Int J Clin Exp Med 2016;9(8):16964-16970
www.ijcem.com /ISSN:1940-5901/IJCEM0027708

Original Article

Effects of light stress on the expression of Kisspeptin/GnRH in rat hypothalamus

Li Luo[1], Youmin Wang[1,2], Datong Deng[1], Yuan Cheng[3], Huanli Zhou[2]

[1]Department of Endocrinology, Institude of Endocrinology and Metabolism, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China; [2]Anhui Provincial Laboratory of Endocrinology and Metabolism, Hefei, Anhui, China; [3]Department of Endocrinology, Institude of Endocrinology and Metabolism, The Second Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China

Received March 8, 2016; Accepted August 15, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Objective: This study aims to explore the effects of light stress on the expression of Kisspeptin/GnRH in rat hypothalamus. Methods: The rat model of chronic stress was established through artificial illumination time. The rats were divided into control group, continuous light group, interval light group and reverse day and night group randomly. The expression levels of Kisspeptin and GnRH in rat hypothalamus were detected by RT-PCR, western-blotting and immunohistochemical methods. Results: The results showed that the expression levels of Kisspeptin and GnRH in rat hypothalamus significantly decreased in continuous light group and reverse day and night group ($P<0.05$), there was no difference between interval light group and control group ($P>0.05$). Conclusions: Long time light stress can decrease the expression levels of Kisspeptin and GnRH in rat hypothalamus.

Keywords: Hypothalamus, kisspeptin, GnRH, chronic stress

Introduction

Stress is a series of reactions regulated by nerve endocrine system when the body encountered adverse stimuli and disrupt the physiological or psychological state. Long chronic stress can lead to sub-health state and even develop to disease [1, 2]. There are many clinical diseases related with stress especially in reproductive endocrine disorders. Chronic stress has become the important pathogenic factor for menstrual disorders, amenorrhea, infertility and sexual dysfunction [3, 4]. Infertility has become a factor affecting social stability with its incidence increasing year by year. Therefore, it is important to investigate the pathogenesis of gonadal disorders induced by chronic stress and find potential targets and effective drugs.

Kisspeptin/GnRH signaling pathway plays an important role in the reproductive endocrine system. Kisspeptin is a neuropeptide of retinoic acid family and encoded by the Kiss-1 gene, its receptor was Gprotein-coupled receptor54 (GPR54). They widely distributed in the brain and body organs of vertebrate. Kisspeptin plays keys role in the activation of gonadotropin releasing hormone (GnRH) neurons in human puberty, the regulation of release of gonadotropin LH and FSH and the development of reproductive organs [5]. The expression of KiSS-1 increased gradually with the mature and GnRH neurons were activated, the release of LH and FSH was promoted and maintained the individual's reproductive ability. Previous studies suggested that the release LH and FSH in hypophyseal portal vessel in rats increased dramatically after the treatment of Kisspeptin, while it could be blocked by the GnRH antagonist [6, 7]. The serum level of Kisspeptin in male infertility patients was significantly lower than that of normal men [8]. At present, the majority of people are in the state of chronic stress because of the pressure of work and life, and the incidence of infertility is rising year by year. Therefore, in this study we explored the effects of light stress on the expression of Kisspeptin/GnRH in rat hypothalamus and the effects of chronic stress on the hypothalamic pituitary gonadal axis.

Table 1. Primers used in this study

Primer	Sequence	Length of product
Kiss-1	F 5'-CACCTGTGGTGAACCCTGAA-3'	192 bp
	R 5'-TTTGCCAGGCATTAACGTCACC-3'	
GnRH	F 5'-TCCAGCCAGCACTGGGTCCTA-3'	208 bp
	R 5'-GGGTTCTGCCATTTGATCCTC-3'	
β-actin	F 5'-GGAGATTACTGCCCTGGCTCCTA-3'	186 bp
	R 5'-GACTCATCGTACTCCTGCTTGCTG-3'	

Materials and methods

Experimental animals

A total of 40 male 7 weeks SD rats (55~70 g) were obtained from laboratory animal center of Anhui Medical University. These animals were pre-feeding for 7 days with free access to food and water to adapt to the environment. They were divided into normal control group (Normal circadian rhythm, light:dark = 12 h:12 h), continuous light group (Direct light with 40 W fluorescent lamp for 10 days, about 2.5 m distance), interval light group (Direct light with 40 W fluorescent lamp once every 3 days), reverse day and night group randomly. The animal behavior was recorded. At the tenth day, the rats were tied to an animal operating table, the head was quickly removed after the blood was taken from the jugular vein. The skin of the head was cut with the tissue shear along the sagittal suture, the interparietal bone and parietal bone were cut and brain tissue was exposed. The hypothalamus was separated and divided into three parts for immunohistochemical staining, RT-PCR and western-blotting experiments separately.

Immunohistochemical staining detection

The expression levels of Kisspeptin and GnRH were detected using immunohistochemical staining method. Briefly, samples fixed in 10% formalin were subsequently embedded in paraffin, and sections of 4-μm thickness were cut from the formalin-fixed samples. The sectioned tissue was deparaffinized in xylene and then rehydrated in a graded ethyl alcohol series. For increased specificity and sensitivity, tissues were microwaved for 10 min for antigen retrieval. Following cooling and rinsing in distilled water, endogenous peroxide activity was blocked with 3% H_2O_2 for 10 min, and the samples were then rinsed in 0.01 mol/l phosphate-buffered saline (PBS, pH 7.4) for 10 min. The sections were subsequently pre-incubated with a protein blocking solution for 10 min, prior to incubation with the primary antibodies at 4°C overnight in a humid chamber. The slides were then washed three times in PBS and incubated with secondary biotinylated antibody for 15 min at room temperature. The streptavidin-peroxidase method was used to detect the antigen-antibody complexes, and diaminobenzidine (DAB) was used as the chromogen substrate. The sections were stained and observed under microscope and analyzed with Image J software.

RNA extraction and real-time PCR

Total RNA was extracted from hypothalamus using trizol Kit according to the manufacturer's protocol. Their concentration and purity were detected with Agilent 2100 Bioanalyzer. 1 μg RNA was subjected to reverse transcription using reverse transcription kit (Promega). Real-time PCR were performed using SYNBR Green PCR Master Mix (Qigen). The primers used in this study were shown in **Table 1**. β-actin gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Reaction parameters were 95°C for 45 sec, 95°C for 5 sec and 60°C for 30 sec with 40 cycles.

Western blotting detection

The tissues were lysed with RIPA lysis buffer and total proteins were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate dilution degree of primary antibody (diluted with TBST containing 1% (w/v) skimmed milk powder). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and gray values was determined using "Image J" software.

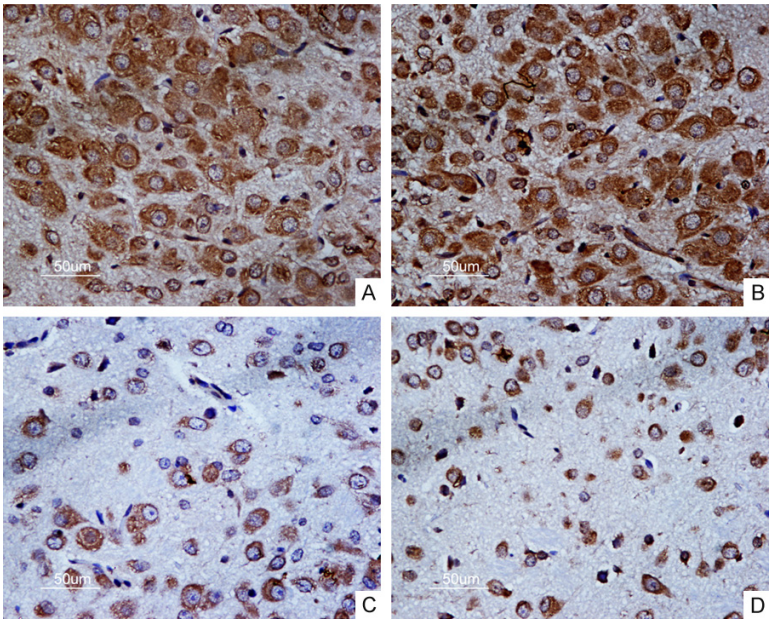

Figure 1. Immunohistochemical results of GnRH expression in different group. A: Control group; B: Interval light group; C: Continuous light group; D: Reverse day and night group.

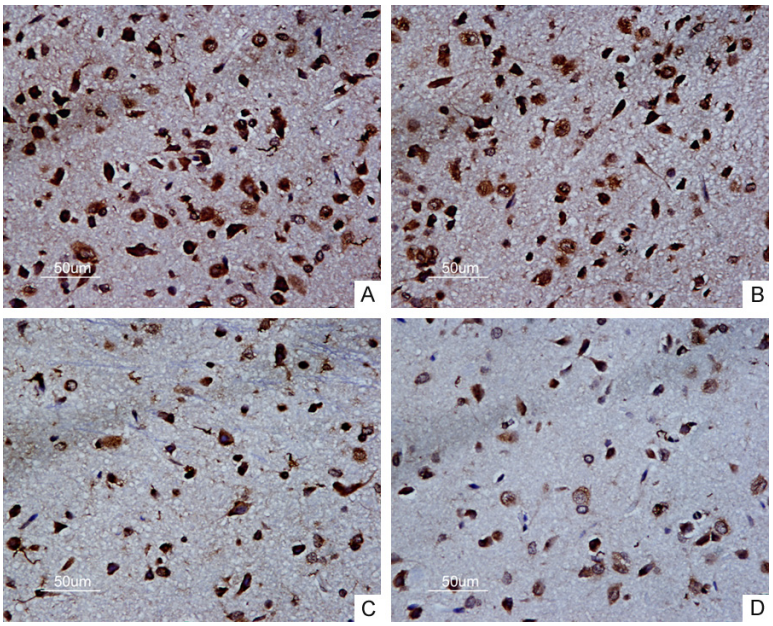

Figure 2. Immunohistochemical results of Kisspeptin expression in different group. A: Control group; B: Interval light group; C: Continuous light group; D: Reverse day and night group.

Statistical analysis

Statistical analysis was performed by SPSS 16.0 software. All data were expressed as mean ± SD, and the expression between groups were examined by t-test. A value of $P<0.05$ and $P<0.01$ was taken to denote statistical significance.

Results

Observation of animal behavior

The rats in the control group screamed and fought noisily at night, while they curled up and sleep quietly during the day. They were in a quiet sleep and curled up in the daytime and at night after continuous light for 1 day. Irritability, excessive vigilance, behavioral inhibition, fight or biting behavior and other behaviors appeared in some rats after continuous light for 1 week.

The immunohistochemical results of expression of Kisspeptin and GnRH in the hypothalamus

The immunohistochemical results were shown in **Figures 1-3**. The expression of Kisspeptin and GnRH in the hypothalamus could be detected in all groups and cell boundary was clear. The positive cells in continuous light group and reverse day and night group were lower than that of control group ($P<0.05$), while there was no significant difference between interval light group and control group ($P>0.05$).

RT-PCR results of Kisspeptin and GnRH mRNA expression

RT-PCR results were shown in **Figure 4**. It showed that the expression levels of Kisspeptin and GnRH in continuous light group and reverse day and night group decreased significantly compared with control group ($P<0.05$), while there was no significant difference between interval light group and control group ($P>0.05$).

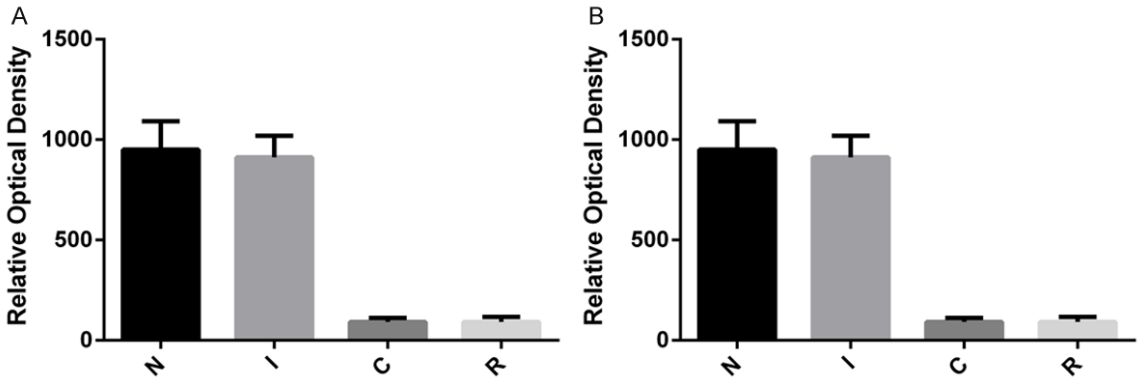

Figure 3. Comparsion of GnRH and Kisspeptin expression in different group. A: GnRH; B: Kisspeptin. N: control group; I: interval light group; C: continuous light group; R: reverse day and night group.

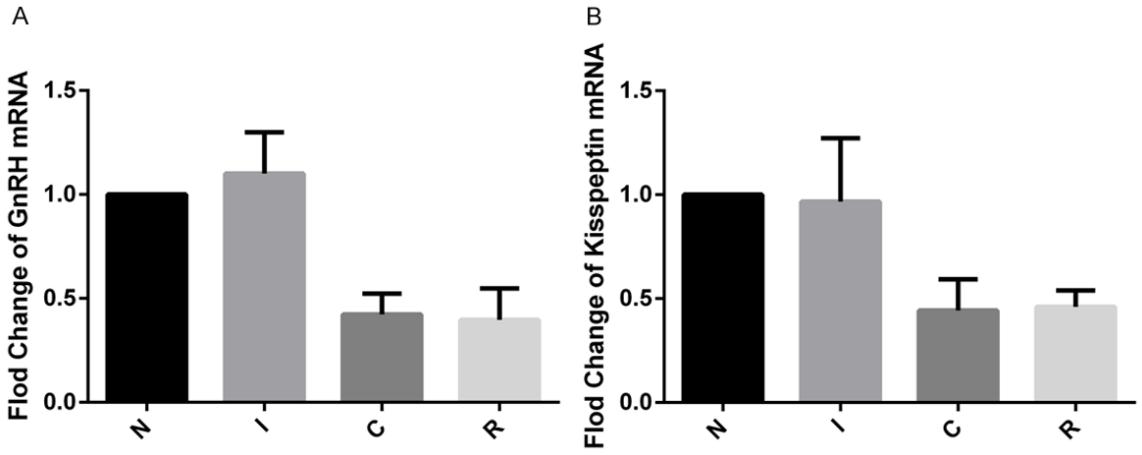

Figure 4. RT-PCR results of Kisspeptin and GnRH mRNA expression. A: GnRH; B: Kisspeptin; N: control group; I: interval light group; C: continuous light group; R: reverse day and night group.

Western blotting results

The western blotting results were shown in **Figure 5**. It showed that the levels of Kisspeptin and GnRH in continuous light group and reverse day and night group decreased significantly when compared with control group ($P<0.05$), while there was no significant difference between interval light group and control group ($P>0.05$).

Discussion

Recent studies showed that Kisspeptin/Kisslr system was involved in regulating the function of HPG axis, and was an important information transmitter of reproductive function maturity and perfection [9]. Kisspeptin was encoded by kissl gene, which played a role by binding and activating its receptor Kisslr. GnRH neurons expressed abundance of kismRNA, Kisspeptin could bind and activate Kisslr to promote the secretion of GnRH in the hypothalamus [10]. Guerriero et al found that the activation of the brain Kisspeptin/Kisslr system and the increase of GnRH were simultaneous, kisspeptin antagonists could inhibit the secretion of GnRH [11]. Kisspeptin/Kisslr system is considered to be the molecular valve of the secretion of GnRH neurons. Many studies showed that Kisspeptin could be a marker for the activation of GnRH neurons, the Kisspeptin/Kisslr system was significantly correlated with the expression of GnRH [12].

In this study, we used artificial light to establish the model of chronic stress in rats. We found that long light stress could decrease the expres-

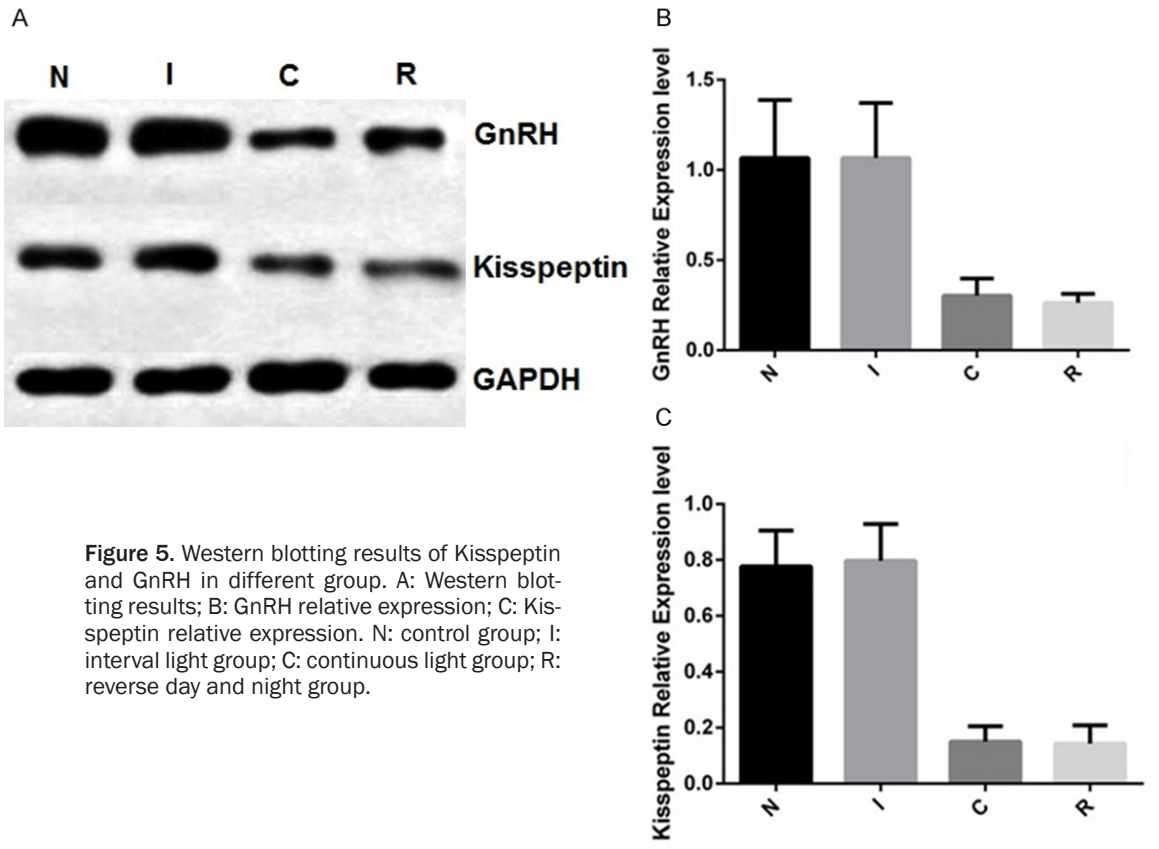

Figure 5. Western blotting results of Kisspeptin and GnRH in different group. A: Western blotting results; B: GnRH relative expression; C: Kisspeptin relative expression. N: control group; I: interval light group; C: continuous light group; R: reverse day and night group.

sion of Kisspeptin and GnRH in rat hypothalamus, while short light stress had no obvious effects on the expression of Kisspeptin and GnRH. The immunohistochemical results showed that Kisspeptin and GnRH expressed in the cytoplasm in all groups and cell boundary was clear. The positive cells in continuous light group and reverse day and night group were lower than that of control group, while there was no significant difference between interval light group and control group. Western blotting and RT-PCR results showed that the expression levels of Kisspeptin and GnRH in continuous light group and reverse day and night group decreased significantly compared with control group, while there was no significant difference between interval light group and control group. All these results suggested that long time in chronic stress could decrease the expression of Kisspeptin and GnRH. After GnRH combined with GnRHR on the cell membrane, GnRH will activate its receptor to stimulate a variety of signaling pathways in the antehypophysis. GnRHR binds to the intracellular Gq/11 protein to activate phospholipase C (PLC), inositol phospholipids was hydrolyzed into diacylglycerol (DAG) and inositol triphosphate (IP3) [13-16]. DAG activates intracellular protein kinase C (PKC) pathway. PKC activation also leads to an enhancement of the mitogen activated protein kinase (MAPK) pathway, including ERK1, 2, 5, p38MAPK and JNK in pituitary cells [17-20]. Activated MAPKs migrated into the nucleus and activated various transcription factors (such as: E ts and/or AP1 family) to regulate gene expression [21-25]. The synthesis and secretion of LHB and FSHB were regulated by these pathways [26]. Decreased expression of GnRH can lead to endocrine disorders such as decreased sexual dysfunction and affect fertility.

In a word, chronic stress induced by artificial light can decrease the expression of Kisspeptin and GnRH in rat hypothalamus, affect the hypothalamic pituitary gonadal axis, and lead to disorders of the endocrine system.

Disclosure of conflict of interest

None.

Address correspondence to: Youmin Wang, Department of Endocrinology, Institude of Endocrinology and Metabolism, The First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei 230022, Anhui, China. Tel: +86-55162922069; Fax: +86-55162922160; E-mail: youminwang@21cn.com

References

[1] Astin JA and Forys K. Psychosocial determinants of health and illness: integrating mind, body, and spirit. Adv Mind Body Med 2004; 20: 14-21.

[2] Mastorakos G, Pavlatou M, Diamanti-Kandarakis E and Chrousos GP. Exercise and the stress system. Hormones (Athens) 2005; 4: 73-89.

[3] Mastorakos G, Pavlatou MG and Mizamtsidi M. The hypothalamic-pituitary-adrenal and the hypothalamic-pituitary-gonadal axes interplay. Pediatr Endocrinol Rev 2006; 3 Suppl 1: 172-181.

[4] Dismukes AR, Shirtcliff EA, Hanson JL and Pollak SD. Context influences the interplay of endocrine axes across the day. Dev Psychobiol 2015; 57: 731-741.

[5] Papaoiconomou E, Msaouel P, Makri A, Diamanti-Kandarakis E and Koutsilieris M. The role of kisspeptin/GPR54 in the reproductive system. In Vivo 2011; 25: 343-354.

[6] Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C, Pinilla L and Tena-Sempere M. Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. Endocrinology 2005; 146: 1689-1697.

[7] Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L and Tena-Sempere M. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology 2005; 146: 156-163.

[8] Ramzan MH, Ramzan M, Ramzan F, Wahab F, Jelani M, Khan MA and Shah M. Insight into the serum kisspeptin levels in infertile males. Arch Iran Med 2015; 18: 12-17.

[9] Dhillo WS, Murphy KG and Bloom SR. The neuroendocrine physiology of kisspeptin in the human. Rev Endocr Metab Disord 2007; 8: 41-46.

[10] Kinsey-Jones JS, Li XF, Knox AM, Wilkinson ES, Zhu XL, Chaudhary AA, Milligan SR, Lightman SL and O'Byrne KT. Down-regulation of hypothalamic kisspeptin and its receptor, Kiss1r, mRNA expression is associated with stress-induced suppression of luteinising hormone secretion in the female rat. J Neuroendocrinol 2009; 21: 20-29.

[11] Guerriero KA, Keen KL, Millar RP and Terasawa E. Developmental changes in GnRH release in response to kisspeptin agonist and antagonist in female rhesus monkeys (Macaca mulatta): implication for the mechanism of puberty. Endocrinology 2012; 153: 825-836.

[12] Terasawa E, Guerriero KA and Plant TM. Kisspeptin and puberty in mammals. Adv Exp Med Biol 2013; 784: 253-273.

[13] Navratil AM, Bliss SP and Roberson MS. Membrane rafts and GnRH receptor signaling. Brain Res 2010; 1364: 53-61.

[14] Stanislaus D, Janovick JA, Brothers S and Conn PM. Regulation of G(q/11)alpha by the gonadotropin-releasing hormone receptor. Mol Endocrinol 1997; 11: 738-746.

[15] Kratzmeier M, Poch A, Mukhopadhyay AK and McArdle CA. Selective translocation of non-conventional protein kinase C isoenzymes by gonadotropin-releasing hormone (GnRH) in the gonadotrope-derived alpha T3-1 cell line. Mol Cell Endocrinol 1996; 118: 103-111.

[16] Kumar D, Periasamy V, Freese M, Voigt A and Boehm U. In Utero Development of Kisspeptin/GnRH Neural Circuitry in Male Mice. Endocrinology 2015; 156: 3084-3090.

[17] Roberson MS, Misra-Press A, Laurance ME, Stork PJ and Maurer RA. A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone alpha-subunit promoter by gonadotropin-releasing hormone. Mol Cell Biol 1995; 15: 3531-3539.

[18] Bonfil D, Chuderland D, Kraus S, Shahbazian D, Friedberg I, Seger R and Naor Z. Extracellular signal-regulated kinase, Jun N-terminal kinase, p38, and c-Src are involved in gonadotropin-releasing hormone-stimulated activity of the glycoprotein hormone follicle-stimulating hormone beta-subunit promoter. Endocrinology 2004; 145: 2228-2244.

[19] Levi NL, Hanoch T, Benard O, Rozenblat M, Harris D, Reiss N, Naor Z and Seger R. Stimulation of Jun N-terminal kinase (JNK) by gonadotropin-releasing hormone in pituitary alpha T3-1 cell line is mediated by protein kinase C, c-Src, and CDC42. Mol Endocrinol 1998; 12: 815-824.

[20] Zhang Q, Xiao X, Li M, Li W, Yu M, Zhang H, Ping F, Wang Z and Zheng J. Berberine moderates glucose metabolism through the GnRH-GLP-1 and MAPK pathways in the intestine. BMC Complement Altern Med 2014; 14: 188.

[21] Mulvaney JM and Roberson MS. Divergent signaling pathways requiring discrete calcium signals mediate concurrent activation of two mi-

togen-activated protein kinases by gonadotropin-releasing hormone. J Biol Chem 2000; 275: 14182-14189.
[22] Roberson MS, Zhang T, Li HL and Mulvaney JM. Activation of the p38 mitogen-activated protein kinase pathway by gonadotropin-releasing hormone. Endocrinology 1999; 140: 1310-1318.
[23] Sundaresan S, Colin IM, Pestell RG and Jameson JL. Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone: evidence for the involvement of protein kinase C. Endocrinology 1996; 137: 304-311.
[24] Mulvaney JM, Zhang T, Fewtrell C and Roberson MS. Calcium influx through L-type channels is required for selective activation of extracellular signal-regulated kinase by gonadotropin-releasing hormone. J Biol Chem 1999; 274: 29796-29804.
[25] Sim PJ, Wolbers WB and Mitchell R. Activation of MAP kinase by the LHRH receptor through a dual mechanism involving protein kinase C and a pertussis toxin-sensitive G protein. Mol Cell Endocrinol 1995; 112: 257-263.
[26] Gharib SD, Wierman ME, Shupnik MA and Chin WW. Molecular biology of the pituitary gonadotropins. Endocr Rev 1990; 11: 177-199.