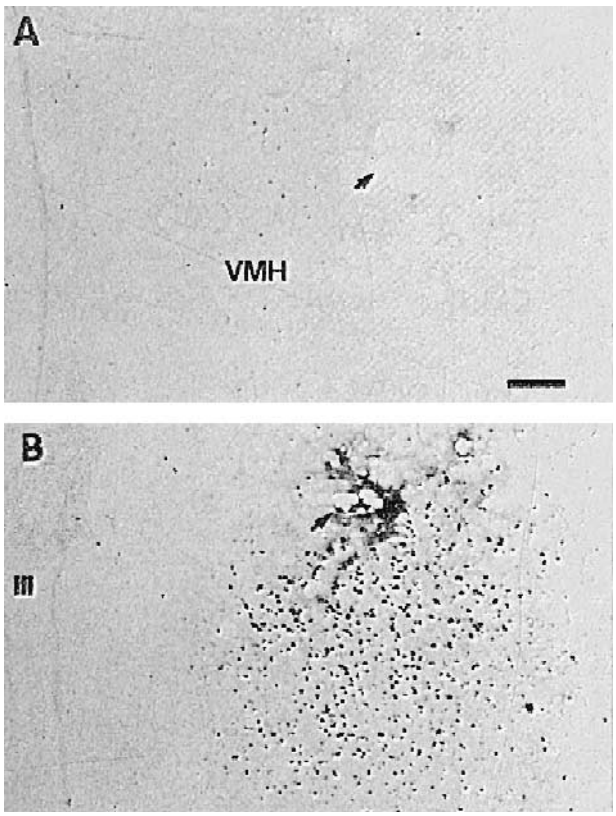

Fig. 2. Photomicrographs showing the effects of c-Jun antisense (A) or sense (B) ODN on EA-induced c-Jun expression in ventromedial nucleus (VMH). III: third ventricle. Arrows point to the lesion sites. Magnifications: A = B, Bar = 0.1 mm.

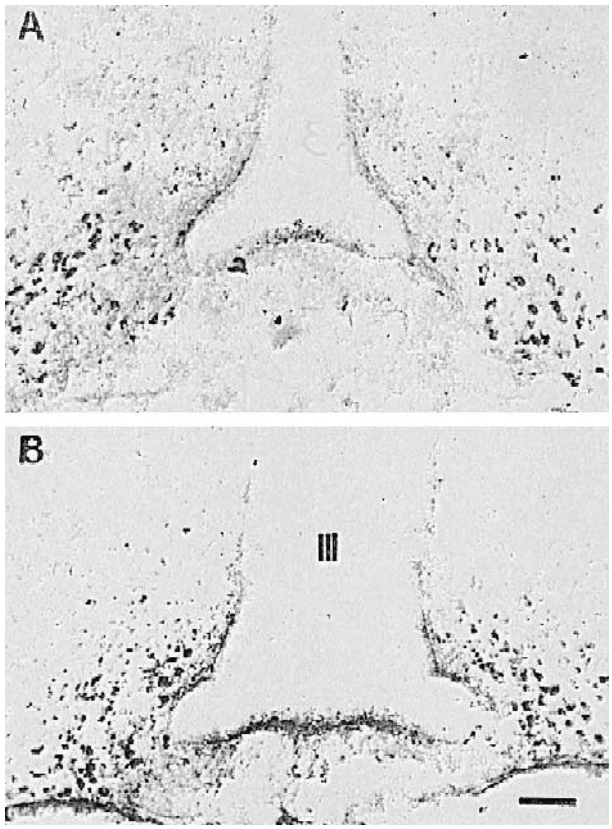

Fig. 3. Photomicrographs showing the influence of injection of c-Fos + c-Jun sense (A) or antisense (B) ODNs on the expression of preproenkephalin mRNA induced by 2 Hz EA stimulation. III: third ventricle. Magnifications: A = B. Bar = 0.1 mm.

ODNs of c-*fos* + c-*jun* into IIIv, given 2 Hz EA for the detection of PPE and POMC mRNA; (b) saline into IIIv ($n = 4$), given 2 Hz EA for the detection of PPE and POMC mRNA; (c) sense ($n = 4$) or antisense ($n = 4$) c-*fos* + c-*jun* ODNs into LPB, given 100 Hz EA for the detection of PPD mRNA; (d) c-*fos* sense (FS, $n = 4$), c-*fos* antisense (FA, $n = 4$), c-*jun* sense (JS, $n = 4$), c-*jun* antisense (JA, $n = 4$) into IIIv, given 100 Hz EA for the detection of PPD mRNA; (e) saline into LPB ($n = 4$) or IIIv ($n = 4$), given 100 Hz EA for the detection of PPD mRNA. All the animals were perfused at 24 h after the termination of EA stimulation for in situ hybridization.

2.8. 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 ± SEM, and the statistical significance was determined by Student's *t*-test.

3. Results

3.1. Effects of c-fos or c-jun antisense ODNs on EA-induced Fos or Jun expression

The purpose of this experiment was to identify the efficiency and specificity of the antisense ODNs in blocking the expression of Fos or Jun proteins. As shown by ICC, injection of saline or sense c-*fos* or c-*jun* ODNs had no effect on EA-induced Fos or Jun expression in the hypothalamus. By contrast, antisense c-*fos* ODN injected into IIIv remarkably reduced the number of FLI cells induced by 2 Hz EA stimulation (Fig. 1, Table 1), but had no influence on EA-induced Jun expression (data not shown). Likewise, injection of c-*jun* antisense ODN into VMH greatly reduced 2 Hz EA-induced Jun expression in VMH (Fig. 2, Table 2) and exerted no effects on Fos expression. These results suggest that the antisense c-*fos* and c-*jun* ODN used in the present study exhibited efficient and specific blocks of c-*fos* or c-*jun* gene expression at the translation level.

3.2. Effects of combined injection of c-fos and c-jun antisense ODNs on opioid gene expression

For the detection of PPE mRNA, the ODNs were injected into the third ventricle. Low frequency (2 Hz) EA

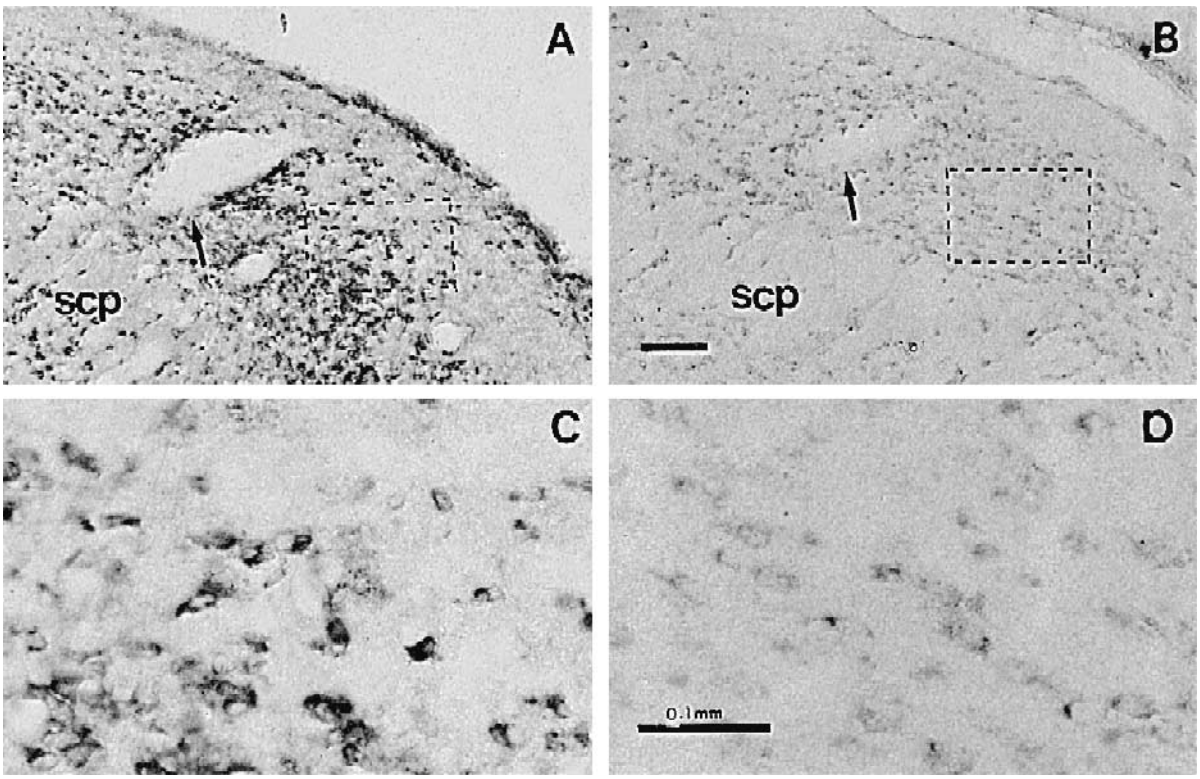

Fig. 4. Photomicrographs showing the effects of combined injection of c-Fos/c-Jun sense (A and C) or antisense (B and D) ODNs on EA-induced PPD mRNA expression in the lateral parabrachial nucleus (LPB). Scp: superior cerebellar peduncle. Arrows show the lesion sites. C and D are high magnifications of A and B, respectively. Magnifications: A = B, C = D. Bars = 0.1 mm.

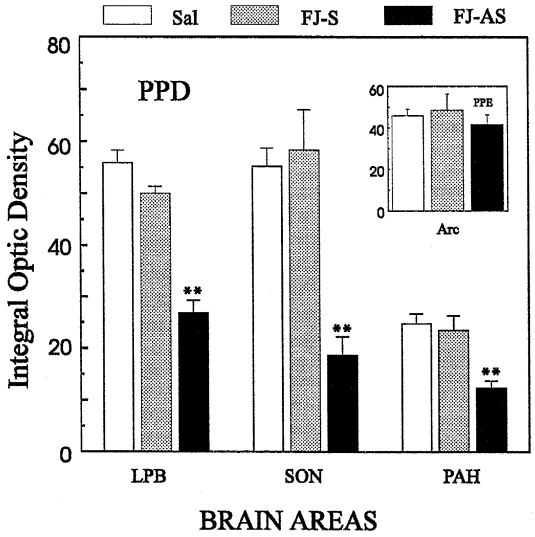

Fig. 5. Diagram showing the effects of combined injection of c-Fos and c-Jun antisense ODNs on EA-induced PPE/PPD mRNA expression. Sal: saline injection; FJ-S: injection of c-Fos + c-Jun sense ODNs; FJ-A: injection of c-Fos + c-Jun antisense ODNs. LPB: lateral parabrachial nucleus. SON: supraoptic nucleus. PAH: paraventricular hypothalamic nucleus.

was administered 8 h after the injection and the animals were perfused 24 h after the termination of EA stimulation for the detection of PPE mRNA. The results showed that combined injection of antisense (or sense) c-*fos* (5 nmol) and c-*jun* (5 nmol) produced no visible influence on EA-induced increase of PPE mRNA in the arcuate nucleus (Fig. 3), nor was there any influence on other hypothalamic areas where EA induced an increase in PPE mRNA expression (data not shown).

As EA was shown to produce an increase in PPD transcription mainly in the hypothalamus and lateral parabrachial (LPB), we delivered c-*fos* and c-*jun* antisense ODNs into the third ventricle (5 nmol for each) or directly to LPB (2 nmol for each). 100 Hz EA was administered 8 h after the injection of ODNs and the animals were perfused at 24 h after. In situ hybridization detection of PPD mRNA showed that combined injection of c-*fos*/c-*jun* antisense ODNs prevented 100 Hz EA from accelerating PPD mRNA expression in a very significant extent no matter it was injected ventricularly (IIIv) or into the nucleus (LPB) (Figs. 4 and 5). The injection of ODNs into the third ventricle, however, did not affect the PPD transcription in LPB, which was blocked only by intranuclear injection. In the saline and sense ODNs control groups, no change in PPD mRNA level was noticed.

Since EA stimulation did not influence the level of POMC mRNA level in rat brain, it is natural to predict that injection of antisense or sense ODNs into the third ventricle would affect POMC mRNA expression in the arcuate nucleus of hypothalamus, which was confirmed experimentally (data not shown).

3.3. Effect of separate injection of c-fos or c-jun antisense ODN on EA-induced PPD mRNA expression

To test whether blockade of the single component of the Fos/Jun dimer would affect EA-induced PPD transcription, we injected c-*fos* or c-*jun* antisense ODNs separately. The ODNs were injected only into the third ventricle in this experiment. The results show clearly that either c-*fos* or c-*jun* antisense ODN is sufficient to block EA-induced PPD transcription (Table 3).

4. Discussion

Although antisense RNA has been shown to be an effective way of specifically blocking gene expression [10,13,14,22], its use in the CNS is not feasible due to the lack of efficient transfer systems. On the other hand, recent data have shown that the use of naked and modified antisense ODNs in the central nervous system was quite successful [3,24,31]. One of the critical factors for these successes was the lack of significant nuclease activity in the cerebrospinal fluid [28], allowing the ODNs to survive longer. These are the reasons we use antisense ODNs to knock out Fos and Jun expression, and as our results show, this approach was successful.

In situ hybridization results clearly showed that EA-induced PPE mRNA increase in the hypothalamus was not influenced by the combined injection of large doses (5 nmol each) of c-*fos* and c-*jun* antisense (ODNs). As ODNs in such dosage have been shown be effective in preventing Fos or Jun expression (Figs. 1 and 2), it is reasonable to draw conclusion that EA-induced Fos and Jun expression

Table 3
Effect of c-Fos or c-Jun ODNs on the PPD mRNA expression induced by 100 Hz electroacupuncture as revealed by in situ hybridization and analyzed by IBAS 2000 image system

	Saline (%)	FS	FA (%)	JS	JA (%)
SON	55.2 ± 3.5 (100)	58.6 ± 6.3	26.0 ± 1.7 (47.0) *	57.9 ± 6.3	27.8 ± 3.2 (50.36) *
PAH	24.8 ± 1.9 (100)	22.8 ± 4.2	12.7 ± 2.8 (51.4) *	29.7 ± 3.6	14.0 ± 1.5 (56.45) *

Values represent integral optic density (I.O.D.) and were shown as $X \pm$ SEM. Numeric data were from 4 animals and 3 sections (rostral, middle and caudal) were taken in each nucleus of each animal. SON: supraoptic nucleus; PAH: paraventricular hypothalamic nucleus; %: percent remaining as compared with the saline control group (100%); FS: sense ODN of c-Fos; FA: antisense ODN of c-Fos; JS: sense ODN of c-Jun; JA: antisense ODN of c-Jun. * $P < 0.01$ as compared with saline or sense ODNs (FS, JS) groups.

was not linked with PPE transcription. These results are in line with other reports which were not able to find the linkage between Fos/Jun and PPE [1,7,8,20,21,34,35]. In another study [12] using double staining of FLI and PPE mRNA we found only a low rate (6–12%) of PPE mRNA positive neurons in the population of neurons displaying FLI nuclei in the Arc, VMH and PAH following EA stimulation, which is also not in favor of the Fos-PPE linkage.

What then are the transcription factors responsible for the PPE expression? Recently, the cAMP-responsive element binding protein (CREB) has come into focus. The earliest report was made by Comb et al. [5] who discovered that a one-base substitution mutation within the CREB element [TGCGTCA] results in a consensus AP-1 site [TGAGTCA] which renders the gene unresponsive to cAMP after transfection to C6-glioma cells. Later it was found that PPE CRE2 site in the extracts from rat striatum [21] or mouse hypothalamus [1] interacts with CREB-like proteins. Similar discoveries were also made by Hyman et al. [17]. Further studies in the present setup adapting the techniques of antisense ODNs of CREB gene might provide deeper insight into the mechanism of the regulation of PPE gene transcription.

In contrast to PPE, EA-induced PPD mRNA expression was significantly prevented by the combined or individual injection of c-*fos* or c-*jun* antisense ODNs, strongly suggesting that EA-induced PPD mRNA expression in SON (except the post-chiasmatic part), PAH, VMH and LPB was activated via Fos and/or Jun pathway. These findings are in accordance with other reports showing that the delivery of antisense c-*fos* ODN into the spinal cord dorsal horn effectively prevented the sequential induction of Fos protein and PPD mRNA expression following the injection of formalin into the plantar foot or administration of serotonin to cultured spinal dorsal horn neurons [16,23]. However, from the present results we could not exclude the possibilities that other transcription factors, e.g., CREB, may also be involved in EA-activated PPD gene transcription, as the investigations done by Hyman et al. [17], Konradi et al. [21] and Cole et al. [4] suggest that CREB may be responsible for basic and induced PPD transcription in striatum and hypothalamus.

In conclusion, the present studies suggest that there exist different neuronal pathways subserving the analgesic effects induced by EA of low and high frequency applied on one and the same body sites. These findings argue against the notion that EA is only a noxious or stressful stimulation. This work is one of the initial steps toward understanding the neuronal pathways of frequency-specific peripheral stimulation. In fact, there are still many mysteries to be disclosed beneath this phenomenon. What determines the response of the CNS to stimuli of different frequencies? What are the connections between the multiple brain structures activated by EA? Are all the structures showing Fos expression involved in EA analgesia? What are the transcription factors of EA-activated PPD mRNA expression in the postchiasmatic part of SON? What fills the time gap between Fos and PPD transcription? Answers to these questions may help elucidate the mechanisms of acupuncture, as well as the mechanisms by which the CNS discriminates peripheral stimulation of different frequencies.

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