UC 577,1; 61 ISSN 0354-3447

Jugoslov. Med. Biohem. 22: 27–32, 2003 *Originalni naučni rad*
Original paper

CHARACTERISTICS OF THE PITUITARY IMMUNOPOSITIVE ACTH CELLS IN RAT FEMALES AFTER CHRONIC EXPOSURE TO CONSTANT LIGHT

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Summary: The effects of chronic exposure to light of adult female Wistar rats on growth and function of pituitary adrenocorticotropes (ACTH cells) were examined. The animals were exposed to continuous light of 600 lux for 95 days, starting on day 30 of age. Control rats were kept under a 12:12 h light-dark cycle, at ambient temperature. ACTH-producing cells were studied using the peroxidase-antiperoxidase immunohistochemical procedure and blood samples were collected for hormone analyses. In animals exposed to a chronic light-treatment all morphometric parameters measured throughout the present study i.e.: ACTH cell volume, nuclear volume and relative volume density were increased by 22% and the differences between this group and the controls were statistically significant ($p<0.05$). The concentration of plasma ACTH was elevated by 13% in light-exposed group in comparison with the control and this difference was statistically significant ($p<0.05$), as well. These findings suggest that continuous exposure to light is specifically involved in growth and secretory activity of ACTH cells of adenohypophysis of rat females.

Key words: Constant light, ACTH cells, ACTH, stress, female rats

Introduction

In photoperiodic mammals such as rats, light is an important regulator of secretion of several pituitary hormones (1). Indeed, shifting of the light phase results in a coincidental shift of the proestrus surge (2), estrogen-induced proestrus-like surge and the mating-induced surges of prolactin in rats (3). In 1987, Hoeffler (4) observed that rat females become acyclic when maintained under conditions of constant light. Besides, it was shown (5) that the exposure of adult rat females to constant light gradually causes polycystic ovaries, similar to that seen in human idiopathic polycystic ovarian disease (PCOD). It was recently reported that the light-dark cycle represents a powerful synchronizer of circadian rhythm in humans, as well as in other mammalian species (6). The suprachiasmatic nucleus (SCN) of the hypothalamus functions as a master pacemaker for the mammalian circadian system (7). The constant-light treatment is noninvasive and seems to be superior for the studies of the hypothalamic-pituitary axis reactivity, particularly in rodents (8).

Chronic exposure of adult rat females to constant light, presumably acts activating the stress system, i.e. paraventricular infundibular CRH (corticotropin-releasing hormone) system (9). The following pituitary cell types were reported to be involved in stress reaction: 1) The cells synthesizing proopiomelanocortin (POMC), a large precursor protein for adrenocorticotropic hormone (ACTH), melanostimulating hormone (MSH) and opioids and 2) the cells producing prolactin (PRL) and growth hormone (GH) (10). The pituitary ACTH cells are under the influence of paraventriculo-infundibular CRH producing neurons, known as the most stress-sensitive neurons (11). ACTH cells are the first differentiated pituitary hormone-producing cell type. These cells control adrenal growth and development, as well as steroidogenic maturation of fetal adrenal glands. During ontogenesis the first immunopositive ACTH cells appear on fetal day 16 in the *pars distalis* and one day later, in the

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pars intermedia (12). ACTH enters the systemic blood and stimulates the adrenal glands to secrete glucocorticoid hormones corticosterone in rats and cortisol in humans (13). Simmons *et al*. (14) demonstrated that exposure to a chronic stress in the early stages of life results in an alteration of noradrenaline content. On the other hand, the action of glucocorticoid hormones is directed toward the modulation of energy metabolism and immune system, allowing an organism an adaptive response to stress (15), thus providing the homeostasis and survival.

The examinations performed throughout the present study were focused on the morphofunctional characteristics of immunopositive ACTH cells after chronic exposure of adult rat females to constant light as a kind of stress.

Material and Methods

Adult rat females of Wistar strain bred at the Institute for Biological Research in Belgrade were used. One group of animals was exposed to continuous light of 600 lux for 95 days, starting on day 30 of age. The control age-matched rats were maintained under a 12:12 h light-dark cycle, at ambient temperature. Food (a product of D.D. Veterinarski zavod, Subotica, Serbia and Montenegro) and drinking water were available *ad libitum*.

Light microscopy and immunocytochemistry

The pituitary glands were quickly excised upon the sacrifice, fixed in Bouin,s solution for 48 h and embedded in paraffin. Serial 5 μm thick tissue sections were deparaffinized in xylol and serial ethanol. Pituitary hormones were localized by the peroxidase-antiperoxidase complex (PAP) method of Stanberger et al. (16). The endogenous peroxidase activity was blocked by the incubation in 9 mmol hydrogen peroxide solution in methanol for 30 min at ambient temperature. Before the application of the specific primary antisera, nonspecific background staining was achieved by incubating the sections with nonimmune, *i.e.* normal porcine serum diluted with phosphate buffered saline (PBS), pH 7.4, for 60 min. After that, the sections were overlaid with the appropriate dilutions of the specific primary antibodies (hACTH antisera, Dako A/S, Glostrup, Denmark) for 48 h at 4 °C. After washing in PBS, the sections were incubated for another 60 min with the second antibody swine-anti-rabbit IgG for 45 min, rinsed again with PBS for 10 min and incubated with rabbit PAP serum for 45 min. Antibody localization was visualized by incubating the sections in Tris-HCl-buffered saline (0.5 mol/L, pH 7.4), supplemented with 3,3-diaminobenzidine tetrachloride (DAB, Serva, Heidelberg, Germany) and 9 mmol/L hydrogen peroxide. The slides were thoroughly washed under running tap water, counterstained with hematoxylin and mounted in Canada balsam (Alkaliod Pharmaceutical Works, Skopje, Macedonia). Control sections were incubated without primary antisera or by substituting nonimmune rabbit serum for the primary antiserum.

Morphometry

The volume densities (Vv) of the nuclei and the cytoplasm of ACTH-immunoreactive cells, as well as numerical density (Na) of their nuclei *per* μm^3 were measured using 50 test areas of the pituitary gland at a magnification of × 1000, using the multipurpose test system M_{42} (17). The number of the nuclei of immunoreactive ACTH-cells *per* mm^3 was estimated using Wiebel and Gomez (18) formula according to Weibel (17). Since rat ACTH cells are mononuclear, the numerical density of the nuclei (Nv) corresponds to the number of cells *per* mm^3.

$$Nv = (k/\beta) \times (Na^{3/2}/Vv^{1/2})$$

On the basis of earlier karyometric studies, the shape coefficient β of the pituitary cells was estimated to be 1.382. It relates Nv (number of cells counted *per* unit volume) to Na (number of cells counted *per* mm^2) and Vv (volume density) and depends on the axial ratio of the nuclei.

The volume densities of ACTH-positive cells were expressed as percentages of total pituitary cells in μm^3.

Biochemical analyses

Blood samples were collected from each animal and separated plasma samples stored at 20 °C until assayed. The plasma levels of ACTH in control and light-exposed rats were measured by radioimmunoassay (ACTH-IMMULITE kit, Diagnostic Products Corporation, Los Angeles, CA, U.S.A.).

Statistical analyses

Biochemical and morphometric data obtained from each rat were averaged *per* experimental group and standard deviation of the mean was calculated using Student's t-test. A probability value of 5% or less was considered statistically significant.

Results

Immunopositive ACTH cells

The characteristic findings for the immunohistochemically labelled ACTH cells in control rat females can be summarized as it follows: localization between the capillaries, stellate in shape with the cytoplasmic processes among neighbouring mostly somatotrophic cells. The nucleus follows the shape of the cell body. Small, specific secretory granules were distributed mainly at the periphery of the cytoplasm. These cells

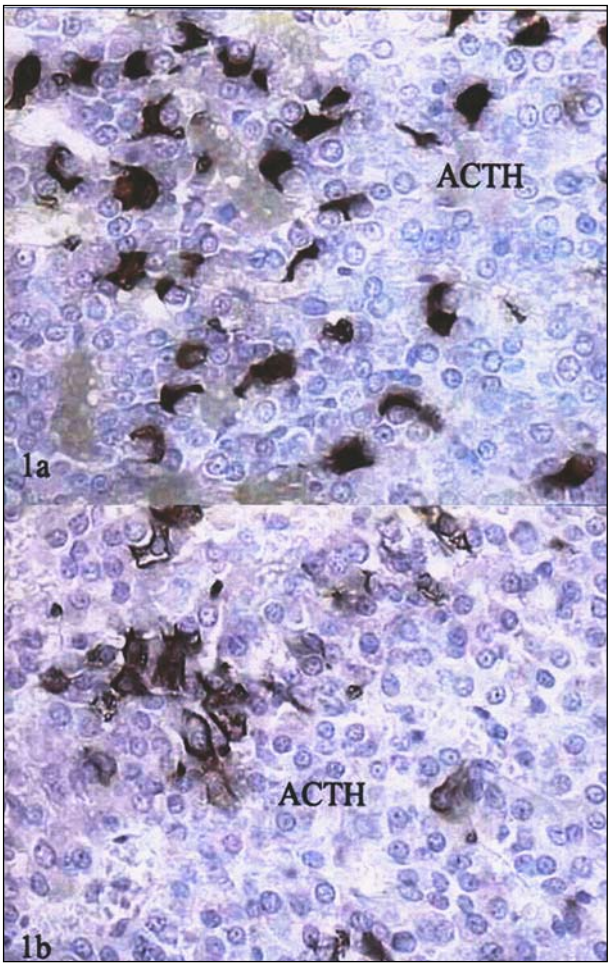

Figure 1. Immunohistochemicaly labelled ACTH cells in: a) control rats, b) in rats chronically exposed to light.

were intensely stained *(Figure 1a)*. In the rats continuously exposed to light, neither the shape nor the localization of ACTH immunoreactive cells were significantly changed in comparison with the controls, but their staining properties were significantly changed *(Figure 1b)*.

The morphometric parameters measured in the present study, i.e. the volume of the ACTH cells and that of their nuclei, as well as volume density are depicted in *Figure 2*. As seen from *Figure 2,* all these morphometric parameters were increased by 22% in rat females chronically exposed to light in comparison with the corresponding controls and the difference was statistically significant ($p < 0.05$).

Also, as shown in *Figure 3,* chronic exposure of rat females to light led to an increase of 13% in plasma ACTH level comparing to the controls and the difference was statistically significant ($p < 0.05$).

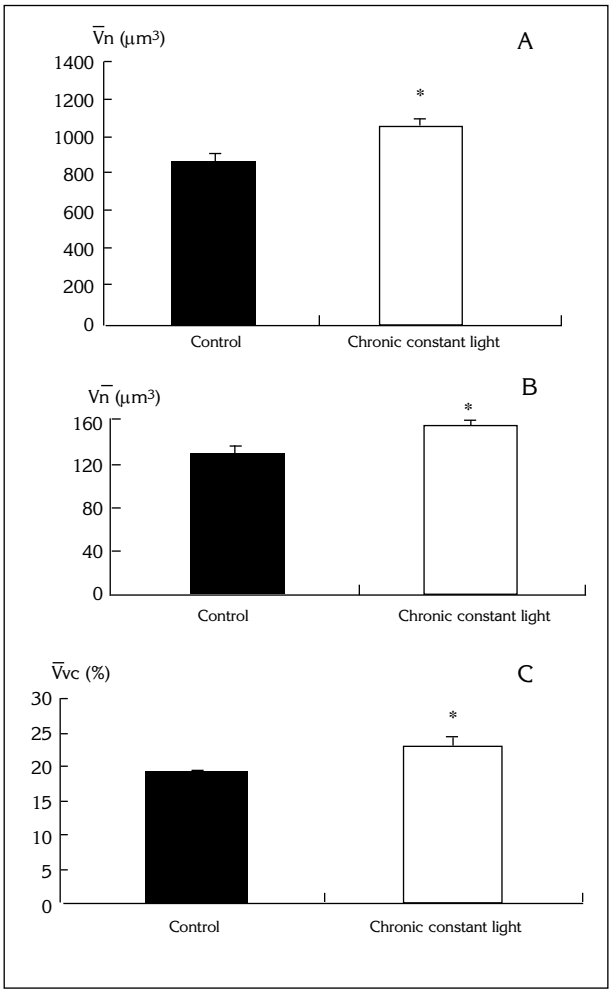

Figure 2. A) Cellular volume ($\bar{V}_c$; μm³) of the immunoreactive ACTH cells B); Nuclear volume ($\bar{V}_n$; μm³) of ACTH cells; C) Relative volume density ($\bar{V}_V$; %) of cells expressed as percentages of total gland tissue. All values are the means ± SD (n=5/group), *p<0.05 v.s. control.

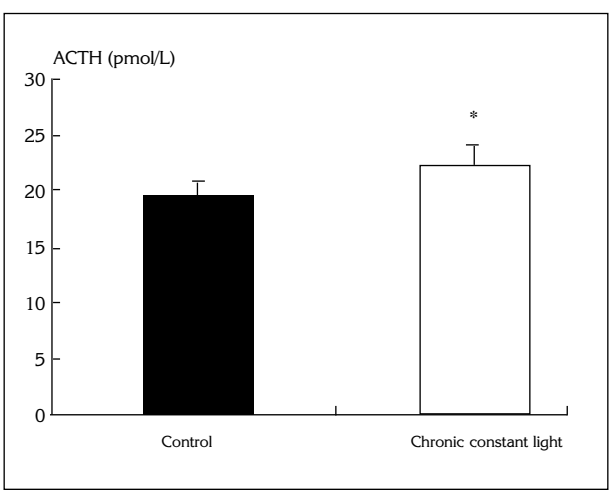

Figure 3. Plasma concentration of ACTH in adult female rats. All values are the means ± SD. (n=5/group), *p<0.05 v.s. control.

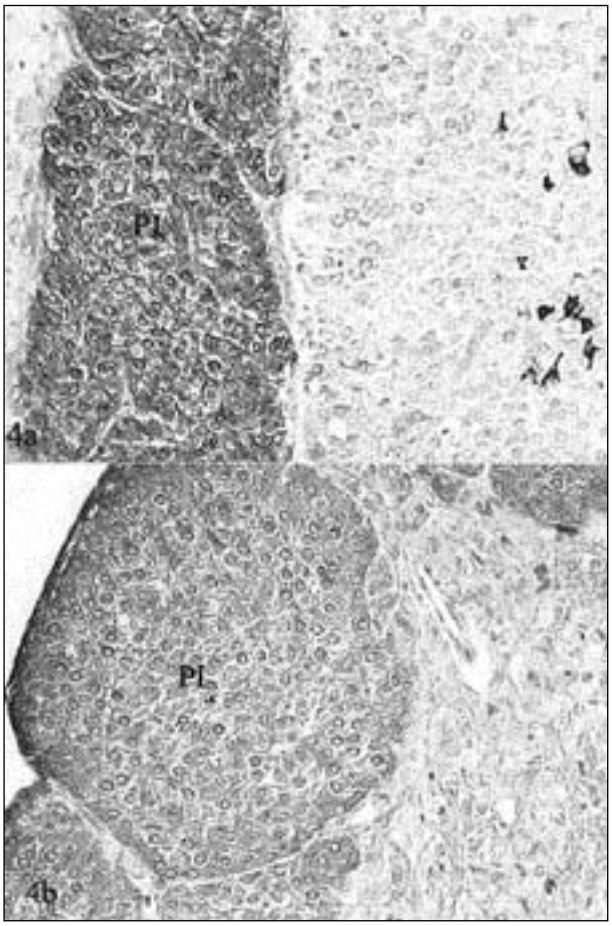

Figure 4. Immunohistochemicaly labelled PI cells in: a) control rats, b) in rats chronically exposed to light.

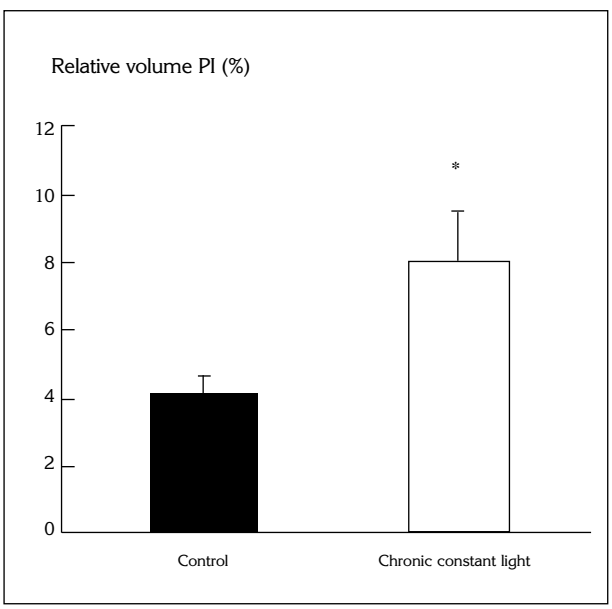

Figure 5. Relative volume (%) of the immunoreactive PI cells in: a) control rats; b) in rats chronically exposed to light. Values are the means ± SD, (n=5/group), *p<0.05 v.s. control

Pars intermedia (PI)

From *Figure 4a,* it can be seen that the PI cells of control rat females are polygonal in shape and contain black granules in various stages of condensation. The PI cells of the rats chronically exposed to light presented in *Figure 4b* were significantly hypertrophic and very light in colour, in comparison with the corresponding controls. Also, dark secretory granules were localized close to the plasma membrane.

Relative volumes of PI cells in light-exposed rat females and the controls are presented in *Figure 5.* It can be seen that this parameter was increased as much as by 95% in animals exposed to continuous light for 95 days comparing to the controls and the difference was statistically significant ($p < 0.05$).

Discussion

The results obtained throughout the present study demonstrate that long-term exposure of adult rat females to light acted significantly increasing all morphometric parameters of pituitary adrenocorticotropes as compared to the controls maintained under conditions of 12/12 h light-dark cycle. Plasma level of ACTH was also significantly increased in the former group of animals in relation to the control value. These results are in accordance with the data of Ivanišević-Milovanović *et al.* (19) who reported that the expo-sure of rat females to constant light for six weeks led to a significant increase of both plasma ACTH level and the synthesis of epinephrine in the adrenal gland. It was observed earlier that chronic exposure of adult rat females to constant light activates the stress system, namely, paraventricular infundibular CRH (corticotropin-releasing hormone) system (9). Besides, the same treatment resulted in various disturbances in the functioning of the reproductive system in rat females (20). The mechanisms responsible for the circadian release of CRH and ACTH are still far from being fully understood, but appear to be controlled by one or more pacemakers, including the suprachiasmic nucleus (SCN) (21). Some studies are consistent in claiming that the neuroendocrine mediation of the stress response is very probably multifactorial (22) and that the SCN output, important for neuroendocrine rhythm, is axonal rather than humoral (23). The ACTH and PRL cells play an important role in the regulation of behavioural adaptation of an organism to environmental changes. The data of Pantić (24) showed a reciprocal interrelationship in the response of PRL and ACTH-corticosterone system to stress.

Some authors reported that exposure of adult rat females to constant light led to development of polycystic ovaries, similar to that seen in human idiopathic polycystic ovarian disease (5, 8). In addition, it was shown that exposure of rat females to continuous light leads to an increased ACTH secretion which is responsible for the appearance of ovarian endocrine dysfunc-

tion, perhaps through the ACTH effect on the adrenal cortex (19, 25). However, it should be mentioned also that Byerley *et al*. (26) reported that the short exposure of animals to bright light does not affect pituitary ACTH production.

Our results showed that the *pars intermedia* (PI) cells of adult rat females chronically exposed to light of 600 lux were significantly hypertrophic and very light in colour, in comparison with the corresponding controls. In these cells, dark secretory granules occur in a very close proximity to the cell plasma membrane. These granules appear to be specific storage for the ACTH molecule precursor, POMC (24). Our data also demonstrated that relative volume of PI cells was significantly increased in rat females chronically exposed to light in comparison with the corresponding controls. It was reported earlier that the pituitary PI cells are the major site of transcription of the gene coding for POMC, a precursor protein molecule of the ACTH, α-melanostimulating hormone (α-MSH), opioids and some other peptides (11).

On the basis of the results obtained throughout this study, it can be concluded that continuous exposure of adult rat females to constant light of 600 lux, significantly activates not only the ACTH cells of the *pars anterior* but also the pituitary PI cells.

Acknowledgements This work was partially supported by the Ministry for Science, Technology and Development of Serbia, grants #1710 and #1385. The authors are especially grateful to Ms Nada Bujišić (»Belladona Laboratories«, Belgrade, Serbia) for her help.

OSOBINE IMUNOPOZITIVNIH ACTH ĆELIJA ŽENKI PACOVA POSLE DUGOTRAJNOG IZLAGANJA STALNOM SVETLU

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Kratak sadržaj: Ispitivani su efekti dugotrajnog izlaganja ženki Wistar pacova stalnom svetlu, na adrenokortikotropne (ACTH) ćelije. Eksperimentalne životinje stare 30 dana izlagane su konstantnom svetlu (600 luksa) tokom 95 dana. Kontrolne životinje odgovarajuće starosti su držane na normalnom, 12:12 h, dnevno-noćnom režimu i sobnoj temperaturi. ACTH ćelije su imunohistohemijski bojene metodom peroksidaza-antiperoksidaza (PAP), a istovremeno je sakupljana krv za određivanje koncentracije ACTH u plazmi. Životinje izložene stalnom svetlu imale su za 22% povećane sve morfometrijske parametre ACTH ćelija merene tokom ovog rada (zapremina ćelija, njihovih jedara i volumenska gustina) u odnosu na kontrole i uočene razlike su bile statistički značajne ($p < 0.05$). Koncentracija ACTH u plazmi je takođe bila veća za 13% ($p < 0.05$) u odnosu na kontrolne vrednosti. Ovi rezultati ukazuju da dugotrajno izlaganje stalnom svetlu na specifičan način stimuliše rast i sekretornu aktivnost ne samo ACTH ćelija adenohipofize već i ćelija *pars intermedia* ženki pacova.

Ključne reči: Stalno svetlo, ACTH ćelije, ACTH, stres, ženke pacova.

References

1. Freeman M, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function and regulation of secretion. Physiological Reviews 2000; 80: 1523 631.
2. Blake CA. Effect of pinealectomy on the rat oestrus cycle and pituitary gonadotrophin release. J Endocrinol 1976; 69: 67 75.
3. Pieper DR Gala RR. The effect of light on the prolactin surges of pseudopregnant and ovariectomized, estrogenized rats. Biol Reprod 1979; 20: 727 32.
4. Hoffman JC, Cullin AM. Effect of pinealectomy and constant light on the estrous cycle of female rats. Neuroendocrinology 1975; 17: 167 74.
5. Mahajan DK. Polycystic ovarian disease: animal models. Endocrinol Metab Clin North Am 1988; 17: 705 32.
6. Leproult R, Colecchia E, L'Hermite-BalŒriaux M, Van Cauter E.: Transition from dim to bright light in the morning induces an immediate elevation of cortisol levels. J Clin Endocrinol Metab 2001; 86: 151 7.
7. Miller JD, Morin LP, Schwarts WJ, Moore RY. New insights into the mammalian circadian clock. Sleep 1996; 19: 641 67.

8. Baldissera SF, Motta LD, Almeida MC Antunes-Rodrigues J. Proposal of an experimental model for the study of polycystic ovaries. Braz J Med Res 1991; 24: 747 51.

9. Liposits ZI, Lengravi I, Vigh S, Gorcs T, Setalo B, Flerko B. The paraventriculo-infudibular corticotropin releasing factor (CRF)-immunoreactive system of the rat-morphological aspects. In: Mc Kerns KW, Pantić V. eds. Endocrine correlates of stress. New York: Plenum press 1984; 1 20.

10. Molitch M. Pituitary response to stress: growth hormone and prolactin. In Ober PK, ed. Endopcrinology of Critical Disease.Totowa NJ: Humana Press 1997; 67 86.

11. Pantić V. Biology of hyphothalamic neurons and pituitary cells. International Rewiew of Cytology 1995; 159: 1 112.

12. Chatelainen A, Dupouy JP, Dubois MP. Ontogenesis of cells producing polypeptide hormones (ACTH, MSH, LPH, GH, Prolactin) in the fetal hypophysis of the rat: influence of the hypothalamus. Cell Tissue Res 1974; 196: 409 27.

13. Nankova B, Kvetnansky R, Hiremagalur B, Sabban B, Rusnak M, Sabban EL. Immobilization stress elevates gene expression for catecholamine biosynthetic enzymes and some neuropeptides in rat sympathetic ganglia: Effects of adrenocorticotropin and glucocorticoids. Endocrinology 1996; 137: 5597 604.

14. Simmons RO, Kellog CK, Miller RK. Prenatal diazepam exposure in rats: long-lasting receptor-mediated effects on hypothalamic norepinefrine-containing neurons. Brein Res 1984; 293:73 83.

15. Ehrhart-Bornstein M, Bornstein SR, Schermbaum WA. Sympathoadrenal system and immune system in the regulation of adrenocortical function. Eur J Endocrinol 1996; 135: 19 26.

16. Sternberger LA, Hardy PHJ, Cuculius JJ & Meyer HG: The unlabelled antibody enzyme method of immunohistochemistry. Preparation and prope-rties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 1970; 18: 315 33.

17. Weibel ER: Stereological methods. 1. Practical methods for biological morphometry. New York: Academic Press, 1979; 1 415.

18. Weibel ER, Gomez DM: A principle for counting tissue structures on random sections. J Appl Physiol 1962; 17: 343 8.

19. Ivanišević-Milovanović O, Demajo M, Karakašević A, Pantić V. The effect of constant light on the concentracion of catecholamins of the hypothalamus and adrenal glands, circulatory adrenocorticotropin hormone and progesterone. J Endocrinol Invest 1995; 18: 378 83.

20. Kasaka T, Sawada T, Ichikawa S. Effect of continous illumination on secretion of pregnane compounds by the rat ovary Experientia 1988; 44: 43 5.

21. Stratakis CA, Chrosusos GP. Hypothalamic hormones: GnRH, TRH, GHRH, SRIF, CRH and dopamine. In: Conn PM, Melmed S. eds. Endocrinology: Basic and Clinical Principles. Totowa: Humana Press Inc, 1997; 185 209.

22. Gala RR. The physiology and mechanisms of the stress induced changes in prolactin secretion in the rat. Life Sci 1990; 46: 1407 20.

23. Mayer-Bernstain E, Jetton A, Matsumoto S-I, Markuns J, Lehman M, Bittman E. Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters. Endocrinology 1999; 140: 207 18.

24. Pantić V. Gonadal steroids as modulators of genetically programmed development of neuroendocrine cells involved in regulation of growth and reproduction. Bulletin T. CII de l Academie Serbe des Sciences et des Arts 1990; 32: 47 66.

25. Cvijić G, Janić-Šibalić V, Demajo M, Karakašević A, Petrović V, Ivanišević-Milovanović O. The effects of continuous light and darkness on the activity of monoamine oxidase A and B in the hypothalamus, ovaries and uterusss of rats. Acta Physiological Hungarica, 1998; 85: 269 76.

26. Byerley WF, Risch Sc, Gillin JC, Janowsky DS, Parker D, Rossman LG, Kripke DF. Biological effect of bright light. Prog Neuropsyhopharmacol Biol Psychiatry 1989; 13: 683 6.

Received: January 10, 2003

Accepted: February 20, 2003