IMMUNOHISTOMORPHOMETRIC CHARACTERISTICS OF PITUITARY GH CELLS IN INFANT AND PERIPUBERTAL FEMALE RATS AFTER TREATMENT WITH ESTRADIOL OR HUMAN CHORIONIC GONADOTROPIN

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IMUNOHISTOMORFOMETRIJSKE KARAKTERISTIKE GH ĆELIJA HIPOFIZE KOD VEOMA MLADIH I PERIPUBRTALNIH ŽENKI PACPVA TRETIRANIH ESTRADIOLOM ILI HUMANIM HORIONSKIM GONADOTROPINOM

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Received / Primljen: 30. 10. 2015. Accepted / Prihvaćen: 06. 11. 2015.

ABSTRACT

The effects of estradiol-dipropionate (EDP) or human chorionic gonadotropin (hCG) on immunohistomorphometric characteristics of pituitary GH cells in infant and peripubertal female rats were investigated. The first group of females received five injections of EDP (0.25 mg/kg b.w.) during the neonatal period of life, and was further divided into two subgroups which were sacrificed at the infantile period (17th day) or at the peripubertal period (38th day). The second group received two doses of hCG (50 IU/kg b.w.) on the 15th and 16th day of life in the first subgroup, and on the 36th and 37th days of life in the second subgroup, while they were sacrificed 24 h after the last treatment, respectively. The control females were injected with an equivalent volume of the vehicle and sacrificed according to the appropriate schedules as the hormone treated rats. EDP treatment decreased GH cell volume density in infant and peripubertal females, by 38% and 76% ($p<0.05$) respectively, in comparison with the controls. The number of GH cells per mm^2 in infantile and peripubertal period was decreased in EDP treated animals by 26% and 53% ($p<0.05$) respectively, compared to the controls. Also, upon EDP treatment in both periods, GH cells were diminished in size and less intensely immunolabelled than in the control groups. The morphometric parameters in animals treated with hCG were insignificantly changed in both analyzed periods, in comparison with the controls. Unlike hCG, EDP manifested clear inhibitory effects on the immunohistomorphometric characteristics of GH cells in examined female rats.

Key words: *female rat, infant, peripubertal, GH cells, estradiol, human chorionic gonadotropin*

SAŽETAK

U studiji su ispitivani efekti estradiol dipropionata (EDP) i humanog horionskog gonadotropina na imunohistomorfometrijske karakteristike hipofiznih GH ćelija veoma mladih i peripubertalnih ženki pacova. Prva grupa ženki je tokom neonatalnog perioda života primila pet injekcija EDP-a (0.25 mg/kg b.w.), a naknadno je podeljena na dve podgrupe koje su žrtvovane kao veoma mlade (17. dan) ili u peripubertalnom periodu (38. dan). Druga grupa je primila dve doze hCG-a (50 IU/kg b.w.) 15. i 16. dana života (prva podgrupa), odnosno 36. i 37. dana života (druga podgrupa), a žrtvovane su 24h nakon poslednjeg tratmana, ponaosob. Kontrolne ženke pacova su primile ekvivalentan volumen rastvarača i žrtvovane su po obrascu koji je važio za hormonima tretirane grupe. Tretman EDP-om je prouzrokovao smanjenje volumenske gustine GH ćelija kod veoma mladih i peripubertalnih ženki pacova za 38% odnosno 76% ($p<0.05$) u poređenju sa kontrolama. Broj GH ćelija po mm^{2}kod veoma mladih i peripubertanih životinja je smanjen nakon EDP tretmana za 26% odnosno 53% ($p<0.05$) poredeći sa kontrolnim vrednostima. Takođe, tretman EDP-om u oba perioda je izazvao smanjenje dimenzija i intenziteta imunobojenja GH ćelija u odnosu na kontrole. Morfometrijski parametri kod životinja tretiranih hCG-om u oba perioda nisu značajno promenjeni u poređenju sa kontrolnim vrednostima. Za razliku od hCG-a, EDP je ispoljio jasne inhibitorne efekte na imunohistomoprfometrijske karakteristike GH ćelija kod ispitivanih ženki pacova.

Ključne reči: *ženke pacova, veoma mlade, peripubertalne, GH ćelije, estradiol, humani horionski gonadotropin*

DE GRUYTER OPEN

UDK: 577.175.322 / Ser J Exp Clin Res 2015; 16 (4): 281-285
DOI: DOI: 10.1515/SJECR-2015-058

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INTRODUCTION

In the present study we aimed to investigate the immunohistomorphometric changes of pituitary growth hormone (GH) producing cells upon application of synthetic hormones, estradiol-dipropionate (EDP) or human chorionic gonadotropin (hCG), to infant and peripubertal female rats. GH cells represent the GH/insulin-like growth factor (IGF1) axis specific operative modul, being controlled by hypothalamic, intrapituitary and peripheral signals in the function of somatic development (1). The period of growth and development of individuals from birth to sexual maturation is particularly sensitive when it comes to GH/IGF1 axis functioning and considers the prolonged phases of GH cells intensive activity as well as their "silencing" (2). Namely, besides the daily-based pulsatile GH secretion in young individuals of both genders (3), maturing females express the luteinizing hormone (LH) increase coinciding decline in circulating GH/IGF1 levels (4, 5), while high circulating estradiol is followed with GH elevation (6, 7, 8). Generally, it is well founded that estradiol has stimulatory effect on the pituitary weight of female rats when applied in critical neonatal period of life (9, 10, 11, 12) as well as that increases the number of chromophobes, prolactin (PRL), luteinizing hormone- (LH) and follicle stimulating hormone- (FSH) producing cells (9, 13, 14, 15, 16). On the other hand, immunohistomorphometric features of pituitary adrenocorticotropic (ACTH) cells of infant and peripubertal female rats appear decreased upon application of estradiol (11, 12). It was reported that, unlike PRL, FSH and LH cells, less than 5% of ACTH cells express estrogen receptor α (ERα) in the human pituitary (17). Also, some novel studies suggest that ERs are substantially involved in GH gene expression (18, 19).

It should be also pointed out that hCG is a heterodimeric glycoprotein, composed of 244 amino acids, with two subunits: α (alpha), identical to that of FSH, LH and thyroid stimulating hormone (TSH); and β (beta), responsible to hormone-specific functions (20). hCG interacts with the luteinizing hormone/choriogonadotropin (LHCG) receptor (21), while at the level of young female rat pituitary significantly increases the number of FSH and LH, and volume of TSH cells (9, 10). ACTH cells upon hCG treatment of juvenile female rats express decreased volume density, while the treatment of peripubertal female rats with the same hormone causes the increase of ACTH cell morphometric parameters (11, 12).

As a continuation of our previous studies in the field of EDP and hCG effects on various pituitary hormone-producing cell histological features in the sensitive periods of female rat life cycle (9, 10, 11, 12), this study is focused on the same, less studied features of developmentally relevant GH cells and relies on up-to-date immunohistomorphometric approach, as a gold standard in modern quantitative histology.

MATERIAL AND METHODS

Animals

In the experiment were used infant (sacrificed at the 17th day of life) and peripubertal (sacrificed at the 38th day of life) Wistar female rats, bred in the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. All animals were individually in cages, under controlled conditions (12:12 light/dark, room temperature - 22°C), with free access to food (a product of the Veterinary Institute Subotica, Subotica, Serbia) and water.

All animal procedures complied with the European Communities Council Directive (86/609/EEC) and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (No 2–12/13).

The first group of female rats received five intraperitoneal (*i.p.*) injections of estradiol dipropionate (EDP; 0.25 mg/kg b.w., ICN-Galenika Pharmaceuticals, Belgrade, Serbia) every second day from the 4th to the 14th day after birth. After the treatment with EDP, the animals were further divided into two subgroups which were sacrificed at the infantile period (17thday) and at the peripubertal period (38thday). The control females were injected with an equivalent volume of sterile olive oil and sacrificed according to the same schedule as the EDP treated rats.

The second group of females *i.p.* received two doses of pregnyl-gonadotrophinumchorionicum (hCG; 50 IU/kg b.w.; N.V. Oregon, Netherlands) on the 15th and 16th day of life in the first subgroup, and on the 36th and 37th days of life in the second subgroup. Treated females from both subgroups were sacrificed 24 h after the last treatment *i.e.* on the days 17th (infantile period) and 38th (peripubertal period). The control females were injected with an equivalent volume of the vehicle and sacrificed according to the same schedule as the hCG treated rats.

Light microscopy and immunocytochemistry

After decapitation the pituitary glands were excised, weighted, fixed in Bouin's solution for 48h, dehydrated in a series of increasing concentrations of ethanol and enlightened in xylol. After embedding in paraplast, each tissue block was serially sectioned at 5 μm thicknesses on a rotary microtome (RM2125 RT Leica Microsystems, Wetzlar, Germany). Sections were deparaffinized in xylol, rehydrated in a series of decreasing concentration of ethanol and immunolabelled using the peroxidase-antiperoxidase (PAP) method of Sternberger et al. (22). The immunohistochemicall procedure is described in detail in our previous work (23). Digital images of the pituitary gland immunolabeled sections were taken using a LEITZ DM RB light microscope (Leica Mikroskopie & Systems GmbH, Wetzlar, Germany), a Leica DFC320 charged coupled device (CCD) Camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and the Leica DFC Twain Software (Leica, Germany).

Morphometry

Morphometric assessment was performed using a light microscope (Olympus, BX-51, Olympus, Japan) equipped with a microcator (Heidenhain MT1201, Heidenhain, USA) to control movements in the z-direction (0.2 µm accuracy), amotorized stage (Prior, Prior Scientifi Inc., USA) for stepwise displacement in the x–y direction (1 µm accuracy), and a CCD video camera (PixeLink, PixeLINK, Canada) connected to a 19" computer monitor. Stage movement was controlled by the newCAST stereological software package (VIS – Visiopharm Integrator System, ver.2.12.1.0; Visiopharm; Denmark) running on a personal computer.

Volume density estimation was used to determine the percentage of immunopositive GH cells in the anterior pituitary gland of experimental and control females. Two pituitary sections from the superior, three from the middle and two from the inferiorpart (seven horizontal sections) of the rat pituitary glands were analyzed (the same sections were used in the subsequent estimation of number of GH cells *per* unit area-mm^2). The counting area was defined using a mask tool. An interactive test grid with uniformly spaced test points for histomorphometric assessment was provided by the newCAST softare.

Test points hitting the immunopositive GH cells and to the uncolored phase of anterior pituitary were determined. Volume densities (V_V) of GH cells were calculated as the ratio of the number of points hitting immunopositive GH cells with nuclei divided by the number of points hitting the uncolored phase of the anterior pituitary:

V_V (%) = Pp / Pt x 100.

Pp - points hitting the immunopositive GH cells with nuclei,

Pt-points of the test system hitting the uncolored phase of anterior pituitary.

Volume density of GH cells was calculated for each analyzed section. Then, the average value for seven analyzed sections was calculated and represents the volume density of GH cells in pituitary gland (23).

The number of GH cells *per* mm^2 was also calculated. In the first step, the areas of analyzed sections were determinated by Measure Properties option (Polygon area) and then, by simple point counting, the number of immunopositive GH cells was estimated. Additionally, the number of GH cells was expressed *per* unit area (mm^2).

RESULTS

Immunopositive GH cells in infant and peripubertal rats were located in *pars distalis* of pituitary gland and appeared ovoid or pyramidal in shape, with pronounced round nuclei (1 A, D). In animals treated with EDP, in both infantile and peripubertal subgroup, GH cells were diminished in size and less intensely immunolabelled than GH cells in the control groups (Fig. 1B, E). In peripubertal EDP treated subgroup of female rats the individual GH cells with cytoplasmatic processes can be observed (Fig. 1E). GH cells in hCG treated animals in both periods were similar in size and shape as in the controls (Fig. 1C, F), but the immunolabelling for GH cells was less intense in peripubertal subgroup than in the controls (Fig.1F, D).

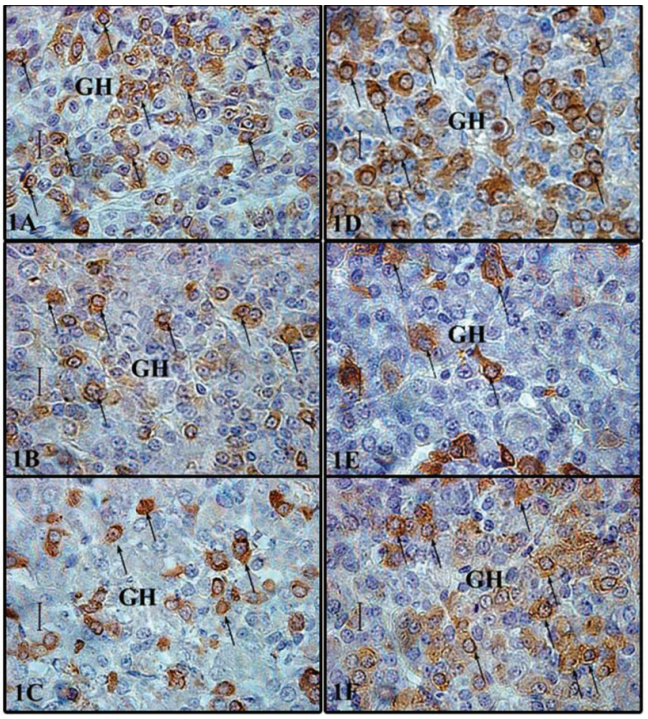

Figure 1. GH-immunopositive cells in the *pars distalis* of pituitary gland from **A**- control infant females, **B** - infant females treated with EDP, **C** – infant females treated with hCG; **D** - peripubertal control females, **E** - peripubertal females treated with EDP, **F** – peripubertal females treated with hCG.

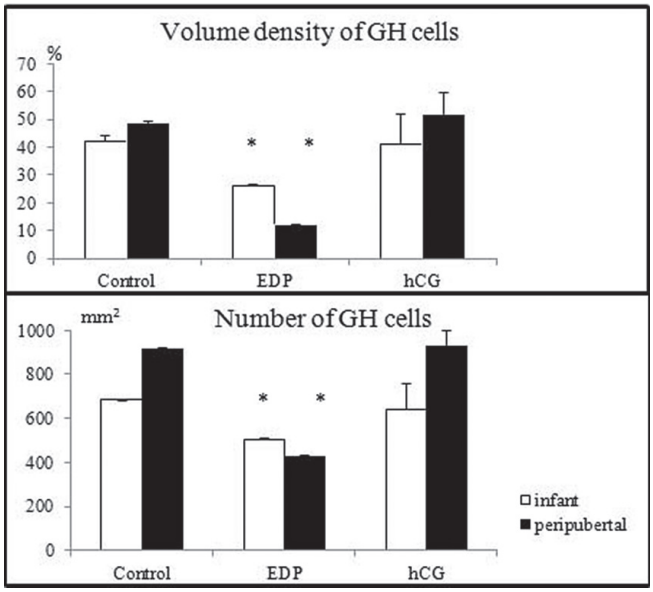

Figure 2. Volume density (A) and number of GH cells per mm^2 (B) in control, estradiol dipropionate (EDP) and human chorionic gonadotropin (hCG) treated infant and peripubertal female rats. All values are means ± standard deviation, n = 5 animals *per* group, *p<0.05 *vs.* control.

Morphometric analysis has shown that EDP treatment significantly decreased volume density in infant (by 38%; $p<0.05$) and peripubertal females (by 76%; $p<0.05$), in comparison with the controls (Fig. 2A). This parameter in animals treated with hCG was insignificantly ($p>0.05$) changed in both analyzed periods, in comparison with the controls (Fig. 2A). The number of GH cells *per* mm^2 in infantile and peripubertal period was significantly decreased in EDP treated animals by 26% and 53% ($p<0.05$) respectively, compared to the controls (Fig. 2B). This parameter in hCG treated subgroups was not significantly ($p>0.05$) changed, in comparison with the adequate control animals (Fig. 2B).

DISCUSSION

The aim of this study was to examine the effects of the treatments with synthetic estradiol or hCG on the immunohistomorphometric features of pituitary GH cells during the infantile and peripubertal period of female rat development. Using a gold-standard approach in modern quantitative histology, we have shown that the EDP treatment caused significant reduction in volume density and number *per* mm^2 of GH cells in infant and peripubertal females. Treatment with hCG didn´t affect on examined immunohistomorphometric parameters in the same stages of development.

The influence of sex steroids on GH/IGF1 axis during puberty is well documented in animal and human studies (24). During that period of life estrogen induces the stimulation of the GH/IGF1 axis and a pubertal growth spurt (25). Although the pubertal rise in sex steroids and activation of GH/IGF1 axis is fundamental for attainment of a normal pubertal growth spurt in both sexes, exposure to sex steroids during the neonatal period is also required (6). Namely, the number and organization of GHRH and SS neurons, GH cells and GH secretory pattern are affected by the neonatal sex steroid environment (24). The number of GHRH mRNA containing neurons in the hypothalamus and GH cells in anterior pituitary of adult male rats is significantly greater compared to that found in females (26). Indeed, the number of GHRH neurons in the early neonatal period does not differ between male and female rats, but in females a progressive loss of neurons occurs until approximately 20 days of age (24). Reduction of GHRH neurons and GH cells is gender-specific and may be caused by the action of estrogen in neonatal period. It is possible that the exogenous estrogen in our experimental conditions exhibits pronounced endogenous estrogen-like effect on the GHRH neurons and GH cells, which caused a reduction in volume density and the number of GH-cells in the anterior pituitary of infant and peripubertal female rats. It was demonstrated that estrogen stimulates the hypothalamic production of somatostatin (27), which also may contribute to the reduction of morphometric parameters of GH cells in our study. These results indicate that the exposure to exogenous estrogen during neonatal period has some permanent effects on the immunostaining properties, volume density and number of GH cells, which partially determines the ability of this gland to produce and secrete growth hormone throughout life.

Human chorionic gonadotropin is a heterodimeric glycoprotein with a numerous functions, mainly related to the maintenance of pregnancy. In our experimental conditions hCG did not affect the morphometric features of GH cells in infant and peripubertal female rats. The available literature data didn't indicate the connection between hCG and GH cells, so we can conclude that GH cells supposedly don't figure as the target cells for applied hCG in the analyzed periods.

Based on these results, it can be concluded that EDP has an inhibitory effect on GH cell immunohistomorphometric characteristics, while the treatment with hCG didn't affect this histological aspect of GH cells in infant and peripubertal female rats.

Acknowledgement

The study was supported by Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant N° ON173009). The authors thank to Mr. Goran Granić (ProMedia d.o.o, Kikinda, Serbia) for technical support.

REFERENCES

1. Melmed S. Idiopathic adult growth hormone deficiency. J Clin Endocrinol Metab 2013; 98(6): 2187-97.
2. McAndrews JM, Stroud CM, MacDonald RD, Hymer WC, Deaver DR. Age-related changes in the secretion of growth hormone in vivo and in vitro in infantile and prepubertal Holstein bull calves. J Endocrinol 1993; 139(2): 307-15.
3. Miller JD, Tannenbaum GS, Colle E, Guyda HJ. Daytime pulsatile growth hormone secretion during childhood and adolescence. J Clin Endocrinol Metab 1982; 55(5): 989-94.
4. Ojeda SR, Jameson HE. Developmental patterns of plasma and pituitary growth hormone (GH) in the female rat. Endocrinology 1977; 100(3): 881-9.
5. Woller MJ, Everson-Binotto G, Nichols E, Acheson A, Keen KL, Bowers CY, Terasawa E. Aging-related changes in release of growth hormone and luteinizing hormone in female rhesus monkeys. J Clin Endocrinol Metab 2002; 87(11): 5160-7.
6. Jansson JO, Edén S, Isaksson O. Sexual dimorphism in the control of growth hormone secretion. Endocr Rev 1985; 6(2): 128-50.
7. Mauras N, Rogol AD, Haymond MW, Veldhuis JD. Sex steroids, growth hormone, insulin-like growth factor-1: neuroendocrine and metabolic regulation in puberty. Horm Res 1996; 45(1-2): 74-80.

8. Meinhardt UJ, Ho KK. Modulation of growth hormone action by sex steroids. Clin Endocrinol (Oxf) 2006; 65(4): 413-22.
9. Sekulić M, Šošić-Jurjević B, Filipović B, Manojlović-Stojanoski M, Milošević V. Immunoreactive TSH cells in juvenile and peripubertal rats after estradiol and human chorionic gonadotropin treatment. Acta Histochem 2006; 108(2): 117-23.
10. Milošević V, Todorović D, Veličković M, Ristić N, Ušćebrka G, Knežević V, Ajdžanović V. Immunohistomorphometric features of ACTH cells in juvenile rats after treatment with estradiol or human chorionic gonadotropin. J Med Biochem 2012; 31(1): 34-9.
11. Milošević V, Todorović D, Šošić-Jurjević B, Medigović I, Pantelić J, Ušćebrka G, Ajdžanović V. The effects of estradiol and human chorionic gonadotropin on ACTH cells in peripubertal female rats: a histological and stereological study. Arch Biol Sci 2014; 66(1): 143-8.
12. Pantić V (1980). Adenohypophyseal cell specificities and gonadal cell steroids. In: M. Jutz & K.W. McKerns (Eds.), *Synthesis and Release of Adenohypophyseal Hormones* (pp. 335-62). New York: Plenum Press.
13. Pantić V. Biology of hypothalamic neurons and pituitary cells. Inter Rev Cytol 1995; 159: 1-112.
14. Van Bael A, Denef C. Evidence for a trophic action of the glycoprotein hormone subunit in rat pituitary. J Neuroendocrinol 1996; 8(2): 99-102.
15. Medigović I, Manojlović-Stojanoski M, Trifunović S, Ristić N, Milošević V, Žikić D, Nestorović N. Effects of genistein on gonadotropic cells in immature female rats. Acta Histochem 2012; 114(3): 270-5.
16. Friend K, Chiou Y, Lopes M, Laws E, Hughes K, Shupnik M. Estrogen receptor expression in human pituitary: correlation with immunohistochemistry in normal tissue, and immunohistochemistry and morphology in macroadenomas. J Clin Endocrinol Metab 1994; 78(6): 1497-504.
17. Zárate S, Seilicovitch A. Estrogen receptors and signaling pathways in lactotropes and somatotropes. Neuroendocrinology 2010; 92(4): 215-23.
18. Avtanski D, Novaira HJ, Wu S, Romero CJ, Kineman R, Luque RM, Wondisford F, Radovick S. Both estrogen receptor α and β stimulate pituitary GH gene expression. Mol Endocrinol 2014; 28(1): 40-52.
19. Pierce J, Parsons T. Glycoprotein hormones: structure and function. Ann Rev Biochem 1981; 50: 466-95.
20. Gregory J, Finlay J. Alpha-fetoprotein and beta-human chorionic gonadotropin: their clinical significance as tumour markers. Drugs 1999; 57(4): 463-7.
21. Sternberger LA, Hardy PHJ, Cuculius JJ, Meyer HG. The enlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J HistochemCytochem 1970; 18(5): 315–33.
22. Ajdžanović V, Medigović I, Živanović J, Šošić-Jurjević B, Trifunović S, Tanić N, Milošević V. Immuno-histomorphometric and – fluorescent characteristics of GH cells after treatment with genistein or daidzein in an animal model of andropause. Acta Vet 2014; 64(1): 93-104.
23. Jurišić M, Manojlović-Stojanoski M, Andrić M, Koković V, Danilović V, Jurišić T, Brković B. Histological and morphometric aspects of Ridge preservation with a moldable, *in situ* hardening bone graft substitute. Arch Biol Sci 2013; 65(2): 429-37.
24. Chowen JA, Frago LM, Argente J. The regulation of GH secretion by sex steroids. Eur J Endocrinol 2004; 151: U95-100.
25. Perry RJ, Farquharson C, Ahmed SF. The role of sex steroids in controlling pubertal growth. Clin Endocrinol (Oxf) 2008; 68(1): 4-15.
26. Chowen JA, Argente J, González-Parra S, García-Segura LM. Differential effects of the neonatal and adult sex steroid environments on the organization and activation of hypothalamic **growth hormone-releasing hormone and somato**statin neurons. Endocrinology1993; 133(6): 2792-802.
27. Frawley LS, Hoeffler JP. Hypothalamic peptides affect the ratios of GH and PRL cells: role of cell division. Peptides 1988; 9(4):825-8.