InVet. 2015, 17 (2): 187-194
ISSN 1514-6634 (impreso)
ISSN 1668-3498 (en línea)

Determination of the neuropeptide Y in the adrenal cortex of stressed pregnant rats

Determinación del neuropéptido Y en la corteza adrenal de ratas gestantes estresadas

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RESUMEN

El estrés crónico durante la gestación puede alterar diversos factores y mecanismos que mantienen la homeostasis del organismo. El neuropéptido Y tiene una función ansiolítica y participa en la modulación de la respuesta al estrés. El objetivo fue determinar los efectos del estrés crónico sobre los niveles plasmáticos de corticosterona y estrógenos, y sobre la inmumarcación del neuropéptido Y en la corteza adrenal de ratas gestantes. El estrés por inmovilización fue aplicado desde el cuarto día de la gestación hasta el sacrificio a los 12, 17 y 21 días de la gestación. Se combinaron técnicas de inmunomarcación, radioinmunoanálisis, análisis estereológico y cuantificación de imágenes. En las ratas estresadas gestantes se presentó un incremento significativo en los niveles plasmáticos de corticosterona a los 12, 17 y 21 días, de estrógenos a los 12 y 17 días y una disminución del índice de neuropéptido Y en los días 12 y 17. Se puede concluir que el incremento de los niveles plasmáticos de corticosterona y estrógenos ejerce un efecto inhibitorio sobre la inmunomarcación del neuropéptido Y adrenal a los 12 y 17 días de la gestación.

Palabras Claves: (corteza adrenal), (estrés crónico), (neuropéptido Y), (ratas gestantes), (corticosterona).

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Recibido: 01/02/2015
Aceptado: 04/09/2015

SUMMARY

Chronic stress during pregnancy can alter many factors and mechanisms that maintain the homeostasis of the organism. Neuropeptide Y has anxiolytic functions and is involved in modulating the stress response. The aim was to determine the effects of chronic stress on the plasmatic corticosterone and estrogen levels and the immunolabeling of the neuropeptide Y in the adrenal cortex of pregnant rats. The immobilization stress was applied from the fourth day of gestation until the sacrifice at days 12, 17 and 21 of the gestation. Immunostaining techniques, radioimmunoassay, stereological analysis and quantification of images were used. In the stressed pregnant rats significant increase in the plasmatic corticosterone levels at 12, 17 and 21 days and in the plasmatic estrogen levels at 12 and 17 days were observed. On the other hand, a decrease in the neuropeptide Y index at 12 and 17 were observed. We can conclude that the increase in the plasmatic corticosterone and estrogen levels exerts an inhibitory effect on the adrenal neuropeptide Y immunostaining at 12 and 17 days of gestation.

Keywords: (adrenal cortex), (chronic stress), (neuropeptide Y), (pregnant rats), (corticosterone).

INTRODUCTION

The exposure to stressful events during different periods such pregnancy has been associated with anxiety disorders, depression, and hormonal imbalances[11]. Acute stress is necessary for the organism to cope with short-term real or imaginary threats. However, chronic stress maintained for long periods, result in a disrupted immune response, neuronal remodeling and memory deficits which resembles some of the symptoms observed in depressed subjects[26, 11, 20]. Most of these processes are associated with alterations on the adrenal-pituitary-hypothalamus axis (HPA). The HPA axis response against different stressors decreases progressively with the advancing gestation. However, it was demonstrated that in situations of chronic stress, pregnant rats could not elicit any adaptation and consequently the corticosterone (CORT) levels remain elevated[21].

Other hormones affected by stress are prolactin (PRL) and estrogen[2]. These hormones increase proportionally with the intensity of the stressful stimuli and it has been demonstrated that the adrenal cortex has specific receptors for these hormones [12, 17]. Also, the neuropeptide Y (NPY) is an anxiolytic peptide that is involved in the modulation of the response to stress and in psychiatric disorders[1]. According to different stimuli that receives, the adrenal gland acts as a potent effector of cell responses[15]. The neural circuits that use the NPY as a signal, are activated when the organism through stressful situations to regulate their homeostasis[28].

In chronic stressed rats, an increase in the plasmatic NPY levels accompanied by the synthesis and secretion of the adrenal NPY was determinate[4]. Both the central and the adrenal NPY interact with the HPA axis; in the central nervous system, the expression and release of NPY increase the vulnerability to a stressor[14].

Currently, there are few studies of models *"in vivo"* under chronic immobilization stress conditions during the gestation[22, 8, 5]. In previous studies, we found that chronic immobilization (IMO) stress applied during the second half of pregnancy produce an increase in the number of immunolabeled NPY neurons in fetal brains, acting as an endogenous alarm system in prenatal

stress conditions[8] but the effect of the chronic stress on the adrenal NPY in pregnant rats is still unknown.

The aim of this study was to determine the effects of chronic stress on the plasmatic CORT and estrogen levels and on the immunolabeling of the NPY in the adrenal cortex of rats at 12, 17 and 21 days of the gestation.

MATERIALS AND METHODS

Animals and laboratory conditions

Thirty Wistar 200–300 g, 90–120-day-old female rats were used for the experiment. Animals were maintained under controlled laboratory conditions at 20 ± 2 °C, with a 12/12 h light/dark cycle and food and water available *ad libitum*. The installations were adapted to the requirements of the layout 6344/96 of the Administración Nacional de Medicamentos, Alimentos y Tecnología Médica, Argentina. For the experiments, the conclusions and recommendation on the Reduction, Refinement and Replacement for the laboratory animals were followed Russell and Burch (1959)[25]. All experiments were conducted according to the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals (NIH Nº 85-23, 1985, http:// www.nih.gov / sigs / bioética). Rats were cycled using colpocytograms and were mated during pro-estrous with a male of the same strain. Pregnancy day zero was defined by the presence of spermatozoa in the vaginal fluid. Pregnant rats were separated into control (CR) and stressed (SR) groups.

Experimental treatment

Experimental rats were subjected to sessions of immobilization stress in a tubular clamp made of perforated plastic. The clamp was anchored to a wood base and padded for animal comfort. Each immobilization session lasted 45 min. Rats were subjected to these sessions every other day beginning with the fourth day of gestation to prevent the embryo resorption and until the day before the sacrifice. The method was adapted from biotherius of the National University of Rio Cuarto under ethical rules for manipulation of experimentation animals. Five animals for each group and for each gestational stage were sacrificed by decapitation without anesthetic at days 12, 17 and 21 of the gestation. After decapitation, the adrenal glands of each pregnant rat were removed, fixed in 10% buffered formaldehyde for 12 h and processed according to the conventional histological technique. Alternate 5 μ m thick sections were cut using a Reichert-Young 2065 microtome and mounted with Vectabond adhesive (Vector Laboratories, Inc., Burlingame, CA). Three microscope slides per animal, each with three sections of the same gland were analyzed. Glomerular, fascicular and reticular zones of the adrenal cortex were identified by their histological characteristics.

Corticosterone detection by radioimmunoassay

After the sacrifice of the animals of both groups in all days of gestation analyzed, the blood was collected in tubes with sodium heparin. The samples were centrifuged at 3000 rpm for 15 minutes at 4 °C. Then, the plasmatic fraction was removed and stored at -20 °C until the corresponding analysis. An anti-corticosterone antiserum (monoclonal, rabbit; Cardiff, UK) and a pattern of CORT were used (4-pregnene-11, 21-diol-3, 20 dione, Sigma, Boston, MA, USA). The intra and interassay coefficients of variation were 8.8%.

Adrenal NPY detection

Histological sections were de-waxed and hydrated in phosphate buffered saline, pH 7.4 (PBS). A 20% solution of hydrogen peroxide was used to block endogenous peroxidases. Nonspecific antibodies were blocked with horse serum (Vector Laboratories, Inc., Burlingame CA) for 30 min. The sections were incubated with an anti-NPY primary polyclonal antibody (1:500, RXB, Merck Milipore, Billerica, USA) overnight in a wet chamber, followed by a biotinylated secondary antibody and the avidin-biotin-peroxidase complex, both for 1 hour in

a wet chamber (Vectastain ABC Elite Kit 6200; Vector Laboratories, Inc., Burlingame CA). The sections were revealed with diaminobenzidine (DAB) (Vector Laboratories, Inc., Burlingame CA). The negative control was the reaction applied to the adrenal cortex without the primary antibody.

Stereological analysis

For each adrenal cortex section, 10 to 12 fields of the glomerular, fascicular and reticular zones were scanned to build an image data base for the CR and SR for each gestational day analyzed. A Zeiss Axiophot microscope with a digital camera and an associated software were used (video-Printer Sony 3000 and Scion stereological image analysis software). Qualitative analysis of the images was performed comparing the distribution of the NPY positive and negative cells in the zones of the adrenal cortex of CR and SR at 12, 17 and 21 days of the gestation. The NPY index (I_{NPY}) was estimated as the ratio of the NPY positive and negative cells multiplied by 100. The morphometric analysis was carried out using the Image J software (NIH). Data were transferred to a data base in Excel software for further statistical analyses[16].

Statistical analysis

One- and three-way ANOVAs were applied. For the comparison of the significant differences between zones or days, we realized a post hoc test (DGC test). Statistical analyses were performed using the InfoStat and SAS 9.1 softwares. Differences were considered significant at $p < 0.05$[24].

RESULTS

The results obtained revealed a significant increased in the plasmatic levels of the CORT in the SR in relation to their controls on the same day of gestation in all ages studied (Table 1).

In the SR, the plasmatic estrogen levels were increased on days 12 (p=0.007) and 17 (p=0.032) of the gestation, with respect to CR. On day 21 of the gestation, no significant differences between groups (Table 2) were observed.

The pattern of distribution of the NPY positive cells was similar in the adrenal cortex of the CR and SR in all gestation days analyzed. NPY positive cells were observed in the reticular and fascicular zones, while in the glomerular zone, NPY positive cells were not observed (Fig. 1).

The three-way ANOVA revealed a triple interaction (p=0.02) in the I_{NPY} between zones, groups and gestation days analyzed. The (DGC) revealed in the fascicular zone a significant decrease of the I_{NPY} in the SR in relation to the CR at 12 days of the gestation (p<0.05), whereas at days 17 and 21 of the gestation, no significant differences were observed. Furthermore, in the reticular zone a significant decrease in the I_{NPY} in the SR in relation to the CR was observed at day 17 of the gestation (p<0.05), while at days 12 and 21 of the gestation, no significant differences were presented (Fig. 2).

Table 1. Plasmatic corticosterone levels in pregnant rats. Data are expressed as mean ± SD. Significant differences between group are shown with asterisks (* mean p<0.05 and **mean p<0.001).

DAYS	CR	SR
12	4.03 ± 0.91	11.36± 0.65**
17	5.70 ± 0.96	10.25 ± 1.25 *
21	6.27 ± 0.86	14.02 ± 1.7 **

Table 2: Plasmatic estrogen levels in pregnant rats. Data are expressed as mean ± SD. Significant differences between group are shown with asterisks (* mean p<0.05 and **mean p<0.001).

DAYS	CR	SR
12	43.08 ± 1.51	72.06± 1.84**
17	61.58 ± 0.57	86.24 ± 0.78 *
21	54.23± 0.72	57.97± 1.21

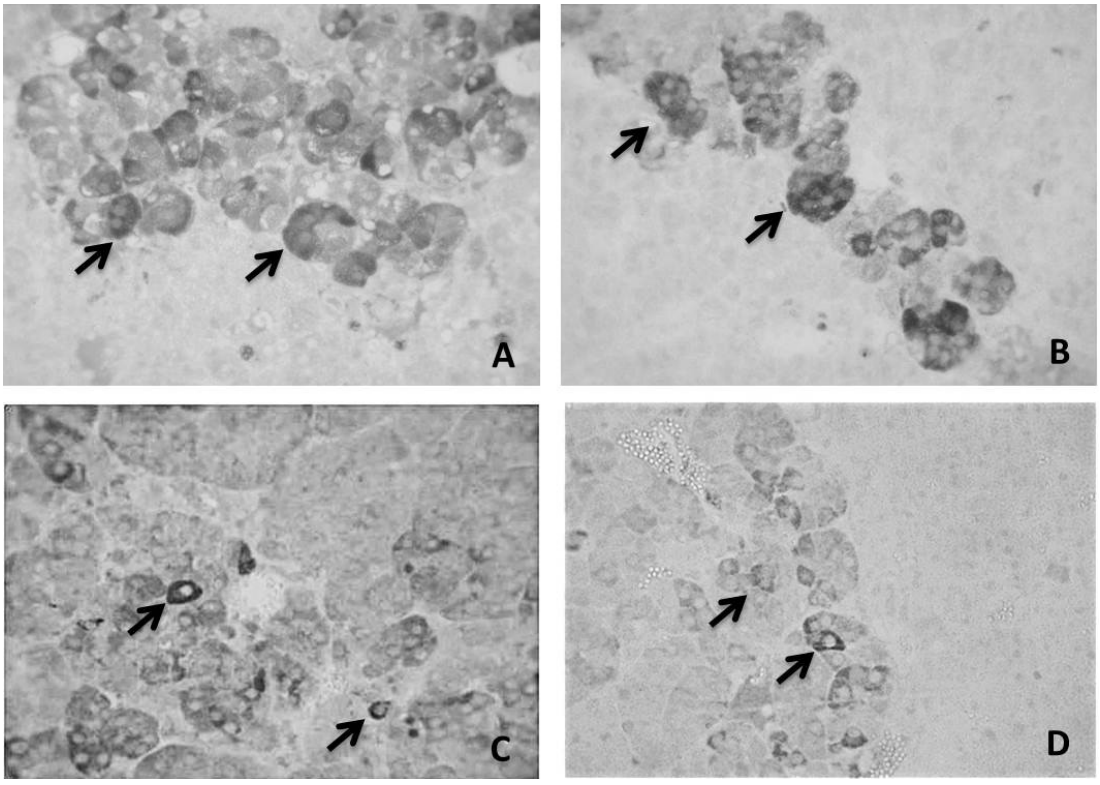

Figure 1. Optical microphotography of the adrenal reticular zone with positive NPY cells (x 400) A: Control rat at gestation day 12. B: Stressed rat at gestation day 12. C: Control rat at gestation day 17. D: Stressed rat at gestation day 17. The arrows indicate positive NPY cells.

DISCUSSION

The adrenal gland responds continuously to repeated and/or transient stimuli, such as stress. The adrenal NPY is involved in the adaptive response to stress; in these situations, changes in the levels of the NPY mRNA and the peptide in the adrenal gland[29] is produced. According to Hinson *et al.* (1995)[13], the adrenal NPY concentration is inversely proportional to the intensity of the stress; a higher intensity of the stress, produce a decrease in the levels of NPY. Accordingly, in this study we found that adrenal NPY showed a decrease in the fascicular and reticular zones in stressed rats at 12 and 17 days of gestation. Also, in stressed rats a decrease in the cell proliferation was observed in the fascicular and reticular zones in the same days of gestation[5].

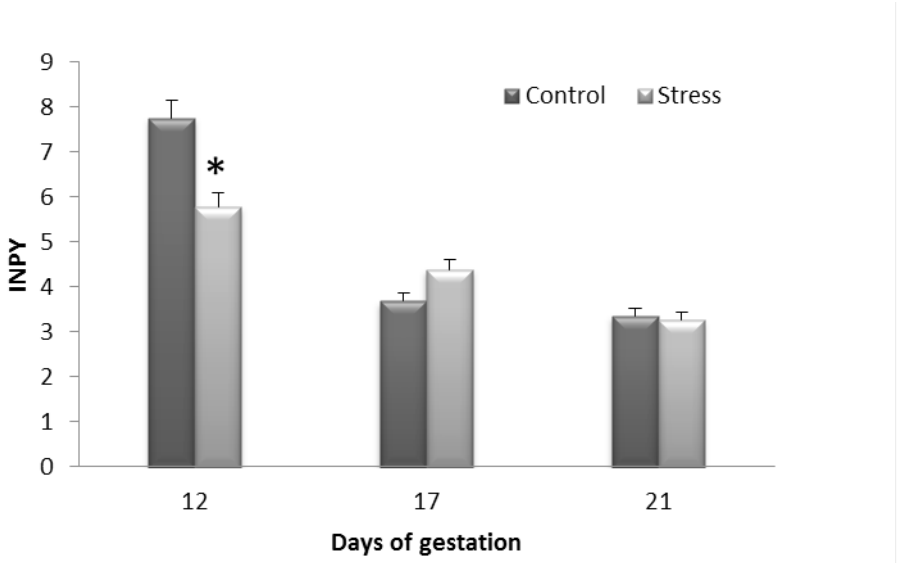

Figure 2. NPY index in the fascicular zone of the adrenal cortex of pregnant rats. Significant differences are indicated with asterisks (* mean p<0.05).

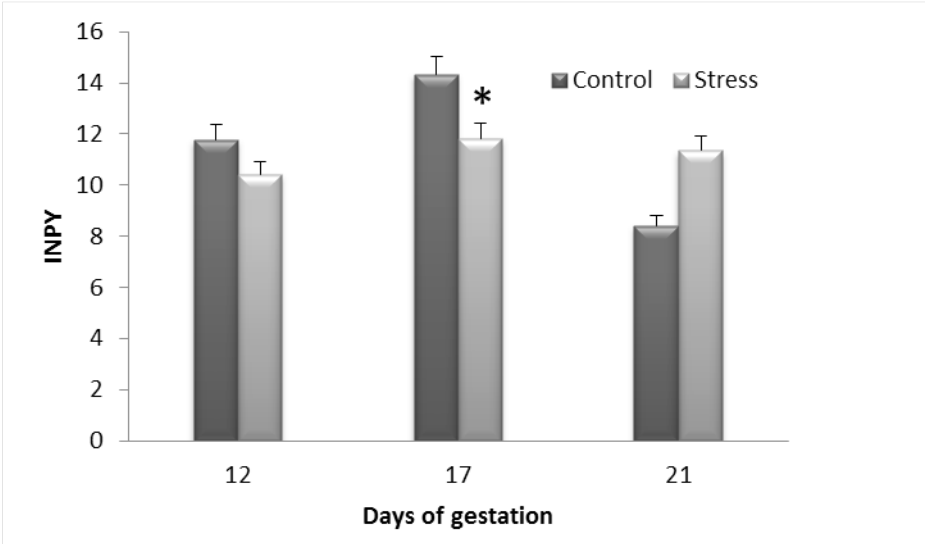

Figure 3. NPY index in the reticular zone of the adrenal cortex of pregnant rats. Significant differences are indicated with asterisks (* mean p<0.05).

Under stress conditions, it was found that the PRL causes an increase in the plasmatic levels of the CORT[18], which could have an indirect effect in the reducing I_{NPY} on days 12 and 17 of gestation.

Recent studies have demonstrated that estrogens act by inhibiting the release of the NPY [10, 9] In the experimental model applied in this study we observed a greater concentration of the plasmatic estrogen levels at days 12 and 17 of the gestation in stressed rats compared to their controls, which might be related to the decreased adrenal I_{NPY}.

The increase in the adrenocorticotropine hormone levels causes a decrease in the adrenal NPY levels, showing that the HPA could regulate the release of NPY, through the release of glucocorticoids[14]. In stressed rats we

observed an increase in the plasmatic CORT levels in all days of the gestation analyzed, which would be associated with the decreased in the adrenal I_{NPY} on days 12 and 17. Also, at day 21 of the gestation, the plasmatic levels of CORT remained high; however, we observed no significant differences in immunostaning pattern of the NPY between groups. This could be due to the masking of the glucocorticoid receptors in response to the sustained increase of the CORT levels[19].

We conclude that chronic exposure to stress with the consequent hyperactivity of the HPA and the increased of the plasmatic CORT and estrogen levels, produce changes in the expression of the adrenal NPY. These factors could be involved in the increasing susceptibility to psychiatric disorders such as depression in chronic stress conditions.

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