Available online at www.sciencedirect.com
SCIENCE DIRECT
Behavioural Brain Research 169 (2006) 48–56
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Corticotropin-releasing factor neurons in the hypothalamic paraventricular nucleus are involved in arousal/yawning response of rats

Ichiro Kita [a,b], Yoshinari Seki [a], Yasushi Nakatani [a], Masaki Fumoto [a], Mitsugu Oguri [a], Ikuko Sato-Suzuki [a], Hideho Arita [a,*]

[a] *Department of Physiology, Toho University School of Medicine, 5-21-16 Omori-nishi, Ohta-ku, Tokyo 143-8540, Japan*
[b] *Department of Kinesiology, Tokyo Metropolitan University, 1-1 Minamiohsawa, Tokyo 192-0397, Japan*

Received 13 September 2005; received in revised form 30 November 2005; accepted 8 December 2005
Available online 18 January 2006

Abstract

Our previous studies have suggested that activation of the hypothalamic paraventricular (PVN) descending oxytocinergic projections is involved in the induction of yawning accompanied by an arousal response, but the possibility that neural systems other than the oxytocinergic system in the PVN also mediate the arousal/yawning response cannot be ruled out. We assessed the activity of corticotropin-releasing factor (CRF) neurons during yawning induced by the PVN stimulation in anesthetized, spontaneously breathing rats using double-staining for c-Fos and CRF. Yawning response was evaluated by monitoring an intercostals electromyogram as an index of inspiratory activity and a digastric electromyogram as an indicator of mouth opening. We also recorded the electrocorticogram (ECoG) to determine the arousal response during yawning. Microinjection of L-glutamate (2–5 nmol) into the PVN produced a frequent yawning accompanied by an arousal shift in the ECoG, and these behavioral effects were associated with a significant increase of c-Fos positive CRF neurons in the medial parvocellular subdivision of the PVN. In addition, a marked enhancement in the c-Fos expression was found in the both locus coeruleus (LC) and global area in the cortex when the frequency of yawning response was increased by the PVN stimulation, suggesting that the arousal response during yawning might be mediated by the activation of LC neurons. The present study suggests that an activation of CRF neurons in the PVN is responsible for the arousal response accompanied by yawning behavior.

Keywords: Yawning; Arousal; Paraventricular nucleus; CRF; c-Fos

1. Introduction

We have reported that a stereotyped yawning response can be evoked by several forms of chemical stimulation of the paraventricular nucleus (PVN) of the hypothalamus in anesthetized, spontaneously breathing rats [14,26,27]. Yawning response in the hypothalamus is typically associated with the induction of arousal response, and in our previous studies, we recorded the electrocorticogram (ECoG) to evaluate arousal responses during yawning and found that an arousal shift in the ECoG, represented by lower voltage and faster rhythm, occurred before the yawning behavior.

It is widely believed that an activation of the descending oxytocinergic system in the PVN is one of the most important mechanisms for mediating the induction of yawning [3,26]. However, the PVN contains various neuropeptides besides oxytocin, such as corticotropin releasing factor (CRF), vasopressin, and dynorphine [34]. Thus, the possibility that not only the oxytocinergic system but also the other neural systems in the PVN could be involved in the arousal/yawning responses cannot be ruled out. In the present study, we focused on CRF neurons as a candidate for the induction of arousal response accompanied by yawning behavior.

CRF neurons in the PVN are generally known to play a critical role in arousal, autonomic, and behavioral responses associated with various stressors [7,15,21,35]. Several studies have reported that mild stresses such as foot shock, forced swimming and REM sleep deprivation modify the induction of yawning response [19,20,36]. We have also indicated that the yawning response occurs by stress-like stimulation such as local hypoxia of the PVN [14] and light stimulation [30], and these manipulations induce not only the yawning response but also an arousal

* Corresponding author. Tel.: +81 3 3762 4151; fax: +81 3 3762 8148.
E-mail address: aritah@med.toho-u.ac.jp (H. Arita).

0166-4328/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2005.12.003

shift in the ECoG, suggesting that the cortical activation accompanied by yawning behavior may be related to stress. Taken together, it is possible that CRF neurons in the PVN are involved in the induction of arousal/yawning response.

Although the mechanism behind the arousal response accompanied by yawning evoked by PVN stimulation or stress is still unknown, the arousal response may be mediated by the widespread projecting neurons in the brainstem reticular formation. We have previously proposed a hypothesis that the arousal response accompanied by yawning evoked by PVN stimulation could be accounted by the projection of CRF neurons from the PVN to the locus coeruleus (LC), which is one of the neuronal aggregates within the brainstem reticular formation [27,29]. In the present study, we first investigated the involvement of CRF neurons in the PVN in the arousal/yawning responses with double-staining for c-Fos and CRF. Then, we observed c-Fos expression to evaluate neuronal activity in the LC and the cortical area in the arousal/yawning response evoked by chemical stimulation of the PVN. The present study suggested that activation of CRF neurons in the PVN is involved in the arousal response during yawning behavior.

2. Materials and methods

2.1. Animals

Eighteen adult male Wistar rats (weighing 300–450 g) were used for the experiments. The rats were caged in groups of 3–4 under controlled conditions of temperature (22–24 °C) and light (12-h light:12-h dark cycle, lights on at 08:00 h) with ad libitum access to food and water. All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Toho University School of Medicine. All efforts were made to minimize animal suffering and the number of animals used.

2.2. Surgery

Surgical procedures were essentially the same as described previously [26]. In brief, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and additional doses were given as needed. Catheters were placed in the femoral artery to monitor arterial blood pressure (BP). Heart rate (HR) was measured from the BP pulse with a tachometer (AT-601G, Nihon Kohden, Japan). To monitor respiratory activity, a pair of twisted wire electrodes, insulated except for 1 mm at the tips, were implanted into the lower intercostal space by way of a 23-gage hypodermic needle; the needle was then withdrawn, leaving the wires in the intercostal muscle. Similarly, pairs of wire electrodes were implanted in the digastric muscle to monitor mouth opening activity. For electrocorticogram (ECoG) recordings, holes were drilled in the skull and two screw electrodes were implanted. Rectal temperature was maintained at 37 °C with a heating lamp.

2.3. Microinjection

The animals were fixed prone in a stereotaxic frame and a parietal craniotomy was performed. For drug microinjections into the paraventricular nucleus of the hypothalamus, we used a glass micropipette connected to a fine cannula. The free end of the cannula was attached to a picopump (PV830 Pneumatic, WPI) for injections. The dura overlying the cortex was opened for advancement of the micropipette, which was inserted to the PVN (coordinates: 1.4 mm posterior to bregma, 0.2–0.3 mm lateral to midline, and 6.0–7.0 mm ventral to the dural surface) [23]. L-Glutamate (0.1 M, 20–50 nl in each injection) dissolved in 0.9% saline was injected into the PVN for induction of frequent spontaneous yawning. The microinjection of L-glutamate was performed several times at intervals of 2–3 min. Control rats were injected with 0.9% saline using the same volume as for L-glutamate.

2.4. Immunohistochemistry

Ninety minutes after microinjection of L-glutamate or saline, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with heparin solution (1000 U/l, 0.9% saline), followed by ice-cooled 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer saline (PBS, pH 7.4). The rat brains were removed and post-fixed in the same fixative without glutaraldehyde for 24 h at 4 °C. The brains were then cryoprotected in a phosphate-buffered 30% sucrose solution with 0.1% sodium azide for 24–48 h. The brains were then frozen and cut in the coronal plane (6 series of 40-μm thick sections) on a microtome and collected in 0.1 M PBS with 0.1% sodium azide.

Immunohistochemical visualization of c-Fos and CRF was carried out on free-floating sections using antibodies and avidin-biotin-peroxidase methods as previously described [24,31]. Similarly, we also performed double-staining for c-Fos and oxytocin to assess activation of oxytocin neurons in the yawning response. Briefly, after blocking endogenous peroxidase and preincubation in 10% normal horse serum, the sections were incubated in primary rabbit polyclonal anti-Fos antiserum (sc-52, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:600 in 0.1 M PBS with 0.1% Triton X-100 (PBS-TX) for 16 h at room temperature. After rinsing three times for 10 min in PBS-TX, the sections were further incubated in secondary biotinylated donkey anti-rabbit IgG (AP182B, Chemicon, Temecula, CA, 1:800) for 90 min at room temperature, rinsed three times for 10 min in PBS-TX, and finally treated with an avidin–biotin peroxidase complex (Vectastain ABC peroxidase kit, Vector Lab Inc., Burlingame, CA, 1:400) for 90 min. The sections were reacted for peroxidase activity in a solution consisting of nickel ammonium sulfate, 0.02% 3,3-diaminobenzidine (DAB) in 0.1 M Tris–HCl buffer (pH 7.6) and 0.01% H_2O_2 for 20 min. The c-Fos immunoreactivity was localized to the cell nuclei and appeared as a dark gray-black stain. For dual immunostaining for CRF or oxytocin, the sections were sequentially incubated in CRF antibody (Peninsula Lab Inc., San Carlos, CA, 1:5000) or oxytocin antibody (Immunostar inc., Hudson, WI, 1:5000). The avidin-biodin peroxidase complex was visualized with DAB in 0.1 M Tris–HCl buffer (pH 7.6) without nickel sulfate. CRF or oxytocin immunoreactivity was localized to the cell cytoplasm and was visible as a brown stain. Then sections were washed in 0.01 M PBS, mounted on gelatin-coated glass slides, air-dried, dehydrated in graded alcohol, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

2.5. Cell counts and quantification

Immunoreactive cells on sections were observed with an Olympus BH-2 microscope equipped with a camera (ELMO, CN42H). The quantitative analysis was performed on all sections containing the PVN. The PVN region includes both parvocellular and magnocellular divisions, and therefore, the distinction between parvocellular and magnocellular subdivisions in the PVN were performed according to the description of Sawchenko and Swanson [28]. The total numbers of c-Fos positive nuclei and double labeled cells were counted bilaterally in each subdivision of the PVN on the sections between −0.8 and −2.1 mm from bregma (corresponding to Plates 21–26 in the atlas of Paxinos and Watson), and the numbers of nuclei and cells per section (total number/sections) were calculated. The percentage of c-Fos positive nuclei in the CRF or oxytocin neurons was also calculated in the subdivision of the PVN. Similarly, the total number of c-Fos positive nuclei was counted bilaterally in the LC on the sections between −9.3 and −10.3 mm from bregma (corresponding to Plates 55–59 in the atlas of Paxinos and Watson); the number per section was then calculated. Furthermore, we calculated the density of c-Fos nuclei in some cortical areas after microinjection of L-glutamate or saline.

2.6. Statistics

Values of immunoreactive cells or nuclei are expressed as the mean ± S.E. Statistical evaluation of the experiments was performed by two-way ANOVA