Neuroscience Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Presentation of noise during acute restraint stress attenuates expression of immediate early genes and arginine vasopressin in the hypothalamic paraventricular nucleus but not corticosterone secretion in rats

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ARTICLE INFO

Article history:
Received 19 September 2014
Received in revised form
12 November 2014
Accepted 28 November 2014
Available online xxx

Keywords:
Acoustic stimulus
White noise
c-Fos
JunB
Arginine vasopressin
Corticotropin-releasing hormone
Corticosterone
Paraventricular nucleus

ABSTRACT

The present study investigated the effect of acoustic stimulation on the activation of the hypothalamic-pituitary-adrenal (HPA) axis in rats submitted to acute restraint stress, through semi-quantitative histochemical analysis of expression of immediate early gene products (c-Fos, JunB and phosphorylated c-Jun) and arginine vasopressin (AVP) hnRNA in the paraventricular nucleus (PVN). Simultaneous presentation of white or pink noise with restraint resulted in a significant attenuation of stress-induced c-Fos and JunB expression in the dorsal body of dorsal medial parvicellular subdivision (mpdd) of the PVN, as compared with restraint without noise. However, this presentation did not change phosphorylation of c-Jun and the plasma corticosterone level. Moreover, white noise presentation during restraint led to a reduction in the number of c-Fos- or JunB-expressing corticotropin-releasing hormone (CRH) neurons and the number of neurons expressing AVP hnRNA in the mpdd. Dual-histochemical labeling revealed co-expression of c-Fos and JunB, as well as JunB and AVP hnRNA in mpdd neurons. These data suggest that acoustic stimuli have an attenuation effect on the restraint-induced activation of neuroendocrine CRH neurons, resulting in the reduction in AVP production as an adaptation of HPA axis to repeated stress.

1. Introduction

As the final common pathway in stress response, the hypothalamic-pituitary-adrenal (HPA) axis drives the neuroendocrine response necessary for survival of organisms to cope with physical and psychological stressors. The neuroendocrine response begins with excitation of corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN), followed by the adrenocorticotropic hormone (ACTH) release from the anterior pituitary gland, and finally ends with the release of the adrenal glucocorticoid, corticosterone (CORT) in the rodents (Watts, 2005). Interestingly, recent studies have reported attenuation effects of an olfactory (Ito et al., 2009) or a gustatory stimulus (Martin and Timofeeva, 2010) on the HPA response to acute restraint stress. As for an acoustic stimulus, however, no study has addressed that effect on the stress response, although one reported no effect of loud noise as a novel heterotypic stressor in the habituation to repeated restraint stress (Masini et al., 2012).

In exploring for the possible effect of acoustic perception on restraint stress response, the most critical choice is that of the stimulus suitable for evaluation of the HPA response. In this sense, white noise may be appropriate, because it has been widely used in previous studies (Day et al., 2005; Spiga et al., 2009). The physiological

Abbreviations: HPA, hypothalamic-pituitary-adrenal; CRH, corticotropin-releasing hormone; PVN, paraventricular nucleus; ACTH, adrenocorticotropic hormone; CORT, corticosterone; AVP, arginine-vasopressin; IEG, immediate early gene; pc-Jun, phosphorylated c-Jun; AP1, activator protein 1; hnRNA, heterogeneous nuclear RNA; mRNA, messenger RNA; mpd, dorsal medial parvicellular part; mpdd, dorsal body of the mpd; mpdv, ventral tail of the mpd; dp, dorsal parvicellular part; mpv, ventral medial parvicellular part; pv, periventricular parvicellular part; pm, posterior magnocellular part.

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http://dx.doi.org/10.1016/j.neures.2014.11.010
0168-0102/

effect of white noise in rats seems to depend on its intensity in presentation. A loud (above 90 dB) white noise is often employed as a stressor to stimulate the HPA axis (Burow et al., 2005; Masini et al., 2012), while white noise at 80 dB has an overshadowing effect, possibly an anxiolytic effect (Zelikowsky et al., 2010).

Also of importance is what indicator is used to assess the HPA response, especially the magnitude of neuroendocrine CRH neuron excitation. Within the PVN, CRH neurons exist principally in the dorsal medial parvicellular part (mpd), and produce arginine vasopressin (AVP) as an ACTH co-secretagogue in the HPA axis (Engelman et al., 2004; Piet and Manzoni, 2011). When stressed, the neurons release these hormones and initiate expression of *crh* and *avp* genes, as well as immediate early genes (IEG) such as *c-fos*, *junB* and *c-jun* (Imaki et al., 1996; Kovács and Sawchenko, 1996; Kovács et al., 1998). In hormone production, CRH heterogeneous nuclear RNA (hnRNA) is more rapidly (within 30-min post-stress) expressed (Liu et al., 2011), whereas AVP hnRNA expression is delayed until 120 min after stress, concomitantly with or later than IEG protein expression (Kovács and Sawchenko, 1996). Although c-Fos does not seem to be a genuine transcription factor of *crh* gene, it has often been used as a cell marker that allows us to assess stress-induced CRH neuron excitation (Imaki et al., 1996; Stamp and Herbert, 1999; Chowdhury et al., 2000; Viau and Sawchenko, 2002; Girotti et al., 2006). Thus, the aim of the present study is to investigate whether or not acoustic stimulus presentation affects the HPA response in rats submitted to acute restraint stress, through semi-quantitative histochemical analysis for IEG and AVP expression as well as an assay of the plasma CORT level.

2. Materials and methods

2.1. Animals

All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was conducted with the approval of the Animal Experiment Committee of the University of Tsukuba, and subjected to the regulations of the university requirements regarding the care and use of laboratory animals for experimental procedures. All efforts were made to minimize the number of animals used and their suffering. Adult male Sprague-Dawley strain rats were purchased (Nippon Clea, Tokyo, Japan), weighing 290–310 g at the time of the experiment, and maintained under a 12-h light/dark cycle (light on 07:30) at 25 °C with food and water *ad libitum*. After they were obtained, the rats were acclimated by handling everyday until the experiment day, and housed individually for 4 days before experiment.

2.2. Restraint stress and presentation of acoustic stimuli

2.2.1. Stress paradigm

Each rat was placed into a transparent plastic tube (5 cm in inner diameter, 25 cm in length), having a front hole 1.3-cm in diameter to avoid accidental injury to the nose, and several lateral sound through-holes 0.5-cm in diameter. Immediately after introducing a rat into the tube, the rat was placed in a soundproof box (50 cm × 50 cm × 40 cm) interiorly at 1000 lux with background noise (50 dB).

2.2.2. Presentation of stimuli

Simultaneously with restraint, rats in the box were presented with either white, pink or blue noise played back with an audio player (20 Hz–20 kHz, XU-D400 MK II, JVC Kenwood Corporation, Japan), through two speakers (ASP-1000N Speaker, OHM Electric Inc., Japan) placed 36 cm above the box floor. The total sound pressure level within the box was always 70 dB during presentation of each noise when measured with a sound level meter (SM-325, As One, Osaka, Japan). White noise was generated using free software KSK Funcgen. Pink and blue noise were made from white noise using free software (SoundEngine Free). These noise included components of 20 Hz–20 kHz frequency bands.

We examined the following five experimental groups: (1) restraint stress without artificial noise (S group); (2) restraint stress plus white noise (W group); (3) restraint stress plus pink noise (P group); (4) restraint stress plus blue noise (B group); and (5) intact, namely without both restraint stress and artificial noise (I group). Rats of all but the groups except the I group were left in the box for 30 min with or without noise presentation, and then brought back to their home cages. The rats of I group were continuously housed in home cages before tissue sampling.

2.3. Tissue preparation

Two hours after the onset of restraint (the point in time when rats were placed in the box) of the S, W, P and B groups, as well as immediately before sacrifice of the I group, the rats were deeply anesthetized with sodium pentobarbital (75 mg/kg, i.p.) and transcardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.5). The hypothalamus was dissected out, postfixed in the same fixative overnight, and immersed in 30% sucrose in PB. The tissues were embedded and frozen, then cut on a cryostat (Cryostat HM560 MV, Carl Zeiss, Oberkochen, Germany) into 10-μm thick coronal serial PVN sections, and stored at −80 °C until analysis.

2.4. Immunostaining

In the analysis of IEG expression, c-Fos and JunB were examined in all groups ($n = 5$ each), while phosphorylation of c-Jun was studied in the S ($n = 5$), W ($n = 5$) and I ($n = 4$) groups. Sections were microwaved for 7 min, treated with 0.3% H_2O_2 in 20 mM phosphate-buffered saline (PBS, pH 7.5) for 30 min, and incubated in blocking reagent (20 mM PBS, 0.1% NaN_3, and 10% normal goat or horse serum) for 1 h. Immunostaining for c-Fos, JunB and phosphorylated c-Jun (pc-Jun) was performed with avidin-biotin-peroxidase complex (ABC) method as follows: the sections were incubated in either an anti-c-Fos antibody (1:7500, Poly6414, Biolegend, San Diego, CA) (24 h, 4 °C), an anti-JunB antibody (1:1000, sc-8051, Santa Cruz, Texas) (72 h, 4 °C), or an anti-pc-Jun (Ser63) antibody (1:100, #9261, Cell Signaling Technology, Danvers, MA) (24 h, 4 °C); then in either biotinylated (b-) goat anti-rabbit or horse anti-mouse IgGs (1:200, Vector Laboratories, Burlingame, CA) (1 h, 36 °C); and finally in ABC (Vectastain® ABC Elite Kit, Vector) (1 h, 36 °C). Immunoreaction was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Nacalai, Kyoto, Japan) in 20 mM PBS containing 0.003% H_2O_2 (8 min, 37 °C). After PBS washing, the sections were coverslipped with 50% glycerol, and photographed with an AxioCam MRc5 equipped on an Axioskop2 plus microscope (Carl Zeiss).

For dual immunostaining of c-Fos and CRH in the S and W groups ($n = 5$ each), c-Fos-stained sections were treated sequentially in H_2O_2, blocking reagent and an anti-CRH antibody (1:15,000, T-4037, Peninsula Laboratories, San Carlos, CA) (24 h, 4 °C). The sections were incubated with b-goat anti-rabbit IgG (1:200, Vector) and ABC (Vector) (1 h, 36 °C each). CRH immunoreaction was visualized using Vector® SG Substrate Kit (Takahashi et al., 2011).

For immunofluorescence staining, sections of S group ($n = 4$) were treated as follows: (1) the c-Fos antibody (1:5000) (24 h, 4 °C); (2) b-secondary antibody (1:200, Vector); (3) the JunB antibody (1:1000) (72 h, 4 °C); (4) streptavidin conjugated with a mixture of Alexa Fluor® 555 (1:200, Invitrogen, Carlsbad, CA) and Alexa Fluor® 633 goat anti-mouse IgG (1:200, Invitrogen) in 20 mM PBS with 10%

Please cite this article in press as: Sugimoto, K., et al., Presentation of noise during acute restraint stress attenuates expression of immediate early genes and arginine vasopressin in the hypothalamic paraventricular nucleus but not corticosterone secretion in rats. Neurosci. Res. (2014), http://dx.doi.org/10.1016/j.neures.2014.11.010