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GABA expression in c-Fos immunoreactive neurons of the rat periaqueductal gray induced by electroacupuncture at the point of Zusanli

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Abstract

Immunofluorescent investigation of the periaqueductal gray (PAG) was made in the rat receiving electroacupuncture (EA) delivered to the acupoint (AP) called Zusanli (ST36) on the hindlimb. The EA led to strong expression of c-Fos- and gamma aminobutylic acid (GABA)- immunoreactivity (IR) mainly in the ventrolateral to lateral subdivision of the PAG. The double immunofluorescent experiments showed GABA expression in c-Fos immunoreactive neurons in the PAG. Morphometric analysis revealed that the number of double-labeled neurons in the AP case is approximately three times higher than that in the non-AP case. The present findings might indicate that PAG neurons activated by the EA at the AP of Zusanli participate in the descending pain control system of GABA.

Key words: GABA, c-Fos, Periaqueductal gray, Electroacupuncture, Pain

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Introduction

The PAG has been indicated to be involved in the influence of EA on the pain control system [1,2]. Activation of this system is characterized by inhibition for nociceptive neurons in the dorsal horn of the spinal cord [3,4,5]. In recent immunofluorescent studies, it is of interest that strong expression of c-Fos as a marker of neuronal activation has been identified in the ventrolateral to lateral subdivision of the PAG following EA at the AP of Zusanli (ST36) [6,7].

However, with respect to neuropeptide playing an important role in pain modulation in the central nervous system, it should be noted that GABA which is one of the major inhibitory neuropeptides is also contained in the similar regions of the PAG [8,9,10]. These findings might raise the possibility that the PAG neurons activated by stimulation of the EA contain GABA. In fact, our previous immunohistochemical study indicated the possibility of double labeling in the PAG [7]. Therefore, the present study was performed to investigate whether c-Fos neurons in the PAG induced by EA at the AP of Zusanli contain GABA using the immunofluorescent method in the rat.

Materials and Methods

For this study 28 adult male Wister rats from SLC (Shizuoka, Japan), weighing 185-220 g, housed in separate cages under controlled conditions at constant temperature (23±1°C) and maintained in a 12:12 light/dark cycle. The animals were anesthetized with intraperitoneal injection of chloral hydrate (490mg/kg) for all surgical procedures and perfusions. The experimental procedures were conducted in accordance with National Institute of Health for Care and Use of Laboratory Animals (NIH publications No. 80-23, revised 1996). The approval of Kagawa University Animal Care and Use Committee was obtained for this study, and all efforts were made to minimize their suffering. The EA was bilaterally applied at the AP of Zusanli (ST36) (N=10) or at the non-AP (N=10). Stainless steel needles were inserted to

a depth of 5 mm into Zusanli, located between the tibia and the fibula, approximately 5 mm lateral to the anterior tubercle of the tibia according to the previous method of the EA [6]. The non-AP was located 5 mm lateral to the midline of the posterior face of the hindlimb. The localization of points was confirmed by measurement of the skin impedance (Lautz, Brazil). Each needle was stimulated using the EA apparatus (SEN-3201, NIHON KOHDEN, JAPAN) for 20 min with electrical pulses at a frequency of 2 Hz and intensity of 2 mA. Sham control animals were undergone by the immunohistochemical procedure without any EA (N=8).

Animals were perfused transcardially with 0.02M phosphate buffered saline (PBS, pH 7.4) followed by fixation with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.02M PBS after 1.5-2.0 h from the onset of the EA stimulation. Serial 20 μm-thick frozen sections were processed for fluorescent immunohistochemical staining for c-Fos and GABA as following brief description. The sections were placed in 1% normal donkey serum (Jackson immunoresearch lab. West Grove, PA, USA) for 1 h and incubated with primary antibodies at 4°C overnight. PBS solution containing the two primary antibodies included a mouse monoclonal anti-c-Fos (1:2000, Sigma) and a rabbit polyclonal anti-GABA (1:1000, Sigma). Sections then were incubated with rhodamine-conjugated donkey anti-mouse antibody and FITC-conjugated donkey anti-rabbit antibody (both 1:100, Jackson immunoresearch lab.) in PBS for 2h. The slides were coverslipped using mounting medium (Vector lab.). Immunofluorescent control studies were performed by omission of the primary antibodies. No respective labeling was detected under these conditions. Sections were analyzed by epi-illumination fluorescence microscope (DP70, Olympus, Japan) and processed by an image analyzer (NIS-Elements D, Nikon, Japan). Confocal images were visualized on Radiance 2100 (Bio-Rad, Hercules, CA) and an optical slice thickness of 0.2 μm.

A morphometric analysis was carried out according to previous report [11,12). Samples were taken every five sections through the PAG and, according to the classification of the PAG, morphometric analysis of double-labeled neurons in the ventrolateral to lateral subdivision was done. A counting square (40 μm× 40μm) was superimposed onto sections at a magnification of ×100 on an image analyzer. c-Fos and GABA immunoreactive neurons were expressed as the percentage of the total number of nuclei. In this study, approximately 500 neurons were counted in the ventrolateral to lateral subdivision of the PAG. Statistical analysis of the data was performed by Student's *t*-test using SigmaStat (Systat Software, Version 3.1). The c-Fos, GABA, merged results were considered statistically significant when $p < 0.01$.

Results

The PAG was located in the area surrounding the cerebral aqueduct at the level from the posterior commissure to the dorsal tegmental nucleus, and consisted of densely packed small neurons which were oval or triangular in shape. In

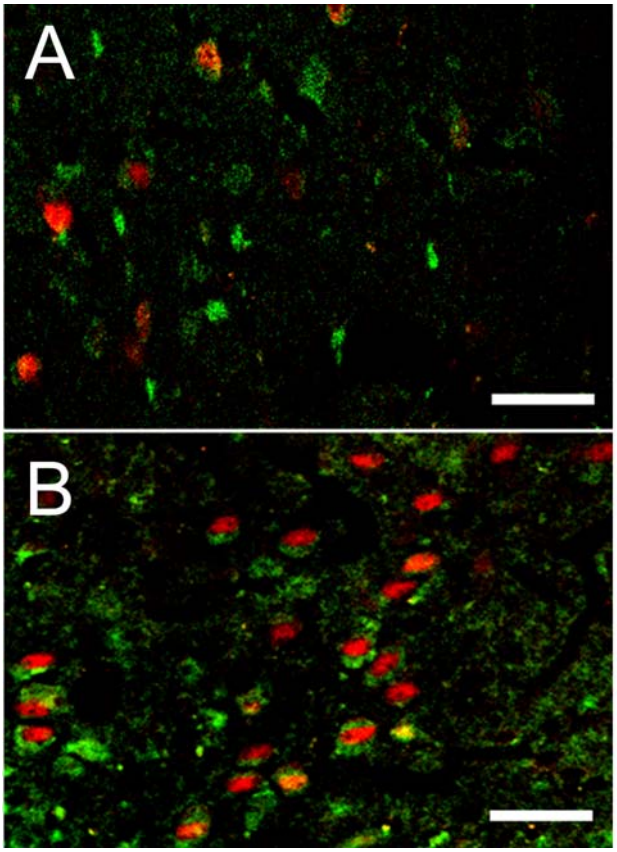

Figure 1: *Photomicrographs of immunofluorescent staining in the ventrolateral portion at the level of rostral one-third of the PAG in the non-AP (A) and AP (B) cases. Note that the number of double-labeled neurons of c-Fos (red colored nucleus) and GABA (green colored cytoplasm) was markedly increased in the AP case compared to that in the non-AP case. Calibration bars = 30 μm in A and B.*

the present experiments, the expression of fluorescent c- Fos-and GABA- immunoreactivity (IR) in sham animals was very weak in the PAG bilaterally. In animals receiving the EA at the non-AP, the expression of c-Fos- and GABA-IR was observed to be increased moderately in all subdivisions of the PAG (Fig. 1A). On the other hand, in animals receiving the EA at the point of Zusanli (ST36), the expression of these IR was characterized by extreme increase in the ventrolateral to lateral subdivision of the PAG. In particular, there was a tendency of stronger expression at the rostral and middle levels of the PAG bilaterally (Figs. 1B and 2A). Double labeling images (Figs. 2A and D) revealed in the AP animals that some of c-Fos immunoreactive neurons with red colored nucleus (Fig. 2C) are GABA-positive neurons with green colored cytoplasm (Fig. 2B) mainly in the ventrolateral to lateral subdivision of the PAG bilaterally. It was of interest that the merged area located in these subdivisions is frequently close to the cerebral aqueduct.

The number of c-Fos and GABA immunoreactive neurons in the ventrolateral to lateral subdivision of the PAG in

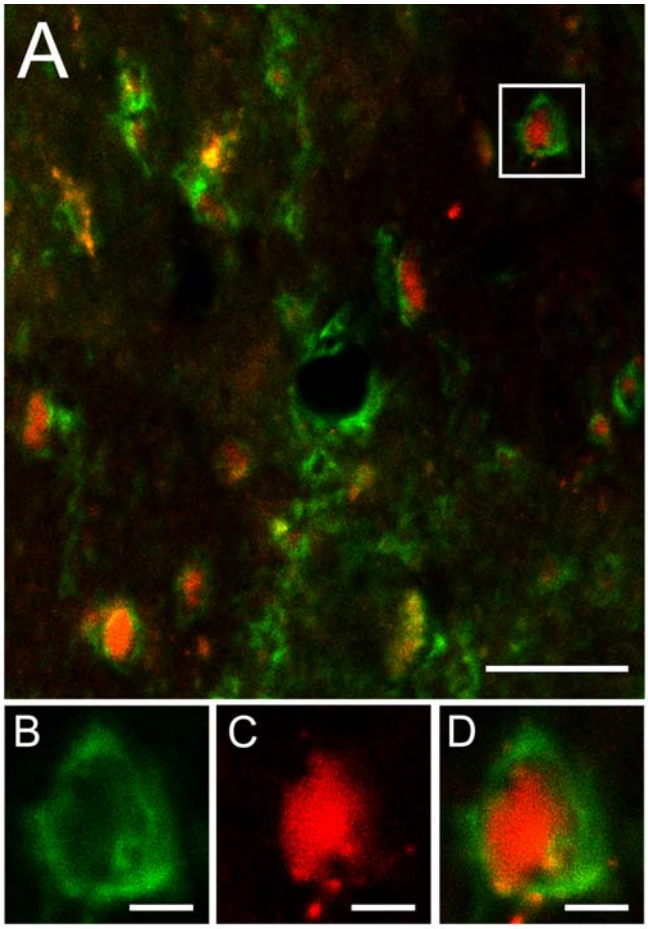

Figure 2: *Photomicrographs of immunofluorescent staining in the lateral portion at the level of rostral one-third of the PAG in the AP case. Note that c-Fos immunoreactive neurons containng GABA are indicated in A. Furthermore, a typical labeling (surrounded area in A) is shown in B (GABA: green colored cytoplasm), C (c-Fos: red colored nucleus) and D (double-labeled neuron). Calibration bars = 20 μm in A and 5 μm in B-D.*

Table 1: Mean ± S.E.M. numbers of c-Fos- and GABA-immunoreactive neurons in the non-AP and AP cases. Note that the number of double-labeled neuron in the AP case is approximately three times higher than that in the non-AP case (by Student's t-test, p < 0.01).

	c-Fos alone	GABA alone	Double Labeling
Non-AP	1.31±0.13	9.24±1.30	3.83±0.42
AP	2.08±0.24*	10.43±0.69	13.36±2.63*

**p < 0.01 (Student's t-test).*

animals receiving the EA at the non-AP and AP was analyzed. The mean ± standard error of the mean (SEM) of non-AP and AP cases together with the results of Student's *t*-test of this data, are shown in Table 1. As shown in Table 1, there were significant differences in the number of double-labeled neurons.

Discussion

GABA expression in c-Fos immunoreactive neurons in PAG

Our previous study specifically examined the responses of the PAG to EA stimulation at the AP of Zusanli by identifying c-Fos expression, and suggested the possibility of GABA expression in c-Fos immunoreactive neurons in the ventrolateral to lateral subdivision of the PAG [7]. The present immunofluorescent experiments showing the double labeling indicated a highly site-specific distribution in the PAG as well as the previous study. The ventrolateral to lateral subdivision has been known to have a significant inhibitory function for dorsal horn nociceptive neurons, particularly spinothalamic neurons [5,13]. Such functional significance containing GABA is considered to play an important role in controlling the output of the PAG. This might mean the existence of inhibitory local circuit in the PAG on the basis of GABA as reported in the cerebellar cortex [14]. However, with respect to the PAG connections with the spinal cord, it was demonstrated that the projections are very sparse and analgesia is mediated through activation of medullary regions [15, 16]. In this point, Reichling and Basbaum [9,17] have shown the medullary regions containing the nucleus raphe magnus and reticular formation to be major relay stations. Taken together, it might be indicated in the present study that PAG neurons activated by the EA at the AP of Zusanli participate in the descending pain control system of GABA.

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