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Emotional stress evoked by classical fear conditioning induces yawning behavior in rats

Natsuko Kubota[a], Seiichiro Amemiya[b], Shinya Yanagita[a], Takeshi Nishijima[b], Ichiro Kita[b,*]

[a] Research Institute for Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
[b] Department of Human Health Science, Tokyo Metropolitan University, 1-1 Minamiohsawa, Hachioji, Tokyo 192-0397, Japan

HIGHLIGHTS

- We investigated the involvement of emotional stress in induction of yawning in rats.
- Emotional stress induced yawning accompanied with neuronal activation of the PVN.
- Emotional stress induced anxiety-related behavior.
- Emotional stress caused neuronal activation of the CeA.

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ABSTRACT

Yawning is often observed not only in a state of boredom or drowsiness but also in stressful emotional situations, suggesting that yawning is an emotional behavior. However, the neural mechanisms for yawning during stressful emotional situations have not been fully determined, though previous studies have suggested that both parvocellular oxytocin (OT) and corticotropin-releasing factor (CRF) neurons in the hypothalamic paraventricular nucleus (PVN) are responsible for induction of yawning. Thus, using ethological observations and c-Fos immunohistochemistry, we examined whether emotional stress evoked by classical fear conditioning is involved in induction of yawning behavior in freely moving rats. Emotional stress induced yawning behavior that was accompanied by anxiety-related behavior, and caused neuronal activation of the central nucleus of the amygdala (CeA), as well as increases in activity of both OT and CRF neurons in the PVN. These results suggest that emotional stress may induce yawning behavior, in which the neuronal activation of the CeA may have a key role.

1. Introduction

Yawning is often observed not only in a state of boredom or drowsiness, but also in stressful emotional situations in humans and other animals. Ethological and psychological studies in primates have reported yawning following neighboring individuals that are vocalizing and showing sexual jealousy [4,5]. Clinical reports have shown that yawning is frequently observed in anxiety disorders, including patients with hysteria, depression, and motion sickness [7,28]. In addition, Major et al. [19] reported that anxiogenic compounds induce anxiety-like behavior in monkeys that is accompanied by yawning. Thus, yawning is considered an emotional behavior.

Previous pharmacological and lesion studies have suggested that the paraventricular nucleus of the hypothalamus (PVN) is essential for the occurrence of yawning [3,26]. Additionally, we have reported that both parvocellular oxytocin (OT) neurons and corticotropin-releasing factor (CRF) neurons in the PVN, each of which sends descending axons to the lower brainstem involved in either arousal, respiratory, cardiovascular, or other autonomic functions, are responsible for yawning that is accompanied by an arousal response [14–16]. Although these results suggest that the stereotyped yawning response is mediated by both parvocellular OT and CRF neurons in the PVN, the mechanisms of yawning during stressful emotional situations have not been fully determined.

The amygdala is generally known to play a pivotal role in emotion [1,2]. The central nucleus of the amygdala (CeA), which is a part of the amygdaloid complex, is particularly known to be essential for integration of behavioral, autonomic, and hormonal responses to emotional stress, and is one of the most important output regions

* Corresponding author. Tel.: +81 42 677 2960; fax: +81 42 677 2961.
E-mail address: kita-ichiro@tmu.ac.jp (I. Kita).

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for the expression of these emotional responses [18,20]. In addition, neuroanatomical and functional studies have suggested the importance of connections from the CeA to the PVN for stress responses [10,12,13]. Thus, yawning during emotional stress may be induced through activation of CeA neurons, as well as both parvocellular OT and CRF neurons in the PVN. In the present study, using ethological observation and c-Fos immunohistochemistry in rats, we investigated whether emotional stress is involved in induction of yawning behavior.

2. Materials and methods

2.1. Animals

Twenty-two adult male Wistar rats (weight, 294–352 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 05:00 h) with ad libitum access to food and water. All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Tokyo Metropolitan University. All efforts were made to minimize animal suffering and the number of animals used.

2.2. Fear conditioning apparatus and paradigm

To produce emotional stress, we used classical fear conditioning, which is a frequently used model that produces a state of anxiety that is mainly mediated by the amygdala [11,20]. Rats were placed in a foot shock chamber (30 cm × 24 cm × 24 cm), which has a grid floor composed of 19 stainless steel rods spaced 1.1 cm apart and wired to a shock generator (ENV-414S, Med Associates Inc., USA). After the rats spent 5 min in the chamber, they were stressed by electric foot shocks (0.7 mA, 3 s, 5 times, 30 s intervals) during the first day of testing. Twenty-four hours after the foot shocks, the rats were again placed in the foot shock chamber (the conditioned context stimulus) without shock delivery for a short period (2.75 min). Control rats were placed in the foot shock chamber for two consecutive days, but did not receive any foot shocks.

2.3. Behavioral observation

Rat was placed in the center of the open-field box immediately following exposure to the conditioned context stimulus. The open-field box consisted of a square, black box (90 cm × 90 cm × 45 cm) in which the floor was divided into nine squares (30 cm × 30 cm). We counted the number of yawning, namely mouth opening, which occurs alone or associated with stretching of the trunk and lacrimation [3] for the 60 min during the light part of cycle. At the same time, we measured anxiety-related behavior and locomotor activity using an open-field test, which is one of the most frequently used procedures to measure anxiety-like behavior. We counted the numbers of entries into the center of the field as indicator of anxiety-related behavior, and the respective numbers of lines crossed (all four paws of the rat crossed a line), and rearing (standing on hind legs) as indicator of locomotor activity. These behaviors of each rat in the open-field box were observed and video recorded in low light conditions (8–13 lx throughout the box).

2.4. Immunohistochemistry

Ninety minutes after the start of the open-field test, 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-buffered saline (PBS, pH 7.4). The 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 containing 0.1% sodium azide for 24–48 h. Next, the brains were frozen and cut in the coronal plane (six 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 was carried out on free-floating sections using antibodies and avidin–biotin–peroxidase methods as previously described [16,23,27]. Briefly, after blocking endogenous peroxidases 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 avidin-biotin peroxidase complex solution (Vectastain ABC peroxidase kit, Vector Lab Inc., Burlingame, CA, 1:400) for 90 min. The sections were reacted for peroxidase activity in a solution containing 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 dark gray-black stain. Similarly, we also performed double staining for c-Fos and OT or CRF to assess activation of OT or CRF neurons in the PVN. Following c-Fos immunostaining, the sections were sequentially incubated in OT antibody (Immunostar Inc., Hudson, WI, 1:5000) or CRF antibody (Peninsula Lab Inc., San Carlos, CA, 1:5000). The avidin–biotin–peroxidase complex was visualized with DAB in 0.1 M Tris–HCl buffer (pH 7.6) without nickel ammonium sulfate. OT or CRF 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 a graded series of alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

2.5. Cell counts and quantification

Immunoreactive cells on the sections were observed with an Olympus BH-2 microscope equipped with a camera (ELMO, CN42H). Quantitative analysis was performed on all sections containing the PVN and CeA. The total numbers of OT or CRF positive cells and double-labeled cells for c-Fos and OT or CRF were manually counted bilaterally in the parvocellular subdivision of the PVN on the sections between −0.8 and −2.1 mm from bregma (corresponding to Plates 40–50 in the atlas of Paxinos and Watson, 5th edition [24]). The percentage of c-Fos positive nuclei in either OT or CRF neurons per section was then calculated in the PVN. Similarly, the total number of c-Fos positive nuclei was counted bilaterally in the CeA on sections between −2.4 and −3.0 mm from bregma (corresponding to Plates 53–58 in the atlas of Paxinos and Watson [24]); the number per section was then calculated.

2.6. Statistics

Behavioral data and values of immunoreactive cells or nuclei are expressed as the mean ± standard error (SE). Statistical evaluation of the experiments was performed using an unpaired t test. A probability value of 0.05 was considered as the level of significance.

3. Results

In the classical fear-conditioning paradigm, each animal showed freezing behavior, such as immobility and absence of vibrissae