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Genistein stimulates the hypothalamo-pituitary-adrenal axis in adult rats: morphological and hormonal study

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Summary. Genistein, the soy isoflavone structurally similar to estradiol, is widely consumed for putative beneficial health effects. However, there is a lack of data about the genisteins' effects in adult males, especially its effects on the hipothalamo-pituitary-adrenal (HPA) axis. Therefore, the present study was carried out to investigate the effects of genistein on the HPA axis in orchidectomized adult rats, and to create a parallel with those of estradiol. Changes in the hypothalamic corticotrophin-releasing hormone (CRH) neurons and pituitary corticotrophs (ACTH cells) were evaluated stereologically, while corticosterone and ACTH levels were determined biochemically. Orchidectomy (Orx) provoked the enlargement ($p<0.05$) of: hypothalamic paraventricular nucleus volume (60%), percentage of CRH neurons (23%), percentage of activated CRH neurons (45%); pituitary weight (15%) and ACTH level (57%). In comparison with Orx, estradiol treatment provoked the enlargement ($p<0.05$) of: percentage of CRH neurons (28%), percentage of activated CRH neurons (2.7-fold), pituitary weight (131%) and volume (82%), ACTH level (69%), the serum (103%) and adrenal tissue (4.8 fold) level of corticosterone. Clearly, Orx has induced the increase in HPA axis activity, which even augments after estradiol treatment. Also, compared to Orx, genistein treatment provoked the enhancement ($p<0.05$) of: percentage of activated CRH neurons (2.3-fold), pituitary weight (28%) and volume (21%), total number of ACTH cells (22%) ACTH level (45%), the serum (2.6-fold) and adrenal tissue (2.8 fold) level of corticosterone. It can be concluded that an identical tendency, concerning the HPA axis parameters, follows estradiol and genistein administration to the orchidectomized adult rats.

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Introduction

Isoflavones are plant derived compounds that are structurally and/or functionally similar to mammalian estrogens and their active metabolites (Whitten et al., 1997). Namely, featuring the polyphenolic, steroid core-like structure, isoflavones bind to estrogen receptors (ER) and affect the gene expression (Wuttke et al., 2007). There are two major ER subtypes in mammals, ERα and ERß. Isoflavones have a higher binding affinity for the ERß than ERα, also their binding affinity for ER is much lower in comparison to 17ß-estradiol (Setchell and Adlercreutz, 1988). Genistein, the most abundant soy-bean derived isoflavone, shows estrogenic and antiestrogenic effects as well as some enzyme binding/inhibiting, independent of ERs (Adlercreutz and Mazur, 1997; Mesiano et al., 1999).

A myriad of published articles consider the effects of isoflavones on female reproductive system and related organs; on the contrary, their effects on different organic systems in adult males have received considerably less attention. The existing literature data mostly elaborate some beneficial effects of isoflavones, as nutritional supplements or alternative therapy, in ageing males i.e. on prostate cancer, osteoporosis and depression, using specific animal models and through limited clinical investigations (Anderson et al., 1999; Setchell, 2001; Sapronov and Kasakova, 2008; Kageyama et al., 2010). On the other hand, their effects on the stress response generating hypothalamo-pituitary-adrenal (HPA) axis have great potential scientific importance. Herein, we examine the effects of genistein on the HPA axis of adult male rats, whose specificity is reflected through preserved central regulation of the stress hormone secretion. Since the HPA axis has far-reaching

psychophysiological significance and bearing in mind the previous studies in ageing males where the central regulation is mostly deprived (Ajdžanović et al., 2009a,b, 2011; Milošević et al., 2009), the present experimental design is promising the step forward.

The corticotrophin-releasing hormone (CRH) and arginin-vasopressin (AVP) neurons in the paraventricular nucleus (PVN) are the central driving force for HPA axis, stimulating the release of the adrenocorticotropic hormone (ACTH) from the pituitary, which in turn acts on the adrenal cortex to trigger the release of glucocorticoids (Sawchenko and Swanson, 1990; Whitnall, 1993). Besides the strong feed-back regulation of HPA activity by glucocorticoids *via* the glucocorticoid receptor (GR), an important modulatory role is exerted by gonadal steroids (Handa et al., 1994). The different levels of circulating gonadal steroids in both genders are considered as essential in sex-specific HPA axis activity and responsivity to stress (Handa et al., 1994). Female rats demonstrate a greater ACTH response to stress, a faster onset of corticosterone (CORT) secretion and a faster rate of CORT rise than males (Burgess and Handa, 1993).

Estrogen treatment of female rats enhances the CORT response to stress and delays recovery from stress in relation to ovariectomized (ovx) females (Burges and Handa, 1993). Additionally, the gonad intact female rats have higher, whereas ovx females have a similar peak of diurnal plasma CORT in comparison to the gonad intact males (Seale et al., 2004), which proves a vital role of estrogen in modulating of the HPA axis function. Based on the identification of ER, in both CRH and AVP neurosecretory neurons in the PVN (Bodo and Rissman, 2006), it is clear that estrogen directly influences cellular activity and gene expression within the PVN, which reflects on HPA axis function. Furthermore, the enhanced responsiveness of HPA axis to a stressor, mediated by estrogen, may be partially due to impairment of GR negative feedback (Patchev et al., 1995) and/or *via* reducing inhibitory GABA-ergic inputs into the PVN (Bodo and Rissman, 2006; McCarthy, 2008; Weiser and Handa, 2009).

In contrast to the action of estrogen, testosterone has an inhibitory influence on HPA axis activity in male rats. Orchidectomy-induced removal of testosterone results in an increase of ACTH and CORT response to physical and psychological stressors, and can be reversed by testosterone replacement (Handa et al., 1994; Seale et al., 2004). The expression of androgen receptor (AR) mRNA in a relatively high level in hippocampus, amygdala and bed nucleus of the stria terminalis, as well as the absence of AR in PVN suggest the possible sites and propose mechanisms by which testosterone can influence the stress response (Simerly et al., 1990; Handa et al., 1994; Zhou et al., 1994; Viau et al., 2001; Murphy et al., 2002). However, androgens may directly act on the PVN through the local conversion to estrogens (Naftolin et al., 1974; Lund et al., 2004).

Estrogen administration to male rats, similarly to female rats, increases the basal CORT secretion, as well as the ACTH and CORT response to physical and psychological stressors (Burges and Handa, 1993), which is followed by the changes in CRH mRNA and protein levels. The mechanism by which estrogen similarly modulates the stress responses in both genders could be a consequence of identical expression of ER mRNA in brains (LaFlamme et al., 1998).

Bearing in mind estrogen effects on the HPA axis in males, the possibility that genistein acts like an estrogen or antiestrogen, with its increasing use as a possible therapy or preventive agent for numerous diseases, we hypothesized that its administration would, in some way, modulate the HPA axis in orchidectomized adult rats. Here, we have used adult male rats to examine the effects of chronic genistein application on the morphological and hormone secreting parameters of the HPA axis. The volume density and activity of CRH neurons in the PVN were determined. Design-based modern stereology was applied to provide an unbiased estimation of the morphological parameters of the pituitary ACTH cells. The ACTH-CORT hormone secretion was determined biochemically.

Material and methods

Animals and diet

The experiments were performed on adult, 2-month-old, male Wistar rats, bred in the facilities of Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The animals were housed in groups of two per cage, under standard environmental conditions (a 12 h light\dark cycle, 22±2°C). For 2 weeks before the experiment, the rats were fed a soy-free diet prepared in cooperation with the Department of Food, School of Veterinary Medicine, Belgrade, Serbia, and INSHRA PKB, Belgrade, Serbia, according to Picherit et al. (2000), with corn oil as the fat source. The diet contained per 100g: 20.3g casein; 65g carbohydrate (45g cornstarch + 20g sucrose); 5.2g corn oil; 3.7g fiber (crystalline cellulose); 1.5g vitamin/mineral mix (Ca-phosphate deficient); 1.8g dibasic calcium-phosphate; 1g calcium carbonate; 1.5g DL-methionine. Casein and crystalline cellulose originated from Alfa Aesar, Johnson Matthey Gmbh & Co. KG, Karlsruhe, Germany; carbohydrate, oil, vitamin/mineral mix, calcium carbonate and calcium phosphate were from INSHRA PKB, Belgrade, Serbia; and DL-methionine from Sigma Chemical Company, St. Louis, MO, USA. During the experiment, animals consumed food that was devoid of any isoflavone content *per se*. Food and water were available *ad libitum*.

Experimental design

The experimental protocols were approved by the Animal Care Committee of the Institute for Biological Research "Siniša Stanković" (Belgrade, Serbia) in

conformity with the recommendations provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A).

The experimental animals were bilaterally orchidectomized (Orx) or sham-operated (So) under ketamine anesthesia (ketamine hydrochloride 15 mg/kg b.w.; Richter Pharma, Wels, Austria). Orchidectomy was performed with a view to eliminating the endogenous gonadal steroids from the hormonal milieu. Two weeks after surgery the first group of Orx rats (n=6) was subcutaneously injected with estradiol-dipropionate (Galenika, Belgrade, Serbia; Orx+E) in a dose of 0.625 mg/kg b.w. every day, except on Sundays, for 3 weeks. The second Orx group (n=6) was subcutaneously injected with genistein (Orx+G) (30 mg/kg b.w.) following the same regime. Genistein (Nutraceutica, Monterenzio, Italy) was dissolved in a minimal volume of absolute ethanol (approximately 0.1 ml) and then mixed with sterile olive oil (about 0.2 ml). Each animal was treated with 0.3 ml of this mixture. The applied dose of genistein (30 mg/kg b.w.) was chosen to mimic human exposure to elevated concentrations of isoflavones when nutritional supplements are used for therapeutic purposes (Doerge and Sheehan, 2002). The injection strategy is equivalent to oral consummation in terms of absorption rate (Jefferson et al., 2007) and provides an easy control of the applied dose. The So and the third Orx group received the same volume of absolute ethanol in sterile olive oil and served as the controls (n=6 per each group). The mode of injection, which excluded Sundays, was postulated in our previous studies (Ajdžanović et al., 2009a,b, 2011; Milošević et al., 2009) and considered the one day "rest" of HPA axis from stress (caused by handling and injections). All animals were killed by decapitation under ether anesthesia (ether ad narcosis Ph. Iug. III., Lek, Ljubljana, Slovenia) 24 h after the last injection.

Tissue preparation

The hypothalamus and pituitary glands were excised, fixed in Bouin's solution for 48 h and dehydrated in increasing concentrations of ethanol and xylene. After embedding in Histowax (Histolab Product AB, Göteborg, Sweden), coronal serial sections of the hypothalamus (5 μm thick) and serial sections of the pituitary (3 μm thick) were obtained with a rotary microtome (RM 2125RT Leica, Glostrup, Denmark). Sakura Tissue-Tek Accu-Edge Low-Profile microtome blades for extremely thin sectioning were used. The slices were placed on silica-coated glass slides (SuperFrost Plus, Prohosp, Denmark).

Double-labeling immunofluorescence

The activity of CRH-containing neurons in the PVN was determined by double-labeling immunofluorescence using cFos. After dewaxing, hydration and rinsing in 0.01M phosphate-buffered saline (PBS; pH 7.4) for 10 min, the sections were exposed to microwaves (700 W) in 0.05M citrate buffered saline (pH 6.0), for 2x10 min for antigen retrieval. Subsequently, the sections were washed in PBS (3x10 min), preincubated in normal donkey serum (1:10) for 30 min to block nonspecific staining and incubated overnight at 4°C with goat anti-cFos polyclonal IgG antibody (dilution 1:50 in PBS; sc-52-G, Santa Cruz Biotechnology, Inc.). After washing in PBS (3x10 min), the sections were incubated with donkey anti-goat Alexa fluor 555 IgG (1:200; Invitrogen) for 2 h. Subsequently, the sections were rinsed in PBS (3x10 min) and incubated in normal donkey serum for 30 min at 1:10 dilution. Primary antibodies of rabbit anti-CRH (dilution 1:500 in PBS; ab8901-100 Abcam) were applied overnight at 4°C. After washing in PBS (3x10 min), the sections were incubated with donkey anti-rabbit Alexa fluor 488 IgG (1:200; Invitrogen) for 2 h, then washed in PBS (2x10 min) and coverslipped with mowiol (Calbiochem) mounting medium. Double labeling procedures were performed with special attention paid to the possible secondary antibody co-interactions. It was determined in separate experiments that secondary antibodies donkey anti-rabbit IgG Alexa Fluor 488 and donkey anti-goat Alexa Fluor 555 do not cross react. In order to confirm the specificity of cFos and CRH immunostaining, the antibody (anti-cFos and anti-CRH) was coincubated with 5-fold excess of blocking peptide (Santa Cruz Biotechnology, Inc.) and the sections were treated in the same way as described above: the antibody (cFos or CRH) was neutralized by incubation with the blocking peptide; the antibody that was bound to the blocking peptide was no longer available to bind to the epitope present in the protein; comparing the staining from the blocked antibody *versus* the antibody alone one can gain insight into which staining was specific. The staining was absent during the procedure performed with the neutralized antibody (Fig. 1).

The sections were examined and photographed using a Zeiss Axiovert fluorescence microscope, equipped with a camera and EC Plan-Apochromat.

Immunohistochemistry

Pituitary ACTH was localized using the peroxidase-antiperoxidase (PAP) method. Antiserum to rat ACTH (NIDDK-anti-r ACTH-IC Lot# AFP-156102789) was obtained from Dr. A.F. Parlow, National Hormone Peptide Program (NHPP), Harbor-UCLA Medical Centre, Carson, CA, USA. The specificity of this antiserum was assessed by the NIDDK. The usable dilution was determined empirically for antiserum.

Endogenous peroxidase activity was blocked by incubation in 9 mM hydrogen peroxide solution in methanol for 15 min. Nonspecific background staining was prevented by incubation of the sections with nonimmune, normal swine serum (Dako, Glostrup, Denmark) diluted in PBS (pH 7.4) for 1 h. After the

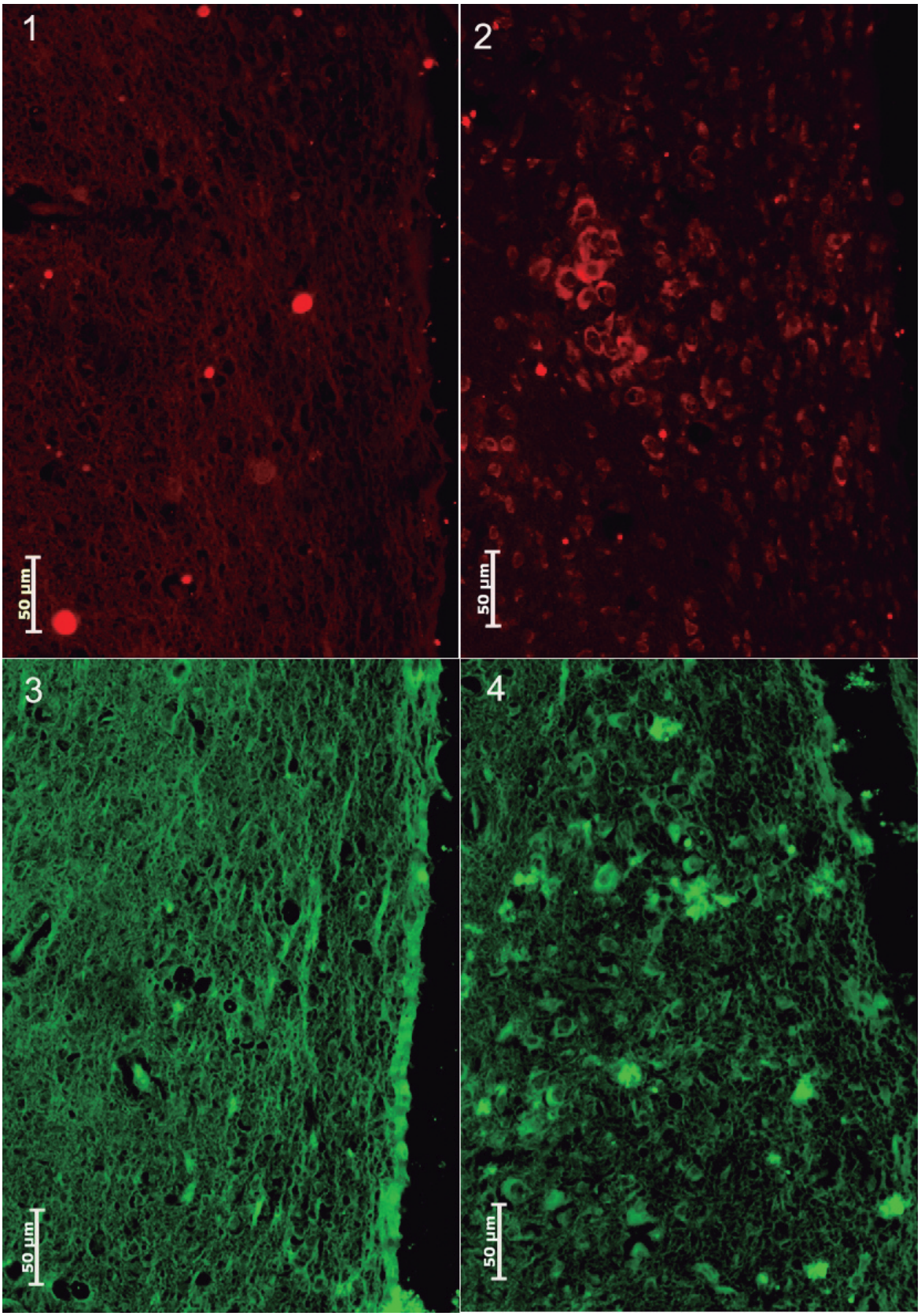

Fig. 1. cFos and CRH immunofluorescent staining in the paraventricular nucleus of adult rat. Photomicrographs **1** and **3** represent neuron immunoreactivity when antibody (c-Fos**(1)** CRH**(3)**) is incubated with blocking peptide. Photomicrographs **2** and **4** represent neuron immunoreactivity when antibody (c-Fos**(2)** CRH**(4)**) is incubated alone. Scale bar: 100 μm.

blocking procedure, the sections were overlaid with the appropriate dilution of ACTH primary antibodies for 24 h at room temperature. After washing in PBS, the sections were incubated for another 1 h with the secondary antibody (IgG/HRP, Dako, Glostrup, Denmark) and again rinsed with PBS. Antibody localization was visualized using 0.05% 3,3-diaminobenzidine tetrachloride (DAB) liquid substrate chromogen system (Dako, Glostrup, Denmark). The sections were thoroughly washed under running tap water, counterstained with hematoxylin and mounted in DPX. The specificity of the immunoreaction products was determined by the omission of primary antibodies during the immunohistochemistry protocol-negative control. This resulted in the complete loss of immuno-reactivity.

Stereological and morphometric measurements - hypothalamus and pituitary gland

All stereological analyses were carried out using a workstation comprised of a microscope (Olympus, BX-51) equipped with a microcator (Heidenhain MT1201) to control movements in the z -direction (0.2 μm accuracy), a motorized stage (Prior) for stepwise displacement in x-y directions (1 μm accuracy), and a CCD video camera (PixeLink) connected to a 19" PC monitor (Dell). The whole system was controlled by the newCAST stereological software package (VIS - Visiopharm Integrator System, ver. 2.12.1.0; Visiopharm; Denmark).

PVN volume estimation

The PVN was identified according to a rat brain atlas (Paxinos and Watson, 2004). The main references used to locate the PVN were the presence of the fornix and the 3rd ventricle. In adult rats, the PVN begins at 1.5 mm and extends to 2.1 mm posterior to the bregma level. The volume of PVN was estimated according to Cavalieri's principle (Gundersen, 1986; Gundersen and Jensen, 1987). Sampling of cresyl violet stained the hypothalamic coronal sections 5 μm thick, was systematically uniform, from the random start (every 10th section from each tissue block was analyzed). The volume (mm^3) of the right and left side of the PVN was determined by multiplying the sum of the areas by the interval between the sections (50 μm), according to the formula:

$$Vpvn = a(p) \cdot BA \cdot \sum_{i=1}^{n} Pi$$

where a(p) is the area associated with each sampling point; BA (the block advance) is the mean distance between two consecutively studied sections (real section thickness 5 μm x 10); n is the number of sections studied for each PVN; $\sum$Pi is the sum of points hitting a given target.

Percentage of CRH-ir and activated CRH-ir neurons

Using photomicrographs of three double-labeled fluorescent sections from three levels of the PVN for each animal, the percentages of CRH and CRH-cFos (activated CRH) neurons were obtained. Care was taken to ensure that corresponding sections from all examined animals were from the same levels. Photomicrographs of three levels of PVN for each animal were imported into the Vis program. By counting points hitting CRH-ir (green) and CRH-cFos-ir (ochre) neurons and dividing these with the points hitting the PVN area x 100, the percentages of immunolabeled CRH and CRH-cFos neurons were calculated.

Pituitary gland volume

Pituitary volumes were estimated using Cavalieri's principle. Sampling of the pituitary sections (3 μm thick) was systematically uniform from the random start. Every 20th section from each tissue block was analyzed (the same sections were used in the subsequent estimation of ACTH cell numbers by the physical dissector method). The mean distance between two consecutively studied sections was 60 μm. The same formula used for PVN volume estimation was employed to determine the pituitary volume and total volume of ACTH cells.

Total number of ACTH cells

A fractionator/physical dissector design with two levels of sampling was used to estimate the total number of ACTH cells from all examined groups, according to the method described recently in detail (Manojlović-Stojanoski et al., 2010). Briefly, we have analyzed every 20th section from each tissue block (1% of the selected

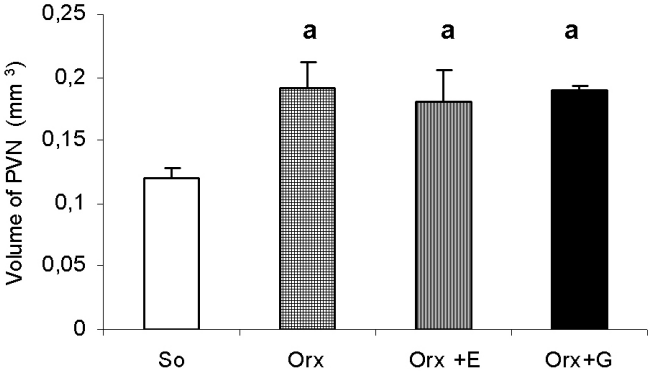

Fig. 2. Volume of the paraventricular nucleus of the hypothalamus in sham-operated (So), orchidectomized (Orx), orchidectomized estradiol-dipropionate (Orx + E) and genistein treated (Orx + G) adult male, n=6 animals per group [a]: p<0.05 vs. So

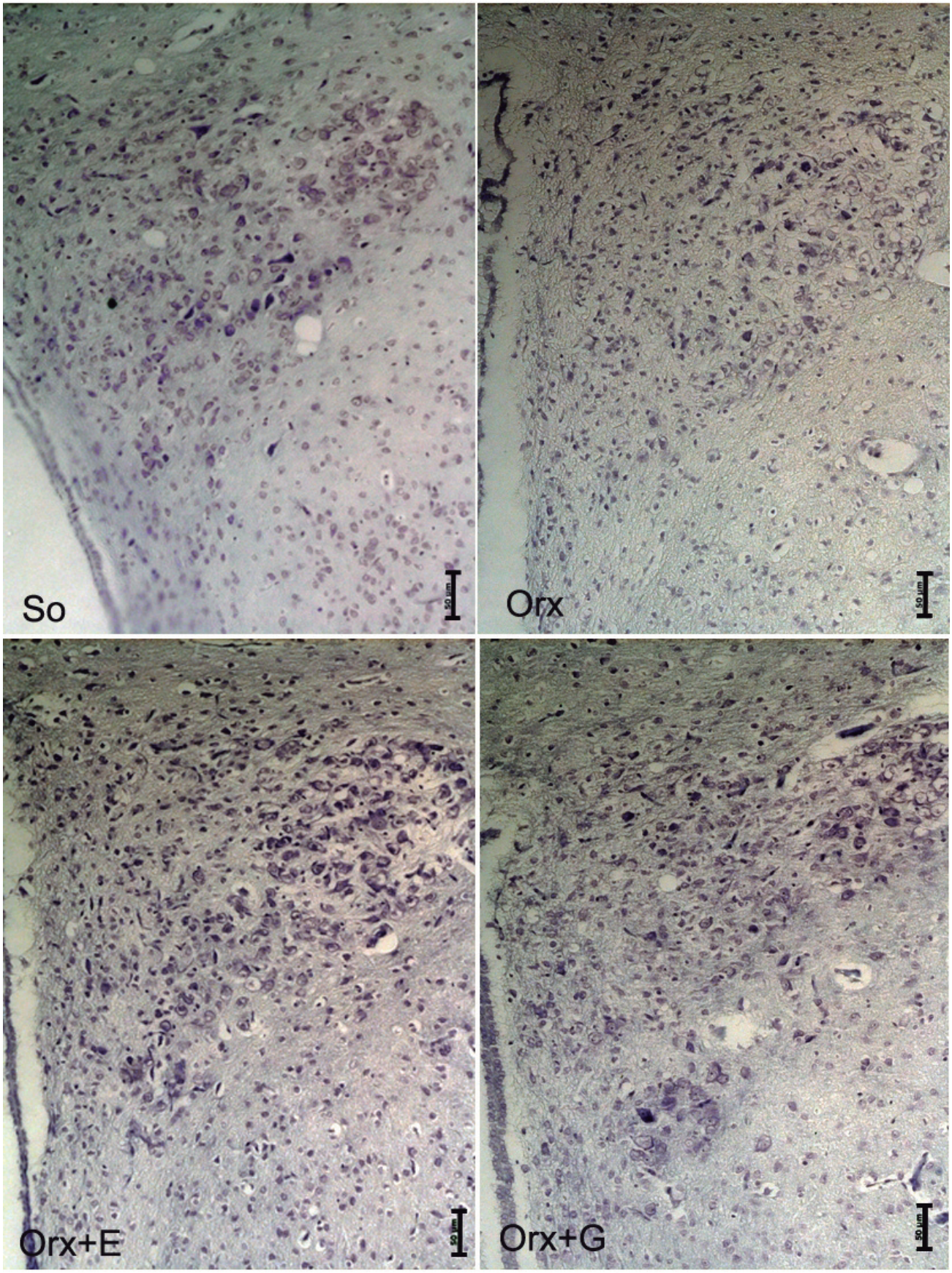

Fig. 3. Cresyl violet stained paraventricular nucleus of the hypothalamus in sham-operated (So), orchidectomized (Orx), orchidectomized estradiol-dipropionate (Orx + E) and genistein treated (Orx + G). Scale bar: 50 μm.

tissue).

Mean ACTH-cell volume

As the mean volume of a single ACTH cell is equivalent to the total volume occupied by ACTH cells divided by their number (de Lima et al., 2007), the size can be calculated.

Blood ACTH and corticosterone assays

All animals were killed by rapid decapitation on the same day. Blood was collected from the trunk in two types of glass tubes (without EDTA for corticosterone measurements and with EDTA for ACTH measurements) and then centrifuged. All the samples were maintained at -70°C until the assays. Each hormone assay of the plasma or serum samples, from the all groups, was analyzed on the same day. Plasma ACTH concentration was determined without dilution, by a chemiluminescence method using an IMMULITE automatic analyzer (DPC, Los Angeles, CA, USA), in duplicate samples within a single assay, with an intra-assay coefficient of variation (CV) of 9.6%. The analytical sensitivity of this assay is 9 pg/ml. This protocol has been approved by the Canadian Council on Animal Care. The corticosterone concentration was determined without dilution by immunoassay (R&D Systems Inc., Minneapolis, USA), in duplicate samples within a single assay, with an intra-assay CV of 8.0%. The sensitivity of this Corticosterone Immunoassay is typically less than 27 pg/ml.

Adrenal tissue corticosterone assay

The right adrenal glands were excised, weighed and immediately shredded on ice. The shredded tissue was then homogenized in Tris-saccharose buffer (pH 7.9; 1 mg of tissue: 1 μl of buffer) using a dispersion system (Ultra - Turax T25, Janke& Kunkel, IKA-Labortechnik) at 8,000 rpm. The homogenate was centrifuged at 35,000 rpm (105,000g) for 1 h (in a Beckman L7-55 ultracentrifuge), and the corticosterone concentration in the supernatant was determined by immunoassay (R&D Systems Inc., Minneapolis, USA).

Statistical analyses

Morphometric and biochemical data obtained for the experimental groups were subjected to one-way analyses of variance (ANOVA). Duncan's multiple range test was used for post hoc comparisons between the groups. A confidence level of $p<0.05$ was considered statistically significant. The data are presented as means ± SD.

Results

Hypothalamus

PVN volume (mm^3)

The mean volumes of PVN were larger ($p<0.05$) in the Orx (0.1929±0.024; or 60%), Orx+E (0.18±0.03; or 50%) and Orx+G (0.1904±0.03 mm^3; or 58%) compared to the So group (0.1201±0.008 mm^3) (Figs. 2,3).

Percentage of CRH-ir neurons (%)

The percentage of CRH-ir in the Orx group (26.78±2,1%) was higher ($p<0.05$) by 23% then that in the So group (22.4±2,3%) while the same parameter in the Orx+E group (34.4±3 %) was higher ($p<0.05$) by 28% then that in the Orx group (Fig. 4a).

Percentage of CRH/cFos -ir neurons (%)

The percentage of CRH/cFos-ir in the Orx group (3.2±0.3%) was larger ($p<0.05$) by 45% than that in So

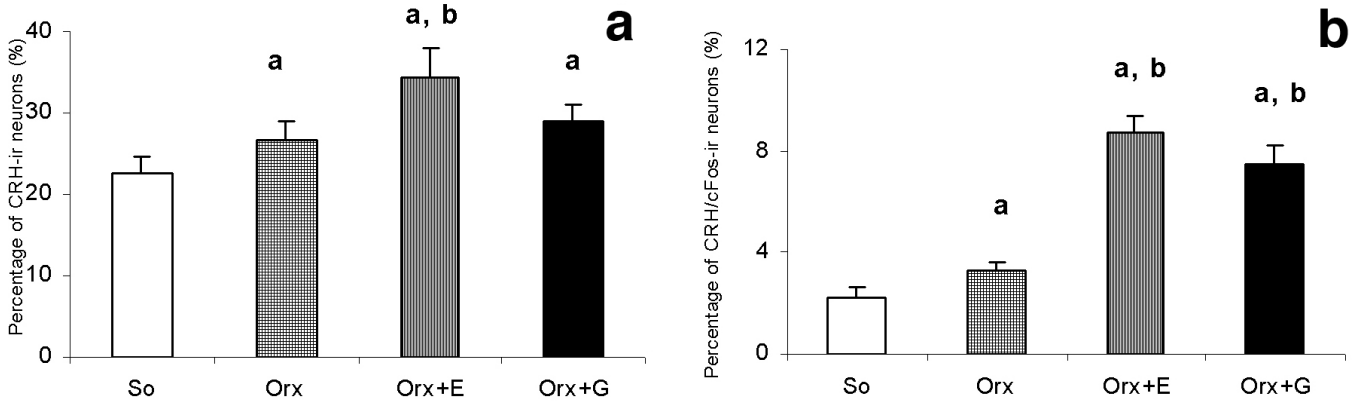

Fig. 4. Morphometric parameters of the paraventricular nucleus of the hypothalamus for sham-operated (So), orchidectomized (Orx), orchidectomized estradiol-dipropionate (Orx + E) and genistein treated (Orx + G) adult male rats. **a.** Percentage of CRH immunoreactive neurons. **b.** Percentage of CRH/cFos immunoreactive neurons. The values are means ± standard deviation, n=6 animals per group [a]: $p<0.05$ vs. So, [b]: $p<0.05$ vs. Orx

group (2.2±0.4%). The same parameter in the Orx+E (8.72±0.6%) was higher ($p<0.05$) by 4-fold and by 2.7-fold in comparison to the So and Orx group, respectively. The percentage of CRH/cFos-ir in the Orx+G (7.5±0.7%) was larger ($p<0.05$) by 3.4-fold and 2.3-fold in comparison to the So and Orx, respectively

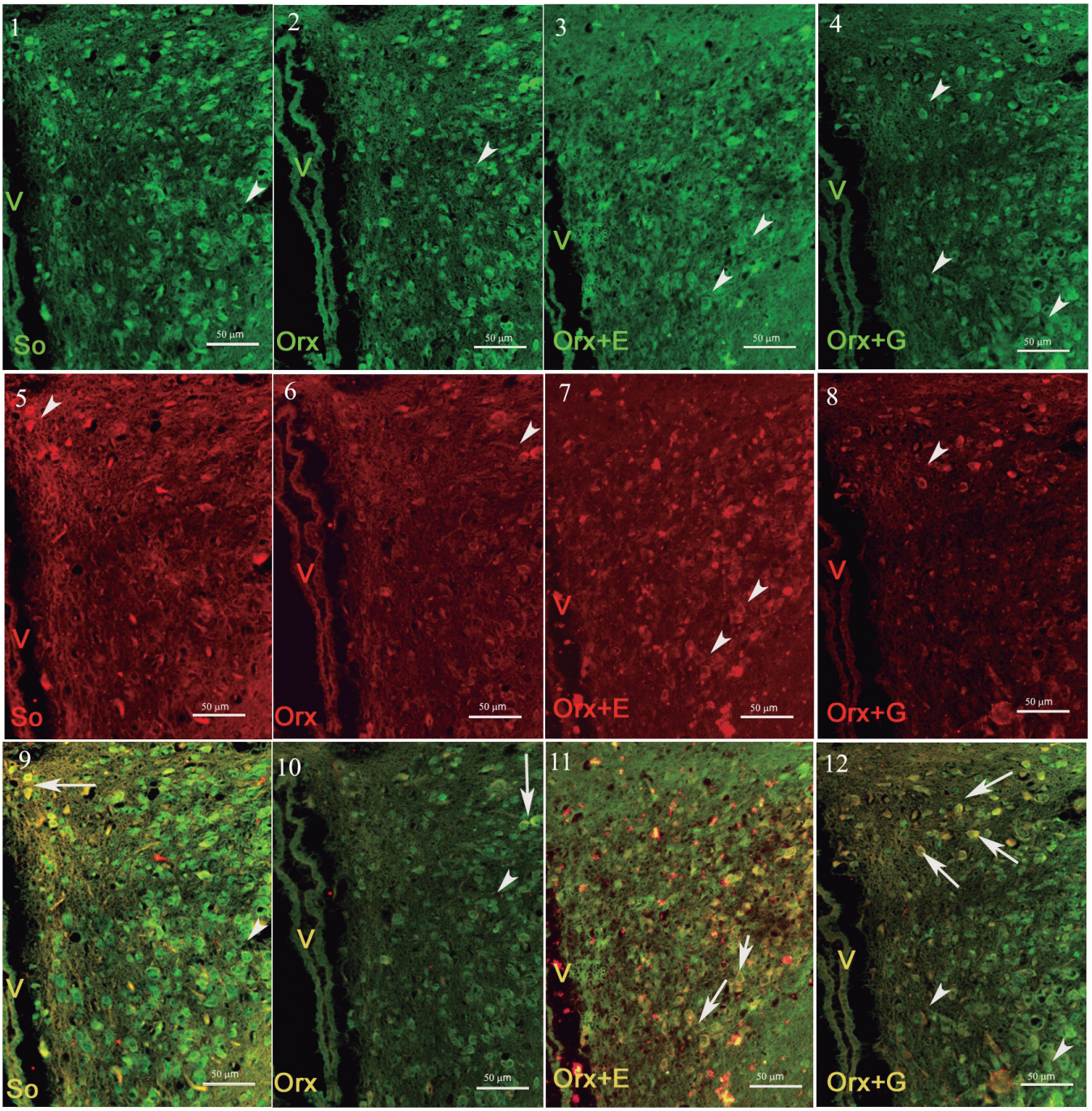

Fig. 5. Double-labeling immunofluorescent histochemistry in the paraventricular nucleus visualizing CRH and cFos in sham-operated (So; **1 and 5**), orchidectomized (Orx; **2 and 6**), orchidectomized estradiol-dipropionate (Orx + E; **3 and 7**) and genistein treated (Orx + G; **4 and 8**) adult male rats. Photomicrographs **1, 2, 3, 4** represent CRH immunoreactive neurons visualized in green; photomicrographs **5, 6, 7, 8** represent cFos immunoreactive neurons visualized in red; photomicrographs **9, 10, 11, 12** represent overlays of photomicrographs **1+5, 2+6, 3+7** and **4+8**. Arrowheads indicate single-labeled CRH or cFos nuclei, while arrows point to the examples of double-labeled neurons. V: third ventricle, scale bar: 50 μm.

(Fig. 4b).

Double immunofluorescence of CRH/cFos neurons

CRH immunoreactive neurons were expressed in all experimental groups. Low cFos immunoreactivity and weak co-localization with CRH immunoreactivity were observed in the PVN of the So and Orx group. Higher cFos immunoreactivity was observed in the PVN of Orx+E and Orx+G rats, in comparison to both the So and Orx. Also, the higher level of co-localization between CRH and cFos immunoreactivity was detected in the Orx+E and Orx+G group in comparison to both the So and Orx rats (Fig. 5).

Pituitary gland

Pituitary weight (mg)

Pituitary weight was larger ($p<0.05$) in the Orx (14.9±1.1 mg; or 15%), Orx+E (34.4±2.9 mg; or 167%) and Orx+G (19±0.8 mg; or 47%) group in comparison to the So (12.9±1.1 mg). The same parameter in the Orx+E group was larger ($p<0.05$) by 131% and 55% respectively, in comparison to the Orx and Orx+G group. The same parameter in the Orx+G group was larger ($p<0.05$) by 28% in comparison to the Orx (Fig. 6a).

Pituitary volume (mm^3)

Pituitary volume was larger ($p<0.05$) in the Orx+E (11.1±1 mm^3; or 102% and 82%) and Orx+G group (7.4±0.4 mm^3; or 34% and 21%) in comparison to the So (5.5±0.6 mm^3) and Orx (6.1±0.6 mm^3) rats, respectively. The same parameter in the Orx+G group was larger ($p<0.05$) by 21% in comparison to the Orx (Fig. 6b).

Total number of ACTH cells

The total number of ACTH cells in Orx+E group (170,000±11,135) was larger ($p<0.05$) by 18% then that in the So group (144,000±4,582). The same parameter in the Orx+G (186,000±4,509) was larger ($p<0.05$) by 30% and 22% respectively, than that in both the So and Orx

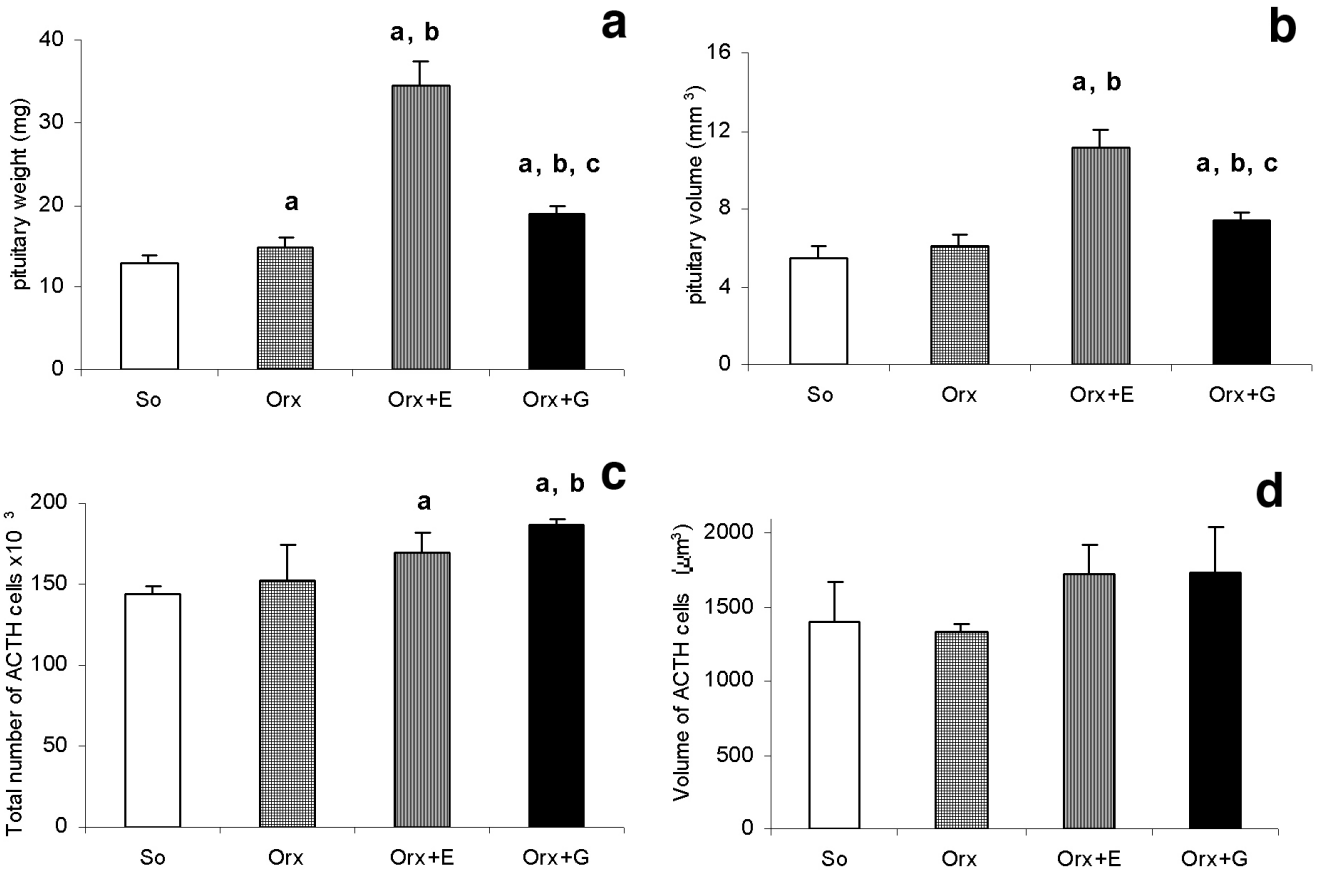

Fig. 6. Pituitary weight **(a)** and morphometric parameters for pituitary ACTH cells in sham-operated (So), orchidectomized (Orx), orchidectomized estradiol-dipropionate (Orx + E) and genistein treated (Orx + G) adult male rats: pituitary volume **(b)**, total number of ACTH cells **(c)**, as well as volume of ACTH cells **(d)**. The values are means ± standard deviation, n=6 animals per group [a]: $p<0.05$ vs. So, [b]: $p<0.05$ vs. Orx.

(170,000±11,135) group (Fig. 6c).

Volume of ACTH cells (μm^3)

There was a tendency for the cell volume to be greater in the Orx+E and Orx+G group in comparison to the Orx rats, but the difference was not statistically significant (Fig. 6d).

Immunohistochemistry of ACTH cells

The ACTH immunoreactive cells in all experimental groups were irregularly shaped, located individually or in groups, between the capillaries in the pituitary *pars distalis*. ACTH cells in the Orx group showed reduced staining intensity than in the So group, while the same properties in the Orx+E and Orx+G group were reduced in comparison to the Orx, as well (Fig. 7).

Biochemical results

The plasma level of ACTH was higher ($p<0.05$) in the Orx (58.6±2.6 ng/L; or 57%), Orx+E (99, 2±9 ng/L; or 166%) and Orx+G (85±9 ng/L; or 127%) group than that in the So group (37.3±2.2 ng/L). The ACTH level was higher ($p<0.05$) in the Orx+E group by 69% and in the Orx+G group by 45% in comparison to the Orx group, as well (Fig. 8a).

The serum level of corticosterone was higher ($p<0.05$) in the Orx+E (24.4±3.01 ng/ml; or 81% and 103%) and Orx+G (34.65±3.78 ng/ml; or 2.8-fold and 2.6-fold) than in both So (12.01±1.04 ng/ml) and Orx (13.46±0.93 ng/ml) groups, respectively. The serum level of corticosterone was higher ($p<0.05$) in the Orx+G group by 41% in comparison to the Orx+E group (Fig. 8b).

The corticosterone in adrenal tissue was higher

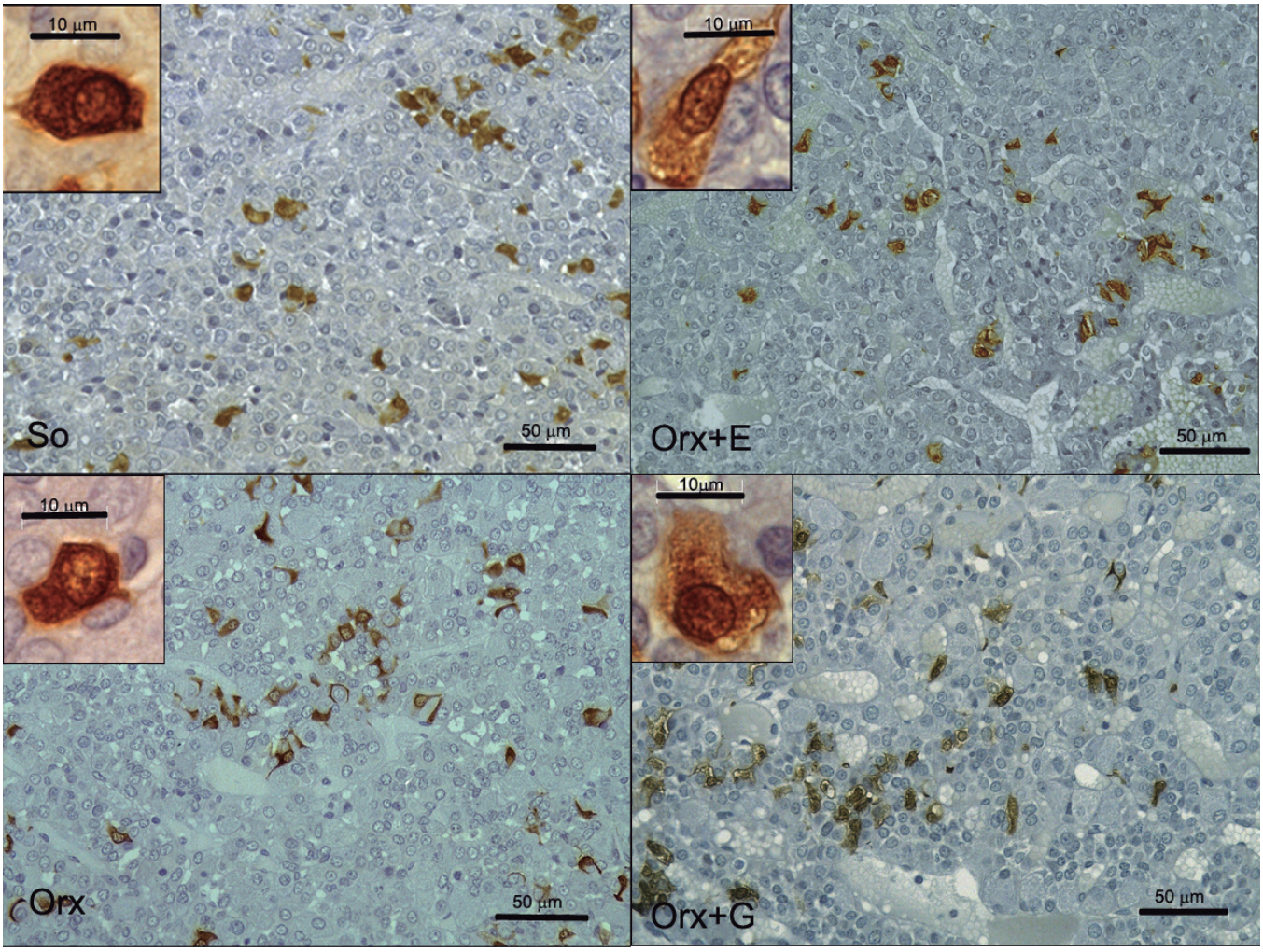

Fig. 7. Immunoreactive ACTH cells in the pituitary gland in sham-operated (So), orchidectomized (Orx) orchidectomized estradiol-dipropionate (Orx + E) and genistein treated (Orx + G) adult male rats. Scale bar: 50 μm, insets scale bar: 10 μm

($p<0.05$) in the Orx+E (7.99±0.35 ng/mg; or 3.3 and 4.8 fold) and Orx+G (3.37±0.28 ng/mg; or 2 and 2.8 fold) group than in both So (1.87±0.06 ng/mg) and Orx (1.36±0.09 ng/mg) groups, respectively. The corticosterone in adrenal tissue was higher ($p<0.05$) in the Orx+E group in comparison to the Orx+G group (Fig. 8c).

Discussion

Genistein is becoming increasingly popular as a therapeutic or food supplement agent, since the intake of this estrogen-like compound has been associated with a myriad of health benefits. In view of these facts, plus the lack of experimental data concerning the effects of genisten on physiologically significant HPA axis in adult males, the present study was designed. In order to get answers about the effects of genistein on the morphological and hormonal parameters of the HPA axis in the hormonal milieu without gonadal steroids, orchidectomy and estradiol-dipropionate treatment were used as the adequate physiological controls to genistein treatment.

Following orchidectomy, higher values of the PVN volume, percentage of CRH neurons and percentage of activated CRH neurons (i.e. neurons double-labeled for CRH/cFos) were observed in this study. It is well known that the functioning of the HPA axis is highly sensitive to gonadal steroids, so orchidectomy as an approach for eliminating testosterone causes a significant disturbance in the function of this axis. Orchidectomy-provoked enhancement of PVN volume represents a consequence of increased CRH-ir, which is in accordance with previous studies (Bingaman et al., 1994; Handa et al., 1994). The significant increase in the examined morphological parameters of CRH neurons after testosterone elimination in our study pointed out a well-known fact: testosterone acts as an inhibitor of HPA axis response (Handa et al., 1994; Evuarherhe et al., 2009). As our current data show, the elimination of inhibitory testosterone action for five weeks, the time necessary for recovery after orchidectomy and chronic treatment, provokes the increase of CRH neuron percentages in the PVN and its activity under basal conditions. Viau and Meaney (1996) found that testosterone suppressed the HPA axis by increasing GR binding within the medical preoptic area (mPOA), suggesting that the mPOA could be a crucial site through which testosterone might suppress the HPA axis. Increased pituitary weights and ACTH levels were observed following orchidectomy, whereas applied modern stereological measurements did not reveal changes in the stereological features of ACTH cells. Hypertrophied gonadotropes and prolactin synthesizing cells are obviously responsible for the

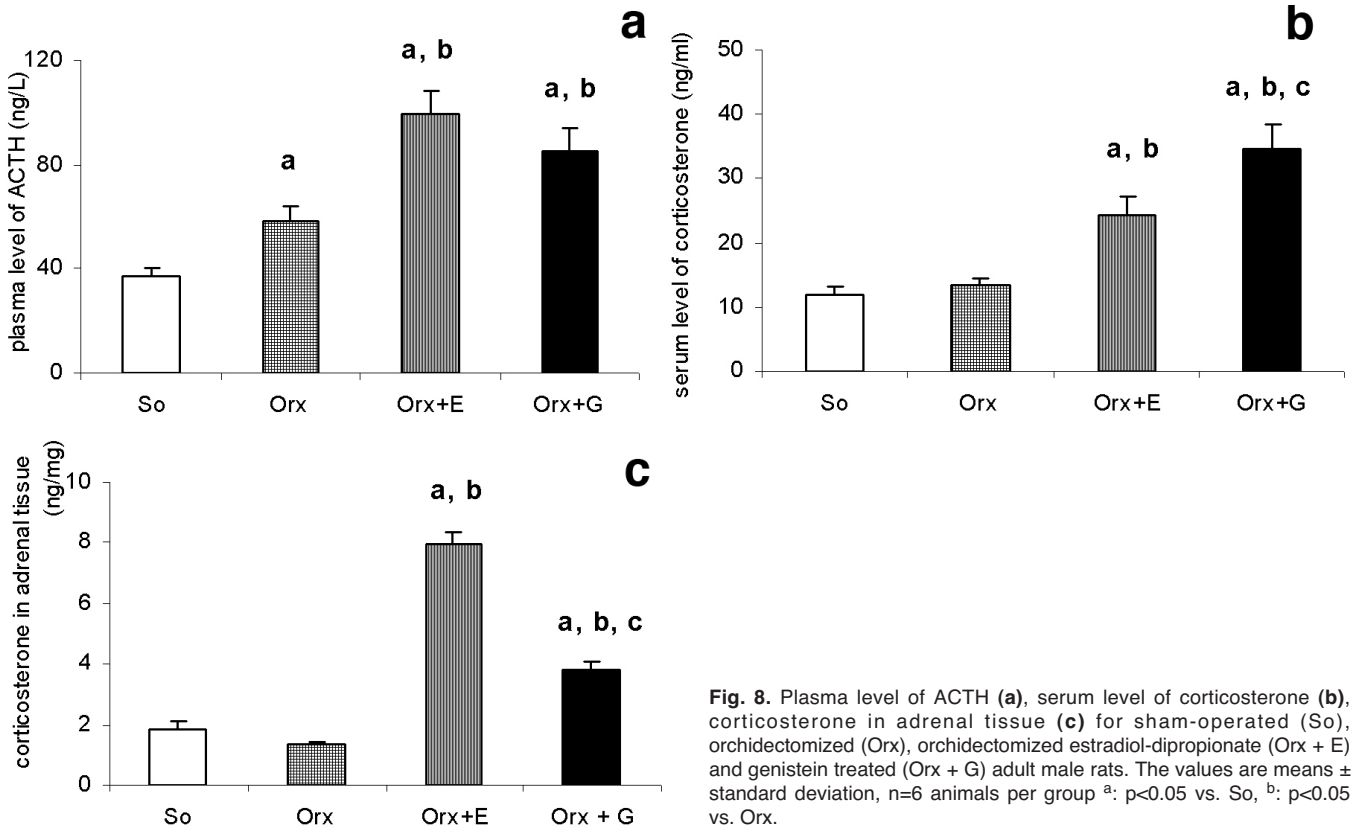

Fig. 8. Plasma level of ACTH **(a)**, serum level of corticosterone **(b)**, corticosterone in adrenal tissue **(c)** for sham-operated (So), orchidectomized (Orx), orchidectomized estradiol-dipropionate (Orx + E) and genistein treated (Orx + G) adult male rats. The values are means ± standard deviation, n=6 animals per group [a]: $p<0.05$ vs. So, [b]: $p<0.05$ vs. Orx.

increase in pituitary weights, since feedback inhibition by testosterone was abolished (Kitay, 1963; Inoue et al., 1985). As orchidectomy in mature male rats did not influence the mitotic activity of ACTH cells (Sakuma et al., 1984), it could be anticipated that their number and size will be unchanged, as we revealed. In our study, the obtained increase in the ACTH level after orchidectomy represents the consequence of the stimulatory influences of activated CRH neurons on ACTH synthesis and secretion, observed in a previous study (Bingaman et al., 1994). The unchanged corticosterone serum and adrenal tissue levels following orchidectomy is in accordance to previous studies (Kitay et al., 1966), and could be correlated with the above mentioned changes in GR binding.

Administration of estradiol-dipropionate to orchidectomized rats provoked enhancement of CRH and CRH/cFos percentage, which indicates the higher basal activity of existing CRH neurons. This result was expected in reference to previous studies supporting estrogen altering the HPA function (Handa et al., 1994; Malendowicz, 1994). It is well known that estrogen administration increases CRH mRNA (Bohler et al., 1990; Patchev et al., 1995; Roy et al., 1999) as well as CRH-ir (Haas and George, 1988) in the PVN. ER receptor mRNA localization within the parvocellular division of the PVN suggests that direct effects of estrogen on CRH cells can occur (Simerly et al., 1990). Estrogen can mediate the changes in HPA activity by influencing the feedback mechanisms at the level of hippocampus as well. Stereological analysis in Orx+E group has shown higher values of pituitary weights and volumes as well as total number of ACTH cells, which was accompanied with the higher ACTH level, indicating the elevated synthesis and secretion of ACTH. The *in vitro* study has shown that estrogen did not modify the ACTH secretion at the pituitary level (Malendowicz, 1994), which supported the estrogen CRH-mediated action on ACTH cells. The elevation of ACTH, serum and adrenal tissue CORT levels over time in Orx+E group is controversial, considering the negative feedback mechanism setup. The down regulation of GR protein levels with the impairment of glucocorticoid negative feedback, following estradiol treatment (Kinyamu et al., 2003; Weiser and Handa, 2009), could be the possible mechanism underlying our result. It could be concluded that orchidectomy induces an increase in HPA axis activity, which persists and even increases with estradiol-diproprionate treatment. On the other hand, treatment with testosterone reverses HPA axis activity and diminishes the effects of orchidectomy (Seale et al., 2004).

The established enhancement of CRH/cFos percentage in Orx+G rats, without change in the CRH percentage in the PVN, indicates the higher basal activity of existing CRH neurons. The most likely action of genistein in brain is *via* the ERs distributed in parvocellular and, as a recent study has confirmed, magnocellular regions of the PVN (Cho et al., 2007). Higher binding affinity of genistein to ERß (Kuiper et al., 1998; Lephart et al., 2002) and their presence in CRH neurons may result in a direct influence of genistein on PVN (LaFlamme et al., 1998; Shughrue et al., 2001; Suzuki and Handa, 2004). At the pituitary level genistein treatment caused an increase in pituitary weights and volumes and total number and volume of ACTH cells, which was accompanied with higher ACTH level, in comparison to the Orx. The increase of ACTH cells total number after genistein treatment could be a consequence of the transdifferentiation of plurihormonal cells, detected in the pituitary, as a response to temporary endocrine demands (Seuntjens et al., 2002). Namely, plurihormonal cells which contain both ACTH and gonadotropic hormones were detected within the population of rat pituitary corticotrophs (Childs et al., 1982). The unchanged volume of particular ACTH cells after chronic genistein application, together with reduced immunostaining intensity and elevated circulating ACTH level, most probably represent the consequence of the continuous synthetic and secretory activity of these cells, due to constant hypothalamic stimulation. Previous studies described the low levels of ER-α and ER-ß mRNA in rat ACTH cells (Mitchner et al., 1998). Coherent with the literature, we suggest that the major effects of genistein on ACTH were realized through the PVN (Couse et al., 1997; Mitchner et al., 1998; Shughrue et al., 1998). The higher values in serum levels of CORT in Orx+G than in Orx+E group could be the result of its more rapid secretion from adrenal cortex, although some further studies are needed to establish the potential mechanisms. A comprehensive increase of the HPA axis morphological and hormonal parameters in Orx+E and Orx+G group could be a link to estrogen-mimetic effects of genistein. The higher values of parameters observed in Orx+E could be the result of significantly higher ER binding affinity of estradiol-dipropionate in comparison to genistein.

In contrast with these results, our previous works in middle-aged male rats have shown the suppressive effects of orchidectomy and chronic genistein exposure on ACTH cells, together with a lowering of serum and tissue CORT levels (Ajdžanović et al., 2009a,b) Also, *in vitro* studies have shown the inhibitory action of genistein on rat pituitary cell proliferation (Zhang et al., 2001), as well as on a decrease in glucocorticoid production from cultured adrenocortical cells (Ohlsson et al., 2010). A likely explanation is the age-dependent decline in pituitary responsiveness to CRH, i.e. in middle-aged rats pituitary responsiveness is about 60% of that observed in young populations (Hauger et al., 1994; Murphy et al., 2002).

The present study, focused on the morphological and hormonal features of the HPA axis in orchidectomized adult rats, provides a parallel between estradiol and genistein effects and shows the genisteins estrogen mimetic activity in the HPA axis. It represents a solid basis for some further investigations in establishing the precise mechanisms of genistein actions at the central

level of the HPA axis and its potential therapeutic usefulness.

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