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Differential immunoreactivity of glucocorticoid receptor and GABA in GABAergic afferents to parvocellular neurons in the paraventricular nucleus

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HIGHLIGHTS

- Glucocorticoid may directly act on GABAergic neurons through GR.
- Different expressions of GABA and GR in anterior and medial parvocellular regions in the PVH.
- GABA mediates an inhibitory compensatory mechanism to modulate the HPA axis.
- Anterior periventricular nucleus relays an indirect inhibition to parvocellular neurons and HPA.

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ABSTRACT

We assessed the distribution of glucocorticoid receptors (GR), GABA (γ-aminobutyric acid) neurons and co-localization of GR in GABA-positive neurons for four hypothalamic sources of GABAergic projections to the parvocellular neurons in the paraventricular nucleus (PVH) from normal, sham-surgery and adrenalectomized male rats subjected to intraperitoneal injections of saline or dexamethasone. Blood samples were collected to measure corticosterone by radioimmunoassay. The distribution of GR, GABA-positive neurons and co-localization of GR in GABA-positive neurons were analyzed by immunofluorescence in sections from the paraventricular nucleus (PVH). In intact and sham rats, dexamethasone induced expression of GABAergic neurons in the regions of the anterior periventricular nucleus (PVa) coincident with anterior (PVHap) and medial (PVHmp) parvocellular subdivisions of thePVH. However, the co-expression of GR in GABAergic neurons was found only in the region of the PVa coincident with PVHmp. These findings confirm that glucocorticoids may directly act on GABAergic neurons through GR. PVHap and PVHmp present differentiated patterns of GABA and GR expression between then. The co-localization of GR in GABA-positive neurons in the region of the PVa coincident with PVHmp demonstrates a critic importance of this region to control the hypothalamus–pituitary–adrenal axis through GABAergic mediation.

1. Introduction

Stress activates the hypothalamus–pituitary–adrenal axis (HPAa), increasing plasma corticosterone that exerts negative feedback control over the HPAa through glucocorticoid receptors (GR) in the pituitary and the hypothalamic paraventricular nucleus (PVH) [6–8,17,23]. Parvocellular neurons in the PVH also receive neuronal inputs from the brainstem and limbic areas [12,23] through a shell of GABAergic interneurons surrounding the PVH [13,21]. This GABAergic input is a strong determinant of HPAa activity [5]. The GABAergic influence for corticosterone secretion under stress is modulated by GABA-A and more specifically GABA-B receptors [19]. Previous studies have identified four main sources of GABAergic afferents to the anterior (PVHap) and medial (PVHmp) parvocellular region of the PVH: anterior hypothalamic area (AHA), anterior periventricular nucleus (PVa), peri-supraoptic area (SO) and anterior perifornical area (APF) [21]. We assessed the distribution of GR, GABA-positive neurons and GR/GABA neurons for these sources in accordance with their coincidence with the PVHap (AHA, PVa and SO) and the PVHmp (PVa, SO and APF).

Abbreviations: HPAa, hypothalamus–pituitary–adrenal axis; PVHap, anterior parvocellular region of the PVH; PVHmp, medial parvocellular region of the PVH; PVa, anterior periventricular nucleus; SO, peri-supraoptic area; APF, anterior perifornical area.

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2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250 ± 10 g were housed under 12 h:12 h light–dark cycle (lights on 07:00 a.m.), at 23 ± 1 °C with free access to food and water. All protocols in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (USA) were previously approved by the Ethics Committee for Experiments on Animals of the Medical School of Ribeirão Preto, University of Sao Paulo.

2.2. Experimental design and surgical procedures

At the end of the fifth day of habituation to housing conditions: two groups of intact animals received (19:00 h) an intraperitoneal injection of either saline (0.15 M NaCl; 0.1 mL/100 g b.w., intact-saline group) or 2.5% dexamethasone (0.1 mL/100 g, Sigma-USA, intact-Dexa group); other two groups subjected to either sham surgery or bilateral adrenalectomy (ADX) under tribromoethanol 2.5% (1 mL/100 g, Aldrich, USA) anesthesia. This surgery was performed via dorsal incisions to gain access to adrenal and remove it or exposed but not excise it (control). Following, the animals received analgesic (50 ug/rat, Benamine®, Schering-Plough, Brazil) and antibiotic (0.1 mL/rat, Pentabiotic®, Fort Dodge, Brazil). Adrenalectomized animals got access to oral 0.9% saline.

At fifth day of surgical recovery (19:00 h), the animals were divided in two groups that received intraperitoneal injections of either saline (0.15 M NaCl; 0.1 mL/100 g b.w., sham-saline group/ADX-saline group) or 2.5% dexamethasone (0.1 mL/100 g, sham-Dexa group/ADX-Dexa group). After twelve hours (at sixth day, 7:00 a.m.), the animals were anesthetized and blood was drawn via an aortic puncture. Plasma samples were stored at ±20 °C until to measure corticosterone. Following, approximately 30 mL of 0.01 M phosphate-buffered saline (PBS, pH 7.4) and 300 mL of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) were perfused through the ascending aorta. Brains were removed, immersed in 30% sucrose in PBS (48 h at 4 °C), frozen in cooled isopentane and stored at −70 °C until sectioning.

2.3. Immunohistochemistry

2.3.1. Tissue preparation

Serial coronal sections (20 μm-thick) (0.9–2.1 mm posterior to bregma) through the anterior (PVHap) and medial (PVHmp) portions of the PVH were cut on a cryostat at −20 °C, and divided into six series of eight sections each. The span between two sections was of 140 μm. We located the PVHap in order to analyze the AHA, PVa and SO coincident with PVHap and the PVHmp in order to analyze the PVa, SO and APF coincident with PVHmp. The slices were thawed and mounted on slides covered with gelatin. Brain sections rinsed in PBS were placed into 0.1 M glycine in PBS (20 min) to remove excess aldehydes. After washes in PBS, sections were blocked (1 h) in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA) and incubated with primary antibodies.

2.3.2. Immunofluorescence

GABA (GAD_{67}) and GR – the synthesis of GABA from glutamate in the brain depends on two isoforms of the enzyme glutamate decarboxylase (GAD). GAD_{65} is most important to synthesis the vesicular pool. GAD_{67} chosen as a marker of GABA neurons is quantitatively more important for GABA synthesis. It participates in the cytoplasmic and vesicular pools [27,28]. The sections were incubated overnight (20 h) at 28 °C with mouse monoclonal anti-GAD_{67} antibody (1:5000, MAB5406, Chemicon, UK) and goat anti-glucocorticoid receptor antibody (1:100, sc-1004, Santa Cruz Biotechnology, USA) diluted in PBS containing 0.1% Triton X-100 and 1% BSA. Following PBS washes, they were incubated (1 h) in a cocktail of fluorescent secondary antibodies: donkey anti-mouse IgG labeled with Alexa Fluor 488 (Molecular Probes, USA) for GABA and donkey anti-rabbit IgG labeled with Alexa Fluor 594 (Molecular Probes, USA) for GR. The slides were washed with PBS and coverslipped. The omission of the primary antibody resulted in no labeling. For each double-label combination, primary antibodies were titrated to determine the maximum dilutions that provided robust signals and a minimum of nonspecific staining (images not shown).

2.3.3. Tissue analysis

Tissue sections were examined using an Axioskope 2 Plus microscope (Carl Zeiss, Germany) with a 20× objective and a Zeiss AxioCam digital camera (Carl Zeiss, Germany) to capture the images. One series of tissue sections through the rostro–caudal extent of the PVH was used per animal (n = 5–9 per group). The series of sections from each brain were compared according of anatomical atlas [22] to define the boundaries of AHA, PVa, SO, APF and different PVH subdivisions. We used the same size of the optical field for all of the photomicrographs. All GABA neurons and GR-immunoreactive (-ir) neurons within the section were quantified bilaterally. Next, sections scored for the co-staining of GABA and GR were re-examined using confocal microscopy (TCS SP2 AOBS, Leica Microsystems, Germany). The quantitative assessment of GABA neurons, GR-ir neurons, and double-labeled cells was obtained from captured microscope images using Image J software (NIH, Bethesda, MD, USA).

2.4. Hormone measurement

Plasma corticosterone was measured by a radioimmunoassay that required extraction with ethanol and used the following reagents: corticosterone H^3 (NEN Life Sciences Products, USA); specific antibody and standard reference (Sigma, USA). All samples from the same experiment were measured in the same assay. The intra-assay error was 5% and the minimum detectable dose was 0.8 ng/mL.

2.5. Statistical analysis

The mean ± SEM of GABA neurons, GR-ir neurons and percent of dual-labeled cells was calculated per region in groups of six animals. Two-way ANOVA followed by the Bonferroni post hoc analyses for multiple comparisons was used to evaluate the number of GR-ir neurons. The number of GABA neurons and the percent of dual-labeled cells were analyzed by Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of adrenalectomy and dexamethasone on plasma corticosterone

Plasma corticosterone was higher in intact-saline (62.2 ± 3.5 ng/mL) and sham-saline (68.9 ± 2.9 ng/mL) groups than intact-Dexa (3.3 ± 0.5 ng/mL) and sham-Dexa (4.8 ± 0.9 ng/mL) groups.

Plasma corticosterone was lower in ADX-saline group (2.6 ± 2.5 ng/mL) than intact-saline group (62.2 ± 8.7 ng/mL), but it was not different of ADX-dexamethasone group (6.7 ± 3.0 ng/mL). Plasma corticosterone decreases significantly after treatment and surgery. The interaction between treatment and surgery reached