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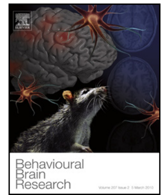

Research report

Chronic corticosterone administration facilitates aversive memory retrieval and increases GR/NOS immunoreactivity

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HIGHLIGHTS

- Chronic corticosterone improves emotional aversive learning.
- Chronic corticosterone increases GR immunoreactivity.
- Chronic corticosterone increases nNOS immunoreactivity.
- Chronic corticosterone increases GR/nNOS double immunostaining.

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ABSTRACT

Glucocorticoids are stress hormones that mediate the organism's reaction to stress. It has been previously proposed that the facilitation of emotional aversive conditioning induced by these hormones may involve nitric oxide-pathways. The purpose of the present study was to address this question. For that, male Wistar rats were surgically implanted with slow-release corticosterone (CORT) pellets (21 days) and tested in a step-down inhibitory avoidance task. Additional groups of animals were also submitted to the same treatment conditions and on the 21st day of treatment assayed for GR (glucocorticoid receptors)-nNOS (neuronal nitric oxide synthase) immunoreactivity (GRi-nNOSi) or measurements of plasma CORT. Results showed that CORT treatment induced facilitation of step-down inhibitory avoidance. This same treatment also significantly increased CORT plasma levels and GRi in the medial, basolateral and basomedial amygdala, in the paraventricular hypothalamic nucleus (PVN), in the ventral and dorsal dentate gyrus, in the ventral CA1 region and in the dorsal CA1 and CA3 regions. Furthermore, nNOSi and GRi-nNOSi were significantly increased by CORT treatment in the medial amygdala and basolateral amygdaloid complex, in the PVN, subiculum, in the dorsal CA3 region and in the ventral CA1 and CA3 regions. These results indicate that the facilitation of aversive conditioning induced by CORT involves GR-nNOS pathways activation, what may be of relevance for a better understanding of stress-related psychiatric conditions.

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1. Introduction

Stress can be described as any challenge to the homeostatic balance that demands an adaptive reaction of the subject [1]. One of the main physiological alterations that follows the stress response, is the activation of the hypothalamic–pituitary–adrenal (HPA) axis [2]. Activation of the HPA axis is controlled by the release of corticotropin-releasing factor (CRF) and arginine–vasopressin (AVP) from the paraventricular hypothalamic nucleus (PVN). CRF and AVP stimulate adrenocorticotropic hormone (ACTH) release, which in turn triggers the release of glucocorticoids (corticosterone and cortisol) from the adrenal cortex.

Glucocorticoids are, therefore, hormones involved in the adaptation of the organism to stressors. After being released, these so-called stress hormones can enter the brain and interact with receptors expressed in regions that mediate HPA axis activity and the organism's responses to stress. There are two types of glucocorticoid receptors: mineralocorticoid (MRs) and glucocorticoid receptors (GRs). Glucocorticoids possess 6-10 times more affinity to MRs [3]. Thus, while MRs seem to mediate the effects of circadian levels of glucocorticoids, GRs are the main receptors activated by glucocorticoids in response to stress or to severe variations in glucocorticoids levels [3].

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GRs are highly concentrated in the pre-frontal cortex, hippocampus and amygdala [3], brain regions involved with emotional conditioning. In fact, it has previously been shown that treatment with glucocorticoids, aside from exerting anxiogenic effects, improves learning and memory associated with aversive stimuli [4–9].

In the last years, a role for nitric oxide (NO) in the modulation, in particular, of emotional aversive conditioning, has also been proposed [10–12]. NO is produced by neuronal nitric oxide synthase (nNOS), in response to the activation of NMDA receptors by glutamate [13,14] and functions as a retrograde neuronal messenger, facilitating synaptic plasticity, long-term potentiation (LTP) and the formation of long-term memories. It has been shown that the pharmacological blockade of NO signaling in rats impairs contextual [11] and cued [10] fear learning. Also, knockout mice with targeted mutation of the nNOS gene showed severe impairment of contextual fear learning [12]. Interestingly, in this same study, an association between increases in plasma corticosterone levels and in the magnitude of contextual freezing responses was also observed.

In fact, previous evidences indicate that glucocorticoid hormones may increase glutamate-mediated neurotransmission and alter NO synthesis/release in the Central Nervous System [15,16]. Also, moderate to high levels of nNOS have been found in regions related to the modulation of fear/anxiety [17], such as the hippocampus and the amygdala. Furthermore, stress-exposure seems to induce the activation of NO neurons in the amygdala, hypothalamus, and periaqueductal grey matter [18], structures that integrate the so-called “Brain Aversive System” [19]. There is also evidence that NO affects the activity of the HPA axis [20,21], exerting both stimulatory and inhibitory effects depending on the brain area involved and the type of stress [for a review, see 21].

Nevertheless, to the best of our knowledge the relationship between NO-pathways activation in different brain regions and glucocorticoid-induced improvement of aversive conditioning has not been thoroughly investigated. The purpose of the present study was to investigate the effect of chronic corticosterone (CORT) treatment (21 days, slow-release pellets) on memory retrieval and GR-nNOS immunoreactivity (GRi-nNOSi) in the amygdala, hippocampus and PVN. In a previous study we have shown that this same treatment procedure induces facilitation of the avoidance task of the elevated T-maze model of anxiety, an anxiogenic effect [9].

For behavioral measurements, a step-down inhibitory avoidance task was chosen. It has been previously shown that with this task, aversive conditioning is rapidly acquired [22,23]. Also, the model has been well studied, in terms of its behavioral and biochemical basis [24–26].

In addition, an independent group of animals was subjected to measurements of plasma CORT on the 21st day of treatment.

2. Materials and methods

2.1. Subjects

Male Wistar rats (CEDEME, Universidade Federal de São Paulo, Brazil), weighing 280–320 g at the beginning of the experiment, were housed in groups of 5–6 per cage. After surgery, animals were housed in pairs in Plexiglas-walled cages until testing. Room temperature was controlled (22 ± 1 °C) and a light–dark cycle was maintained on a 12-h on–off cycle (07:00–19:00 h lights on). Food and water were available all throughout the experiments. The study was approved by the Ethical Committee for Animal Research of the Federal University of São Paulo (number 0064/12) and was performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behavior, which are based on the conditions stated by the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, 1996).

2.2. Apparatus – step-down inhibitory avoidance

The apparatus was a 40 cm × 25 cm × 20 cm box with a 5-cm high, 8-cm wide and a 25-cm long platform on the left end of a grid composed of a series of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart. Luminosity at the center of box was 60 lux. After each experimental session, the apparatus was cleaned with a 10% ethanol solution.

2.3. Surgery

One day after their arrival, rats were anaesthetized with an IP injection of ketamine hydrochloride (80 mg/kg; Agribrands, Brazil) and xylazine (10 mg/kg; Agribrands, Brazil). A 1-cm incision through the skin of the upper back of the animal was made and a 2-cm subcutaneous pocket was open horizontally with a pair of forceps to allow the implant of a slow-release CORT pellet (200 mg, 21-day release, Innovative Research of America, USA – this procedure induces the daily release of approximately 10 mg/kg of CORT). The pellet was inserted into the pocket and the incision sutured. Sham rats were subjected to the same surgical procedure except that a pellet was not implanted. To prevent infections, at the end of the surgery, all animals were injected (IM) with 0.2 ml of a pentabiotic preparation (Pentabiotico Veterinário Pequeno Porte; Forte Dodge, Brazil).

2.4. Procedure

Training was performed on the 20th day of treatment. For that, animals (*N* = 8–9) were placed on the platform of the step-down inhibitory avoidance apparatus and the latency to step down placing the four paws on the grid was measured. In training sessions, after stepping down onto the grid, animals received a 2-s, 0.8-mA footshock. This procedure induced an escape response back to the platform. Animals that did not remain for at least 50 s on the platform after shock were excluded. Twenty-four hours later animals were tested. In test sessions, no footshock was given and the step-down latency was cut off at 300 s.

2.4.1. Double staining immunohistochemistry: GR and nNOS

On the 21st day of treatment, independent groups of CORT and sham animals (*N* = 6) were anesthetized with ketamine/xylazine as described above and perfused with 100 ml of 0.9% saline for 1 min, followed by 500–700 ml of 4% paraformaldehyde and H_2O at 4 °C, pH 9.5, for approximately 25 min. The brains were post-fixed for 1 h in the same fixative at 4 °C and stored in phosphate buffered saline (PBS) plus 20% sucrose, at 4 °C. Regularly spaced series (5 × 1-in-5) of 30 μm-thick frozen sections were cut in the frontal plane, collected in ethylene glycol-based cryoprotectant solution and stored at −20 °C for later determination of double staining.

For identification of neurons with immunoreactivity for the GR receptor (GR-ir) polyclonal antibody raised in rabbits against GR (anti-GR, 1:1000; Abcam, Cambridge, United Kingdom) was used. Immunohistochemistry was performed using a conventional avidin–biotin immunoperoxidase protocol [27] and Vectastain Elite reagents (Vector Laboratories, USA). Tissue was pretreated with hydrogen peroxide (0.3%; Sigma, USA) before addition of the primary antibody to squelch endogenous peroxidase activity. The reaction with diaminobenzidine-DAB (0.05%; Sigma, USA) was performed for 40 s and was amplified using nickel ammonium sulfate,

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