Original Article

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

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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.
Light stress and Kisspeptin/GnRH

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% H₂O₂ 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.

<table>
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<th>Table 1. Primers used in this study</th>
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<td><strong>Primer</strong></td>
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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 ($P>0.05$).
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-
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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.

Expression 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 anterohypophysis. 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: Ets 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.
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