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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

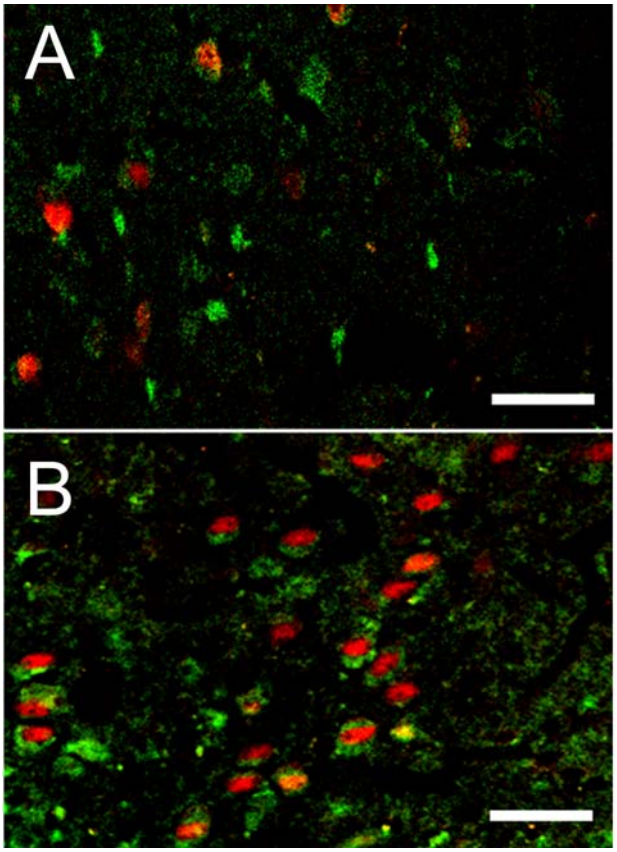

Figure 1: *Photomicrographs of immunofluorescent staining in the ventrolateral portion at the level of rostral one-third of the PAG in the non-AP (A) and AP (B) cases. Note that the number of double-labeled neurons of c-Fos (red colored nucleus) and GABA (green colored cytoplasm) was markedly increased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

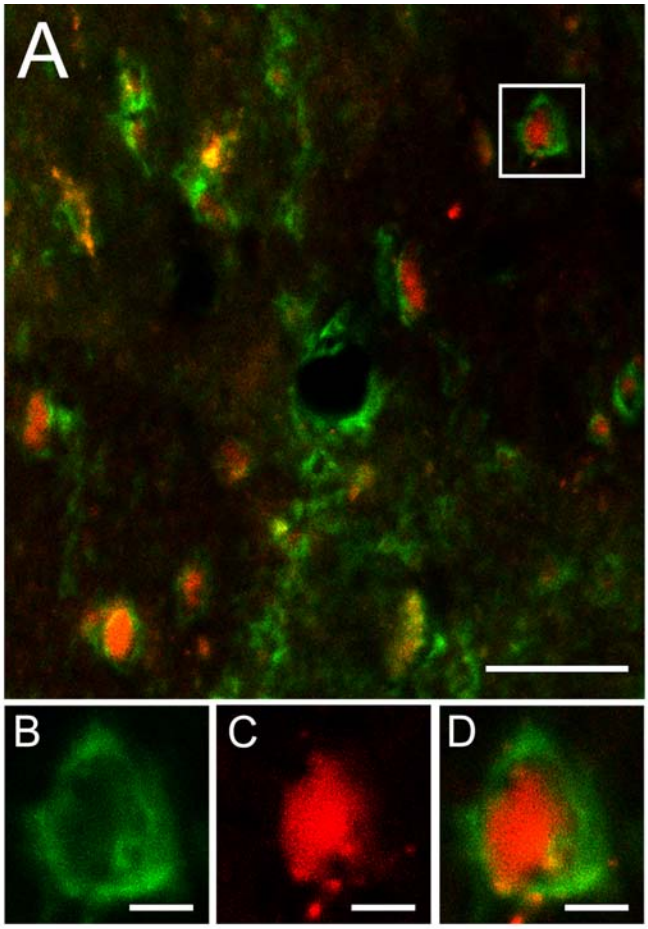

Figure 2: *Photomicrographs of immunofluorescent staining in the lateral portion at the level of rostral one-third of the PAG in the AP case. Note that c-Fos immunoreactive neurons containng GABA are indicated in A. Furthermore, a typical labeling (surrounded area in A) is shown in B (GABA: green colored 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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References

1. Lee J-H, Beitz AJ. The distribution of brain-stem and spinal cord nuclei associated with difference frequencies of electroacupuncture analgesia. Pain 1993; 52: 11-28.
2. Guo HF, J. Tian J, Wang X, Fang Y, Hou Y, Han J. 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. Brain Res Mol Brain Res 1996; 43: 157-166.
3. Aimone LD, Gebhart GF. Stimulation-produced spinal inhibition from the midbrain in the rat is mediated by an excitatory amino acid neurotransmitter in the medial medulla. J Neurosci 1986; 6: 1803-1813.
4. Dostrovsky JO, Shah Y, Gray BG. Descending inhibitory influences from periaqueductal gray, nucleus raphe magnus, and adjacent reticular formation. II. Effects on medullary dorsal horn nociceptive and non-nociceptive neurons. J Neurophysiol 1983; 49: 948-960.
5. Zang D, Owens CM, Willis WD. Two forms of inhibition of spinothalamic tract neurons produced by stimulation of the periaqueductal gray and cerebral cortex. J Neurophysiol 1991; 65: 1567-1579.
6. Medeiros MAD, Canteras NS, Suchecki D, Mello LEAM. Analgesia and c-Fos expression in the periaqueductal gray induced by electroacupuncture at the Zusanli point in rats. Brain Res 2003; 973: 196-204.
7. Fusumada K, Yokoyama T, Miki T, Wang ZY, Yang W, Lee NS, et al. c-Fos expression in the periaqueductal gray is induced by electroacupuncture in the rat, with possible reference to GABAergic neurons. Okajimas Folia Anat Jpn 2007; 84: 1-9.
8. Belin MF, Aguera M, Tappaz M, McRae DA, Bobillier P, Pujol JF. GABA-accumulating neurons in the nucleus raphe dorsalis and periaqueductal gray in the rat: a biochemical and radioautographic study. Brain Res 1979; 170: 279-297.
9. Reichling DB, Basbaum AI. Contribution of brainstem GABAergic circuitry to descending antinociceptive controls: I GABA-immunoreactive projection neurons in the periaqueductal gray and nucleus raphe magnus. J Comp Neurol 1990; 302: 370-377.
10. Sandkuhler J, Willmann E, Fu QG. Blockade of GABA (A) receptors in the midbrain periaqueductal gray abolishes nociceptive spinal dorsal horn neuronal activity. Eur J Pharmacol 1989; 160: 163-166.
11. Matsumoto Y, Tsukamoto Y, Miki T, Ogawa K, Lee KY, Yokoyama T, et al. Age-related changes in growth hormone-immunoreactive cells in the anterior pituitary gland of Jcl: Wistar-TgN (ARGHGEN) 1Nts rats (Mini rats). Congenit Anom (Kyoto). 2006; 46: 188-193.
12. Dougherty KJ, Sawchuk MA, Hochman S. Phenotypic Diversity and expression of GABAergic inhibitory interneurons during postnatal development in lumbar spinal cord of GAD67-GFP mice. Neuroscience. 2009; 163 (3): 909-919.
13. Liebeskind JC, Guilbaud G, Besson JM, Oliveras JL. Analgesia from electrical stimulation of the periaqueductal gray matter in the cat: Behavioral observations and inhibitory effects on spinal cord interneurons. Brain Res 1973; 50: 441-446.
14. Saito K, Barber R, Wu J, Matsuda T, Roberts E, Vaughn JE. Immunohistochemical Localization of glu- tamate decarboxylase in the rat cerebellum. Proc Natl Acad Sci. 1974; 71: 269-273
15. Basbaum AI, Fields HL. The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: Further studies on anatomy of pain modulation. J Comp Neurol 1979; 187: 513-531.

16. Behbehani MM. Functional characteristics of the midbrain periaqueductal gray. Neurobiol 1995; 46: 575-605.
17. Reichling DB, Basbaum AI. Contribution of brainstem GABAergic circuitry to descending antinociceptive controls: II. Electron microscopic immunocytochemical evidence of GABAergic control over the projection from the periaqueductal gray to the nucleus raphe magnus in the rat. J Comp Neurol 1990; 302: 378-393.

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