Expression of Luteinizing Hormone in Rat Brain

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흰쥐 뇌에서 황체형성 호르몬의 발현

지도교수 : 이 영 기

김 광 식

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ABSTRACT

Luteinizing hormone, a glycoprotein hormone which mainly synthesized in anterier pituitary. To find the present of LH hormone and the expression LH gene in rat brain region, the present study was performed immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). LH immunoreactivity was detected in hippocampus and cerebral cortex of middle-aged (12 months) and old (24 months) rat brain, but not young (4~6 months) rat. LH-β subunit transcript are also detected in the brain, and the middle-aged rat brain highly expressed then young rat brain retion. It seems that LH immunoreactivity found in the brain results from intra-neuronal synthesis not uptake from the extracellular space. We infer these neuronal LH immunoreactivity might result from lack of negative feedback regulation by steroid hormones and suggest that the decrease in steroids levels in combination with the LH in the pyramidal neurons of elderly brain could play an important role in aging process or development of Alzheimer's disease.

Key ward : Luteinizing hormone (LH), LH-β subunit transcript, Alzheimer's disease, RT-PCR

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INTRODUCTION

Luteinizing hormone (LH), a glycoprotein hormone which is mainly synthesized in the anterior pituitary, is secreted as blood-borne hormone to exert its effects in the gonads. The processes of ovulation, spermatogenesis and the secretion of sex steroids are all regulated by the action of this hormone together with follicle stimulating hormone on the testes and ovaries. The sex steroids, the male androgens (e.g. testosterone), the female estrogens (e.g. estradiol) and progesterone have diverse actions, not only on the reproductive systems in maintaining normal function, but also on sexual differentiation, growth and development. In the fetus differentiation of the reproductive tracts and the brain are controlled by steroids secreted by the developing gonads, and subsequent sexual development is dependent on gonadal steroid secretions. The feedback actions of the sex steroids on the brain and pituitary are important in the regulation of gonadotropin secretion and sexual behaviour in the adult. Steroid hormones has been known to have a neuroprotective role in diverse neurodegenerative diseases such as Parkinsonism, Alzheimer disease and fetal alcohol syndrome.

The rate of secretion of LH is controlled by gonadotrophin releasing hormone (GnRH) whose neuronal cell body is located in the preoptic area of hypothalamus. GnRH is released from hypothalamic

nerve terminals into the hypophyseal portal vessels and transported to the anterior pituitary where it stimulates the synthesis and release of LH and FSH. There has been much debate as to whether there are two releasing factors, one specific for LH and the other for FSH but, to date, only one has been identified and it is widely thought that the differential release of LH and FSH may be determined by other factors.

It has been demonstrated that LH is localized in extra-pituitary regions. Moreover LH receptor are also localized in tissues other than gonads, including brain. This raises the possibility that LH may be synthesized and act locally in the brain since other substances of anterior pituitary origin have been shown by a variety of methods to be present in many areas of the brain.

A few decades ago LH-like activity was reported in the rat amygdala (Pacold *et al.*, 1978). Immunoreactive LH was detected in the nerve processes and terminals of forebrain, and in some neuronal perikarya in the arcuate nucleus of rat hypothalamus (Hostetter *et al.*, 1981). A recent report demonstrated in human aging brain after menopause/andropause that LH is localized in the cytoplasm of pyramidal neurons of hippocampus (Bowen *et al.*, 2002). In addition, they showed a significant increase in LH in the cytoplasm of pyramidal neurons and neurofibrillary tangles of Alzheimer's disease brain compared to age-matched control brain. It was suggested that the

decreased steroid hormone production and the resulting LH expression in the neurons vulnerable to Alzheimer's disease pathology may have some relevance to the development of the Alzheimer's disease. It is, however, unclear whether the presence of LH in neurons of human aging and AD brain is due to intracellular LH expression or to LH uptake from extracellular sources, since gonadotropins are known to cross the blood brain barrier (Lukacs *et al.*, 1995). Moreover there is no report by using the brain of experimental animal whether LH is expressed in such neurons as found in the human brain. In the present study, therefore, we decided to elucidate whether 1) LH is localized in rat brain 2) there is any difference between old and young rat brain in LH immunoreactivity as is evident in human brain (Bowen *et al.*, 2002) 3) immunoreactive LH detected in neurons is de novo synthesized or is simply the result of uptake from extracellular space.

MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats were maintained in the Experimental Animal Center at University of Cheju National University under 14-h light, 10-h dark photoperiod (lights on from 06:00 to 20:00 h). Food and water were available continuously. Three age groups were studied: young (3-4 months old), middle-aged (12-14 months old), old (22-24).

2. Immunohistochemical staining

The rats were anesthetized with pentobarbital sodium (100 mg/kg, i.p.) and perfused with freshly prepared 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. The brains were removed and postfixed in the same fixative overnight and subsequently cryoprotected with 20% sucrose in 50 mM phosphate buffered saline (PBS), pH 7.4 for 48 h. Frozen sections were cut at 40 um in the coronal plane. Immunohistochemical staining was performed with free floating ABC method according to the kit manufacturer (Vector Lab, USA). Briefly, sections were treated with 1% H_2O_2 for 30 min to eliminate endogenous peroxidase activity and nonspecific binding sites were blocked with 10%

normal goat serum in PBS containing 0.1% Triton X-100 (PBSTx) for 30 min. Polyclonal LH antibody (1:500 dilution; obtained from NIAH and Chemicon, USA) was applied to the sections and incubated overnight at 4°C. Sections were then incubated with biotinylated goat anti-rabbit secondary antibodies for 90 min and with avidin-biotin peroxidase complex for 1 hr at room temperature. Sections were reacted with DAB solution until staining is optimal as determined by light microscope. Immunostaining specificity was confirmed with adjacent sections in which the primary antibody was omitted or preabsorbed with LH (negative control) and with sections of anterior pituitary (positive control).

3. Total RNA extraction

Total RNA was extracted from frozen tissue using TriZol reagent (Invitrogen, USA) according to manufacturer's protocol. The tissues were homogenated in 1 ml of TriZol-reagent per 100 mg of tissue using glass homogenazer and then incubated for 5min at room temperature. The homogenized samples were added 0.2 ml of chloroform and mixed vigorously by vortex, and incubated for 3 min at RT. The mixture was centrifuged at 12,000 ×g for 15 min at 4 ℃. The aqueous phase was transfered to new tube. The RNA form aqueous phase was precipitated by mixing with 0.5 ml of isoprophanol, incubated for 10 min at RT and then centrifused at 12,000 ×g for 10 min at 4℃. The supernatant was

removed and the pellet was washed with 75% DEPC-treated ethanol, and centrifused at 7,000 ×g for 5 min at 4℃. After The ethanol was removed, the pellet was dried at room temperature for 5 min and dissolved with DEPC-treated H_2O. To calculate the amount and degree of purity of the RNA, the absorbance was measured at 260 nm and 280 nm. The RNA samples with an A260/A280 ratio from 1.6-2.0 were selected for synthesis of cDNA.

4. Synthesis of cDNA and RT-PCR analysis

The reverse transcription was carried out using reverse transcriptase, oligo (dT) 18 primer, dNTP and 1 U RNase inhibitor. After incubation 70℃ for 5min, 37℃ for 5 min, 37℃ for 60 min. Subsequently, the polymerase chain reaction (PCR) was performed using cDNA, the reaction mixture [0.5 U Taq DNA polymerase, dNTP mixture and reaction buffer containing $MgCl_2$ (TaKaRa, Japan)] and gene specific primers under the following thermal conditions; one cycle at 94℃ for 5 min, 35 cycles of 94℃ for 1 min (pre-denaturation), 59℃ for 1 min (annealing), and 72℃ (elongation) for 1 min, and finally, and one cycle at 72℃ for 10 min carried out with a peltier thermal cycler (MJ research, USA). The primers used in PCR were designed with conserved region (Figure 1) of rat specific LH-β cDNA sequence, and the actin primer was used at internal control. Two primer pair was designed and the sequence of each primer

is shown in table 1. The PCR products were analyzed on a 1.2% agarose gel containing ethidium bromide, and measured the density using image analyzer.

Table 1. Sequences of primers used PT-PCR analysis and product size.

Primer	Primer sequences	Product size
rLH u002	5'-CCGGATCCAATGGAGAGGCTCCAGGGGC-3'	507 bp
rLH d507	5'-GGAATTCGCAGTTGTAAAGCCTTTATTGGGAG-3'	
rLH u018	5'-CGGGATCCGGGCTGCTGCTGTGGCTGCT-3'	497 bp
rLH d507	5'-GGAATTCGCAGTTGTAAAGCCTTTATTGGGAG-3'	
β-Actin F	5'-GAGATCATGTTTGAGACCTT-3'	509 bp
β-Actin R	5'-CGGATGTCMACGTCACACTT-3'	

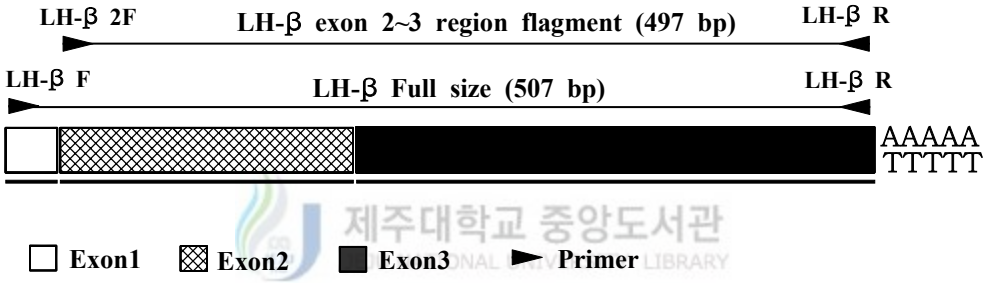

Figure 1. Structure of LH-β cDNA and region of each primer.

RESULTS

Both antisera obtained from NIAH and Chemicon company showed similar immunostaining results within the same experimental group throughout all the immunohistochemical staining carried out in the present study. As positive control, many LH immunoreactive cells were observed in sections of anterior pituitary of all age groups but not of posterior pituitary (Fig. 2). LH immunoreactive cells in the anterior pituitary were ovoid and found in the vicinity of capillaries, which is in accordance with the well-known shape and distribution of gonadotrophs. There was no specific staining in any brain area and pituitary of young, middle aged and old rats in which LH antiserum was replaced by normal rabbit serum or preabsorbed with LH peptide (Fig. 1).

In the brain of young adult rats LH immunoreactivity was not found in any region of brain (Fig. 3B, 4B). However LH immunoreactive neuronal perikarya and processes were detected in the hippocampus, cerebral cortex and hypothalamus of middle aged and old rat brain. The most intense immunostaining for LH was found in the cerebral cortex. In the hippocampus LH immunoreactivity was localized in the pyramidal neurons of CA1 and CA2 while cells of polymorphic and molecular layers did not reveal any positive immunostaining (Fig. 4B, 4C). Immunoreactivity in pyramidal cell was confined to the thin cytoplasm

sparing the large nucleus, which is the morphological feature of pyramidal cell (Fig. 4E, F). In the cerebral cortex some pyramidal neurons in both the external and internal pyramidal layer revealed LH positive immunostaining, however, more numerous immunopositive neurons were found in the external layer and no staining was observed in other neuronal cell types and layers of cerebral cortex (Fig. 3). These LH immunoreactive neurons were found mainly in the frontal lobe, to the lesser extent in the parietal lobe and almost none in the temporal or occipital lobe of cerebral cortex. In the hypothalamus very faintly immunostained neurons in the arcuate nucleus were detected in only one animal (middle-aged) among 6 rats tested.

To verify the detected LH immmunoreactivity in the hippocampus and cerebral cortex results from LH mRNA expression, we performed RT-PCR. Two primer pairs revealed similar results in anterior pituitary, hippocampus and cerebral cortex. Amplified PCR products for the LH beta subunit were observed in both the young and middle-aged rats. However the middle-aged rats contained higher abundance of amplified products than young rats (Fig. 5).

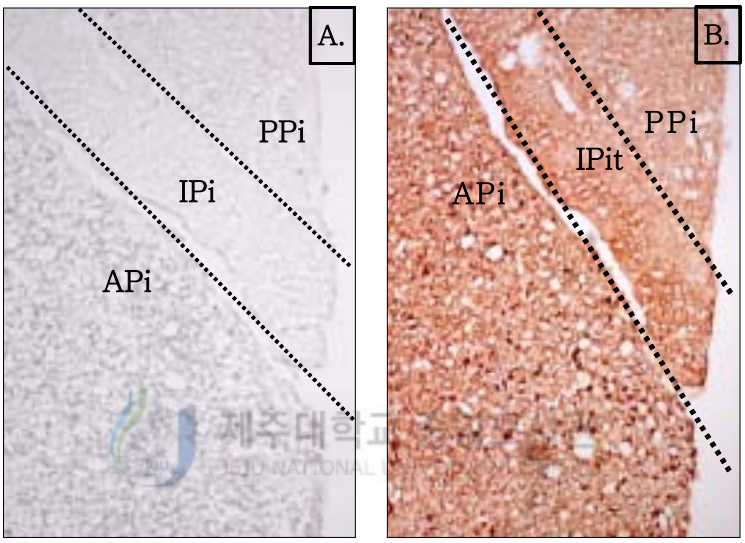

Figure 2. The immunoreactivity of LH negative control (A) and positive control (B) in tissue section of rat pituitary.

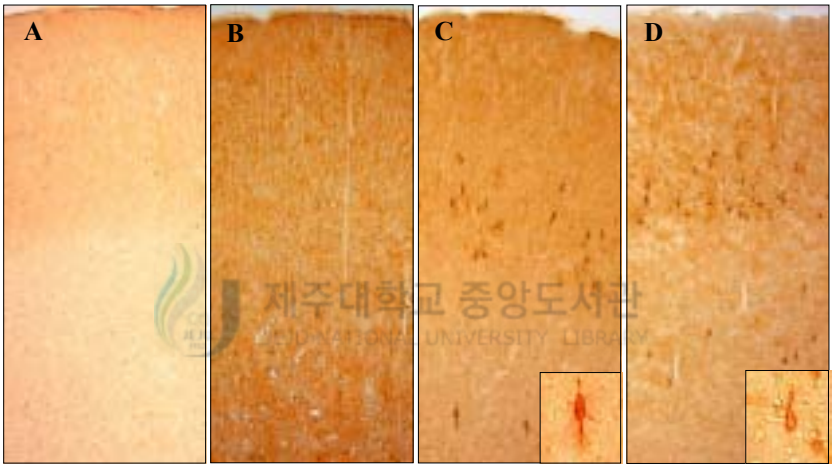

Figure 3. The LH immunoreactivity in cerebral cortex. Negative control (A), young (B), middle-aged(C) and old (D) rat brain.

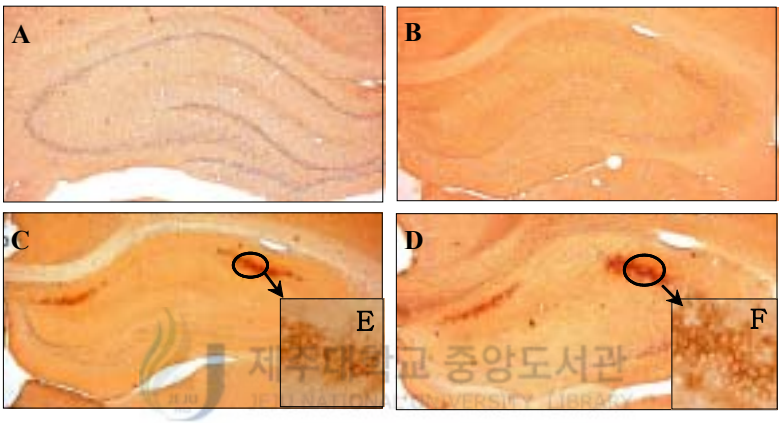

Figure 4. The LH immunoreactivity in hippocampus. Negative control (A), young (B), middle-aged (C) and old (D) rat brain.

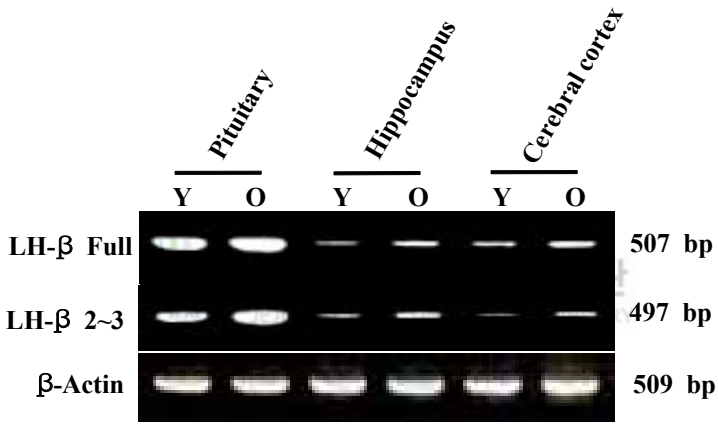

Figure 5. Detection of the transcripts for rat LH-β in the pituitary and rat brain region by RT-PCR.

DISCUSSION

LH, a gonadotropin of anterior pituitary origin which is under the control of GnRH synthesized in the hypothalamus, is a glycoprotein hormone mediating sex steroid synthesis by the gonads. In addition to its synthesis in the anterior pituitary, LH-like protein and mRNA have been shown to be present in other peripheral tissues such as leukocytes, ovary, testis, epididymis, and uterus by using radioimmunoassay and RT-PCR (*Chen et al.*, 1999; Lee and Lee, 1999). However little attention was put on its presence in the brain (Petrusz 1975; Antunes *et al.*, 1979; Gross and Page, 1979; Hostetter *et al.*, 1981; Emanuele *et al.*, 1981) despite the reports that GnRH and LH receptors are present in various regions of brain (Lei *et al.*, 1993; Jennes *et al.*, 1997).

In the present study we report the LH immunoreactivity in the middle-aged and old rat brain but not in the young rat. To our knowledge this is the first report to detect LH immunoreactivity in the pyramidal neurons of cerebral cortex. The LH immunoreactivity detected have the possibility to originate from uptake from extracellular sources since LH is known to cross the blood brain barrier (Lukacs *et al.*, 1995). However, since LH-β subunit transcript are also detected in the brain, it seems that LH immunoreactivity found in the brain results from intra-neuronal synthesis not uptake from the extracellular space. The

physiological significance of LH in the rat brain is not known at present although it was reported that pyramidal neurons of hippocampus show intense LH immunoreactivity in Alzheimer's disease and mild immunoreactivity in age-matched control brain and suggested that the increased levels of LH in hippocampal pyramidal neurons, together with the decline in steroid hormone production, could play an important role in the pathogenesis of Alzheimer's disease (Bowen *et al.*, 2002). There is, however, no direct evidence that LH in rat brain play any causative role in developing Alzheimer's disease or aging process although the hippocampus and cerebral cortex are known to have neurons vulnerable to Alzheimer's disease. The onset of age-related changes, such as memory, occurred gradually in males, with a very mild impairment at the age of 12 months and progressively greater decline at the age of 18 months to an even more severe impairment at the age of 24 months (Markowska, 1999). This time course of aging process is well matched with our results showing that LH immunoreactivity in rat brain is detected at the age of 12 months and more numerous LH immunoreactive pyramidal cells are localized at the age of 24 months. Thus we tentatively suggest that LH may play a role in aging processes such as loss of memory.

In human the loss of negative feedback by estrogen on gonadotropin production after menopause results in a three- to four-fold and a four- to 18-fold increase in the concentrations of serum LH and FSH, respectively

(Chakravarti *et al.*, 1976). Likewise, men also experience a greater than two- and three-fold in LH and FSH, respectively (Neaves *et al.*, 1984). However the situation is quite different in rat. The serum concentrations of LH, testosterone and estrogen is decreased with aging (Karpas *et al.*, 1983; Anzalone et al., 2001; Kim *et al.*, 2002). We have no idea of the underlying mechanism about these difference between elderly human and rat in serum concentrations of LH, and it is unknown that animal model of Alzheimer's disease show increased serum concentration of LH and the presence of LH immunoreactive pyramidal neurons in the cerebral cortex and hippocampus as is shown in human Alzheimer's disease (Bowen *et al.*, 2002). Further studies to answer these questions are required to obtain more concrete evidence about relevance of neuronal LH to the pathogenesis of Alzheimer's disease and aging process of brain.

It is well established that estrogens affect brain throughout the life span. Moreover, the effects of those hormones are not limited to the areas primarily involved in reproduction but also include areas relevant to memory, such as the basal forebrain, the hippocampus, and the cortex. These regions influenced by gonadal hormones also are affected strongly by aging (Geinisman *et al.*, 1986) and are sites of extensive neural degeneration in Alzheimer's disease (Terry and Katzman, 1983; Perry, 1986; Price and Sisodia, 1994). Some epidemiologic study suggest that estrogen and testosterone have a protective effect in Alzheimer's disease (

Henderson *et al.*, 1994; Tang *et al.*, 1996; Kawas *et al.*, 1997; Bowen *et al.*, 2000). For example long-term consequence of decreased estrogen after menopause is increased risk for Alzheimer disease and this risk can be reduced by estrogen replacement therapy (Kawas *et al.*, 1997; Paganini-Hill and Henderson, 1996). However, little attention has been made on the LH which is regulated by feedback action of estrogen (Bowen *et al.*, 2000). While the decreased level of serum concentration of LH has been demonstrated, our study show that LH immunoreactivity is newly appeared from 12 months old in the hippocampus and cerebral cortex. We infer these neuronal LH immunoreactivity might result from lack of negative feedback regulation by steroid hormones and suggest that the decrease in steroids levels in combination with the LH in the pyramidal neurons of elderly brain could play an important role in aging process or development of Alzheimer's disease.

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국 문 요 약

Luteinizing hormone (LH)는 주로 뇌하수체전엽에서 합성되어지는 glycoprotein으로 뇌하수체이외의 다른 조직에서 발현 되고, LH 유사 분자로서 기능을 수행한다고 최근 연구들에서 잘 밝혀졌다. 그러나, 아직까지 뇌에서 노화가 진행됨에 따른 LH의 발현에 관하여 아직까지 잘 보고 되어져 있지 않다.

본 실험은 노화가 진행됨에 따라 rat brain에서 LH가 발현이 되는지를 확인하기 위하여 면역조직화학염색기법과 RT-PCR을 수행하였다. 면역조직 화학 염색 방법을 수행한 결과 노화가 진행되는 12개월 흰쥐의 hippocampus와 cerebral cortex의 pyramidal cells에서 면역양성반응을 나타내었으며, 젊은 흰쥐에서는 나타나지 않았음을 확인 하였다. 그리고, RT-PCR을 수행한 결과 뇌하수체에서 뿐만 아니라 hippocampus와 cerebral cortex에서도 LH mRNA가 발현됨을 확인하였고, mRNA 발현 양상은 젊은 흰쥐에서보다 노화가 진행되는 흰쥐에서 많은 양의 mRNA가 발현 되고 있음을 확인 할 수 있었다. 면역조직화학염색과 RT-PCR을 수행한 결과들을 종합하여 볼 때, 노화가 진행됨에 따라 흰쥐 뇌의 hippocampus 와 cerebral cortex에서 LH 발현이 증가함을 확인 하였으며, 이들 LH는 외부에서 합성되어 뇌로 유입되는 것이 아니라, 뇌의 특정 세포내에서 합성된 것일 지도 모른다. 따라서 이러한 LH의 증가가 노화의 진행과정과 퇴행성 뇌신경질환의 형성에서 중요한 기능을 수행 할 것이라 사료되어진다.