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Expressions of preproenkephalin mRNA during electroacupuncture analgesia enhanced by fenfluramine[1]

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AIM: To study the changes of preproenkephalin (PPE) mRNA in rat brain in response to electroacupuncture (EA) combined with fenfluramine (Fen), a releaser of 5-HT. **METHODS:** *In situ* hybridization histochemistry technique was used to observe the expression of PPE mRNA in rat brain during EA analgesia potentiated by Fen. **RESULTS:** The greatest relative increase of PPE mRNA was seen in lumbar spinal cord (laminae I & II), nucleus raphe magnus, dorsal raphe nucleus, periaqueductal gray, interpeduncular nucleus, preoptic lateral area, amygdala nucleus and caudate-putamen ($P<0.01$, *vs* NS + EA). Moderate increases were found in lateral septal, preoptic medial area, hypothalamus ventromedial nucleus, lumbar spinal laminae III & IV ($P<0.05$, *vs* NS + EA). Thalamus showed no statistical significant change in PPE mRNA. **CONCLUSION:** The enhancing of PPE mRNA in relative brain nuclei is involved in potentiating action of Fen on EA.

KEY WORDS enkephalins; messenger RNA; fenfluramine; electroacupuncture; *in situ* hybridization; central nervous system

Serotonin (5-HT) is involved in and beneficial to electroacupuncture (EA) analgesia (EAA)[1,3]. Fenfluramine (Fen), a releaser of 5-HT, enhances acupuncture analgesia markedly[2]. The effect of Fen was related to endogenous opioid system[4,5] which may play a key role. However, the mechanism of potentiating effect of Fen on EAA is not clear. The present study was to observe the expression of PPE mRNA in EA with/without Fen in certain rat brain, thus to get some new understanding on the relationship between opioidergic and serotoninergic systems.

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MATERIALS AND METHODS

Rats Sprague-Dawley rats (♂, $n=20$, 215±35 g) were bred by the Department of Experimental Animals, Shanghai Medical University.

Reagents Digoxigenin (Dig) RNA labelling Kit and Dig detection Kit were from Boehriger Mannheim; Restriction endonucleases Sac 1 and Hinf 1 RNAase were from Sino-American Biotechnology Company; Proteinase K was from Sigma; Other reagents were all AR.

Nociceptive test Nociceptive test was undertaken in quiet surrounding at 18 ℃. The rat tail was inserted subcutaneously with needles which were connected with pain test apparatus (Model WO-9E Pain Threshold Meter, Beijing). The least intensity of stimulating current that induced the tail flick was recorded as pain threshold. The pain threshold test was repeated thrice as the preadministration control, which ranged from 0.1 to 0.2 mA in normal rats.

EA EA was applied unilaterally at points (the needles were inserted 5 mm) of "Zu-San-Li" (ST 36, between muscle anterior tibialis and muscle extensor digitorum longus) and "Kun-Lun" (B60, in the depression between the tip of the external malleolus and tendo calcuneus) by EA apparatus (Model 6805-2, Shanghai with the dense-sparse frequency of wave and 20 V intensity, which provoked slight twitches of hindlimb.

Tissue preparation The rats were treated with (1) normal saline (NS) ip; (2) Fen 4.5 mg kg^{-1} ip

(Fen); (3) EA for 20 min (NS+EA); (4) Fen 4.5 mg kg^{-1} ip for 10 min, then EA for 20 min (Fen+EA). After 10 h, rats were deeply anesthetized, and perfused intracardial with 4 % paraformaldelyde. The brain and spinal cord were sliced on freezing microtome (30 μm). The sections were stored in the modified cryoprotectant solution (500 mL phosphate buffer solution 0.1 mol L^{-1} pH 7.2, 300 g sucrose, 300 mL ethylene glycol, with the final volume adjusted to 1 L with H_2O) at −20 C.

***In situ* hybridization histochemistry method** For labelling PPE RNA probe with Dig and *in situ* hybridization were reported[6]. The sections were put into a sterile culture dish containing PBS 0.1 mol L^{-1} and rinsed, then placed in 1.5 mL epintorph with 500 ng L^{-1} PPE probe and hybridization buffer (50 % formamide, 5 × SSC, 2 % (w/v) blocking reagent, 0.02 % (w/v) SDS) and incubated at 37 C for 16 h. After hybridization, the sections were rinsed in 2 × SSC, treated with RNAase (20 μg L^{-1}) and rinsed 0.5 × SSC. Finally, immunological detection was proceeded and the sections were incubated in color solution (levamisole 0.24 g L^{-1}, nitroblue tetrazolium salt 0.34 g L^{-1}, 5-bromo-4-chloro-3-indolylphosphate toluidinium salt 0.18 g L^{-1} in NaCl 100 mmol L^{-1}, MgCl 50 mmol L^{-1} pH 9.5) for 6 h at 25 C. The section was mounted and examined under light microscope (Nikon).

Using computer-assisted image processing system (FG-100-AT, Imaging Technology Inc, and TV-Camera, RCA Inc, USA), the density of PPE mRNA positive neurons was measured by the gray level of cell image. Data were analyzed by *t*-test between 2 samples.

RESULTS

Effect of Fen on EAA Changes of pain threshold in NS group was observed for 90 min. When unilateral EA was applied, the pain threshold increased. There was no significant change in response of ip Fen 4.5 mg kg^{-1}. After Fen + EA, the pain threshold was further increased and prolonged *vs* NS+EA group ($P<0.01$), with the peak appearing at 30 min following EA (Fig 1).

Expression of PPE mRNA In NS group,

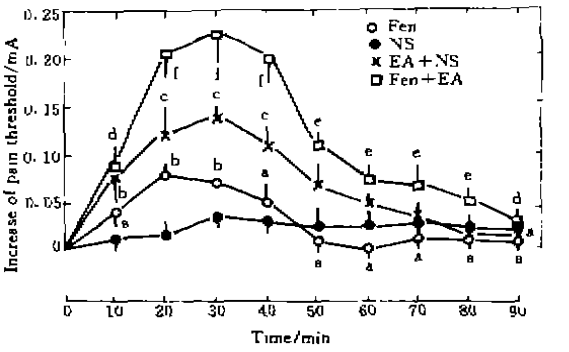

Fig 1. Effect of fenfluramine (Fen) of electroacupuncture analgesis (EAA). $n=5$, $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* NS group; $^dP>0.05$, $^eP<0.05$, $^fP<0.01$ *vs* EA group.

PPE mRNA positive neurons were seen in caudate putamen, accumben nucleus, preoptic area, periaqueductal gray, dorsal raphe nucleus, and lumbar spinal cord. In rats treated with Fen + EA, the highest increase of PPE mRNA content was seen in caudate putamen, amygdala nucleus, accumbens nucleus, preoptic lateral area, interpeduncular nucleus, periaqueductal gray, dorsal raphe nucleus, nucleus raphe magnus, and lumabr spinal cord (laminae I and II), compared with that of NS +EA group ($P<0.01$). Moderate increases of PPE mRNA content in Fen + EA group were detected in lateral septal, preoptic medial area, hypothalamus ventromedical nucleus, and lumbar spinal laminae III IV, compared with those of NS+EA ($P<0.05$). Thalamus showed little change in PPE mRNA *vs* NS group ($P>0.05$). In NS+EA group, there were increases in above areas, but the increases were less than those in Fen + EA group. In Fen group, the increase of PPE mRNA was seen in caudate putamen and amygdala nucleus, dorsal raphe nucleus, raphe magnus nucleus and lumbar spinal cord (laminea I and II) (Tab 1).

The neuroanatomical localizations of PPE-positive cells showed different degree

Tab 1. Density of PPE mRNA positive neurons in brain. ($n=4$, $\bar{x}\pm s$). [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* NS (normal saline); [d]$P>0.05$, [e]$P<0.05$, [f]$P<0.01$ *vs* Fen (fenfluramine); [g]$P>0.05$, [h]$P<0.05$, [i]$P<0.01$ *vs* NS+EA (electroacupuncture).

	NS	FFM	NS+EA	FFM+EA
Telencephalon				
Caudate-putamen	10.9±1.3	17.7±1.5[b]	25.1±3.3[c]	50.5±3.5[cfh]
Amygdala				
Lateral nucleus	12.0±2.3	20.5±0.7[b]	30.7±1.0[c]	47.0±2.6[cfi]
Accumbens	12.3±1.0	13.2±1.0[a]	31.6±1.3[c]	48.6±1.8[cfi]
Preoptic area				
Lateral area	10.5±1.7	13.0±2.0[a]	29.2±1.2[c]	46.8±2.5[cfi]
Medial area	11.0±1.1	11.6±2.7[a]	29.3±1.0[c]	33.6±2.1[cfh]
Lateral septal nucleus (dorsal part)	10.7±2.2	13.7±1.8[a]	20.7±1.3[c]	27.0±2.9[cfh]
Diencephalon				
Hypothalamus				
Ventromedial nucleus	16.5±0.7	18.3±2.3[a]	25.7±2.2[c]	33.8±2.4[cfh]
Mesencephalon				
Periaqueductal gray (ventral part)	8.0±1.1	10.0±1.4[a]	15.8±2.1[c]	27.5±2.8[cfh]
Interpeducular nucleus (central)	16.0±1.0	19.0±2.3[a]	23.0±2.1[c]	50.8±3.0[cfh]
Dorsal raphe nucleus	9.3±1.8	24.7±2.0[c]	21.1±2.1[c]	48.6±2.4[cfi]
Pons/medulla				
Raphe magnus nucleus	15.1±1.8	21.6±1.1[b]	15.3±2.4[c]	43.1±3.0[cfi]
Lumbar spinal cord				
Laminae I—II	17.2±1.4	26.0±1.1[b]	31.1±1.3[c]	48.1±3.0[cfh]
Laminae III—IV	11.3±0.8	12.9±0.9[a]	15.1±1.0[b]	20.1±1.2[cfh]

changes between Fen+EA group and NS+EA group in caudate putamen, interpeduncular nucleus, and lumbar spinal dorsal horn. The density of PPE mRNA was markedly increased in the brain treated with Fen+EA (Fig 2B, D, Plate 2) *vs* EA (Fig 2A, C). Marked change of PPE mRNA contents were seen in different layers of lumbar spinal cord. When Fen was combined with EA, laminae I showed labelled PPE neuron cells (Fig 2F). Whereas in EA group, the levels of PPE mRNA in lamina III and IV were low (Fig 2E).

DISCUSSION

In our results, the increase of PPE mRNA positive neurons in areasclosely related to nociceptive modulation such as caudate putamen, accumben nucleus, preoptic area, periaqueductal gray and the dorsal horn of spinal cord was seen in EA group. It is generally accepted that the opioidergic system play an important role in nociception and analgesia[5,8]. These results are in line with such a view.

Our previous work showed the fentanyl could further increase the Fen potentiating effect on EAA[2]. The finding that 10 h after EA combined with Fen, the levels of PPE mRNA significantly increased in areas mentioned above including dorsal raphe nucleus, nucleus raphe magnus and the dorsal horn of spinal cord, and the degree of increase was higher than that of EA or Fen along, further demonstrated that the opioidergic system was involved in Fen potentiation in EAA. The enhanced PPE mRNA content in above areas was related to after-effect of Fen potentiation on EAA and reflected the long-term or chronic readiness of a cell to synthesize PPE precursor which had lost.

Recently we reported that using autoradiographic technique, the increased density of opioid receptors induced by EA combined with Fen occurred in brain nuclei mentioned above[9]. These result are also strongly consistent, indicating the opioidergic system is involved in serotonin potentiation in EAA.

In summary, EA and EA combined with Fen all induced the increasing expression of PPE mRNA in certain central areas closely related to nociceptive modulation. The degree of expression by EA combined with Fen was far stronger than that of EA. The results suggested that the endogenous opioid peptides be involved in EAA potentiated by Fen.

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氯氟拉明加强针刺镇痛时前脑啡肽原 mRNA 的表达

R965.2

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目的：研究大鼠脑内前脑啡肽原(PPE) mRNA的变化与5-HT的释放剂芬氟拉明加强针刺镇痛的关系. **方法**：用原位杂交组织化学法观察PPE mRNA的表达. **结果**：脊髓背角(I，II层)，中缝大核，中缝背核，中央灰质，脚间核，视前外侧区，杏仁核和尾壳核内的PPE mRNA的含量大量升高($P<0.01$，*vs* NS+EA)，而在隔外侧区，视前内侧区，下丘脑腹内侧核以及脊髓背角III—IV层的PPE mRNA的水平也有中等程度增高($P<0.05$，*vs* NS+EA). 但在丘脑却没有明显的变化. **结论**：PPE mRNA含量在与痛相关的区域里增高，是芬氟拉明加强针效的机制之一.

关键词 脑啡肽；信使RNA；芬氟拉明；电针；原位杂交；中枢神经系统