Localization of Immunoreactive Luteinizing Hormone in Aging Rat Brain

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ABSTRACT : A recent report demonstrated that in human aging brain after menopause/andropause luteinizing hormone (LH) is localized in the cytoplasm of pyramidal neurons of hippocampus and a significant increase of LH is also detected 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 Alzheimer's disease. It is, however, unclear whether the presence of LH in neurons of human aging and Alzheimer's disease brain is due to intracellular LH expression or to LH uptake from extracellular sources, since gonadotropins are known to cross the blood brain barrier. Moreover, there is no report by using the brain of experimental animal that LH is expressed in such neurons as found in the human brain. In the present study, we found that LH immunoreactivity is localized in the pyramidal neurons of cerebral cortex and hippocampus of 12 and 18 months old rats but can not detect any immunoreactivity for LH in the young adult (3-5 months old) rats. To confirm that these LH immunoreactivity results from *de novo* synthesis in the brain but not the uptake from extracellular space, we performed RT-PCR and found that mRNA for LH is detected in several regions of brain including cerebral cortex and hippocampus. These findings suggest us that LH expression in old rat brain may play an important role in aging process of rat brain.

Key words : Luteinizing hormone (LH), Alzheimer's disease, Cerebral cortex, Hippocampus.

INTRODUCTION

Luteinizing hormone (LH), which is mainly synthesized in the pituitary and secreted as blood-borne hormone to exert its effects in gonads, has been known to be found in extra-pituitary regions. Moreover LH receptor are also localized in tissues other than gonads, including brain (Lei et al., 1993; Thompson et al., 1998; Bukovsky et al., 2003). 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 (Hostetter et al., 1981).

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 more 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

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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 that 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 the human brain (Bowen et al., 2002), and 3) immunoreactive LH detected in neurons is de novo synthesized or is simply the result of uptake from extracellular space. To accomplish this objectives, we used immunohistochemistry for the LH protein and RT-PCR analysis to demonstrate LH gene expression directly.

MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats were maintained in the Experimental Animal Center at University of Jeju 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. Four age groups were studied: young (3-5 months old, n=5), middle-aged (12-14 months old, n=7), old (22-24 months old, n=6). All experimental protocols in this study were approved by the Institutional Animal Care and Use Committee at the Jeju National University in accordance with the guidelines of Korea Food and Drug Administration.

2. Immunohistochemical Staining

The rats were anesthetized with pentobarbital sodium (100 ㎎/㎏, 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 ㎛ 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 or Chemicon Ltd, USA) was applied to the sections and incubated overnight at 4℃. Sections were then incubated with biotinylated goat anti-rabbit secondary antibodies for 90 min and with avidin-biotin peroxidase complex for 1 h 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 ㎖ of Trizol reagent per 100 ㎎ of tissue using glass homogenizer and then incubated for 5 min at room temperature. The homogenized samples were added 0.2 ㎖ of chloroform and mixed vigorously by vortex, and incubated for 3 min at room temperature. The mixture was centrifuged at 12,000 ×g for 15 min at 4℃. The aqueous phase was transferred to new tube. The RNA from aqueous was precipitated by mixing with 0.5 ㎖ of isopropanol, incubated for 10 min at RT and then centrifuged at 12,000×g for 10 min at 4℃. The supernatant was removed and the pellet was washed with 75% DEPC-treated ethanol, and centrifuged at 7,000×

g for 5 min at 4℃. After 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 RNA, the absorbance was measured at 260 nm and 280 ㎚. RNA samples with an A260/280 from 1.6-2.0 were selected for the synthesis of cDNA.

4. Synthesis of cDNA and RT-PCR Analysis

The reverse transcription was carried out using transcriptase, oligo (dT) 18 primer, dNTP and 1 U RNase inhibitor. After incubation at 70℃ for 5 min, 37℃ for 5 min, 37℃ for 60 min. Subsequently, the polymerase chain reaction (PCR) was performed using cDNA, the reaction mixture, 0.5U 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℃ for 1 min (elongation), and finally one cycle at 72℃ for 10 min carried out with Peltier thermal cycler (MJ Research, USA). The primers used in PCR were designed with the conserved region of rat specific LH-β cDNA sequence (Fig. 1), and the actin primer was used as 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.

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 intermediate and posterior pituitary (Fig. 2). LH immunoreactive cells in the anterior pituitary were ovoid and found in the vicinity of capillaries, which is in accordance

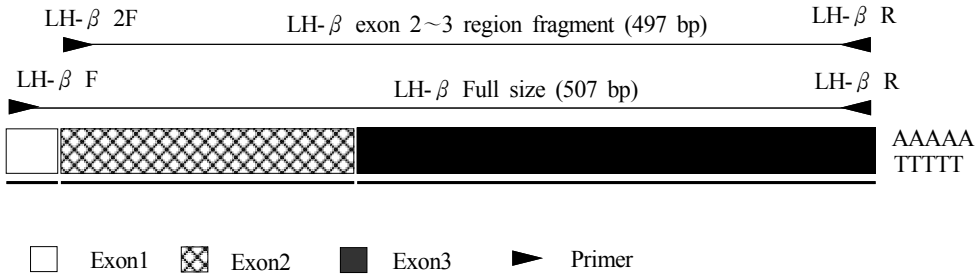

Fig. 1. Structure of LH-β cDNA and regions of each primer.

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

Primer		Primer sequences	Pimer size	Product size(bp)
rLH-β	F	5'-CCGGATCCAATGGAGAGGCTCCAGGGGC-3'	28	507
rLH-β	R	5'-GGAATTCGCAGTTGTAAAGCCTTTATTGGGAG-3'	32	
rLH-β	F	5'-CGGGATCCGGGCTGCTGCTGTGGCTGCT-3'	27	497
rLH-β	R	5'-GGAATTCGCAGTTGTAAAGCCTTTATTGGGAG-3'	32	
β-Actin	F	5'-GAGATCATGTTTGAGACCTT-3'	20	509
β-Actin	R	5'-CGGATGTCMACGTCACACTT-3'	20	

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 pre-absorbed with LH peptide (Fig. 2).

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). Immunoreactvity in pyramidal cell was confined to the thin cytoplasm sparing the large nucleus, which is the morphological feature of pyramidal cell. In the cerebral cortex some pyramidal neurons in both the external and internal pyramidal layers revealed LH positive staining. However, more numerous immunopositive neurons were found in the external pyramidal 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 lesser extent in the parietal lobe and almost none

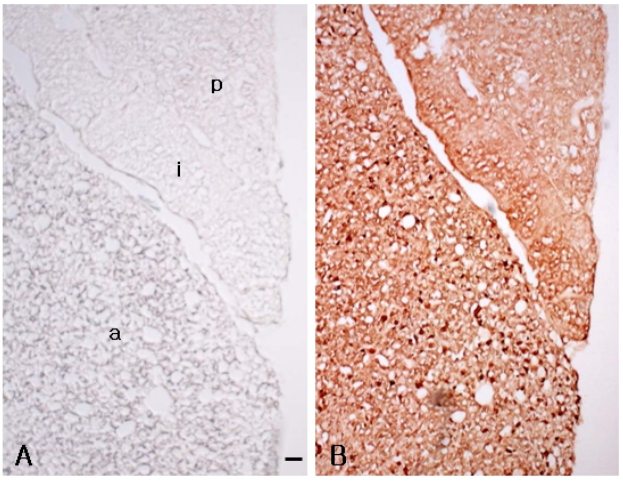

Fig. 2. Localization of LH immunoreactivity in the rat pituitary gland. There is no immunoreactivity in which LH antiserum was replaced by normal serum (A). Many LH immunoreactive cells were shown in the anterior lobe but not in the intermediate and posterior lobes (B). a, anterior lobe; i, intermediate lobe, p, posterior lobe. Scale bar = 50 ㎛.

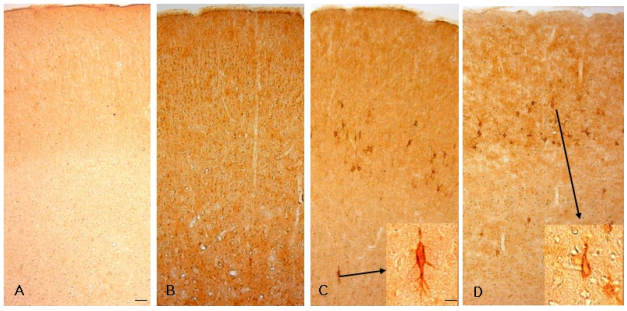

Fig. 3. Immunohistochemical localization of LH immunoreactivity in the cerebral cortex. There is no immunoreactivity in any cotrical area in which LH antiserum was replaced by normal serum (A) and in young adult rat (B). LH immunoreactive pyramidal neurons were found in the middle aged (C) and old rats (D). Insets are the photomicrographs of high magnification, which show the typical pyramidal neurons. Scale bars = 200 ㎛ in A-D; 30 ㎛ in inset.

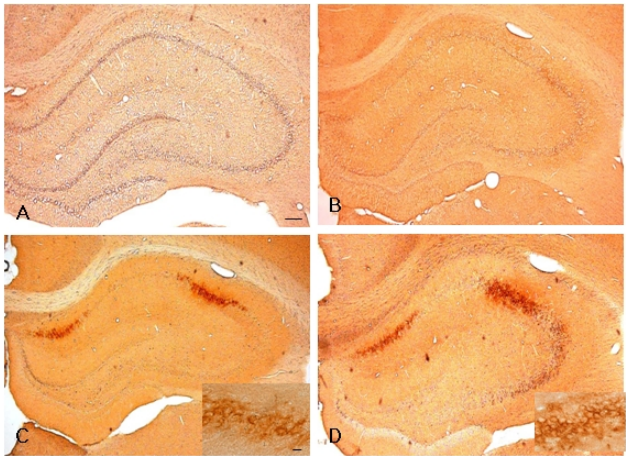

Fig. 4. LH immunoireactivity in hippocampal tissue sections. No immunostaining was observed in negative control section (A) and in young adult rat (B). In middle aged (C) and old rat (D) LH immunoreactivity was localized in the pyramidal neurons of CA1 and CA2. Scale bars = 200 ㎛ in A-D; 10 ㎛ in inset.

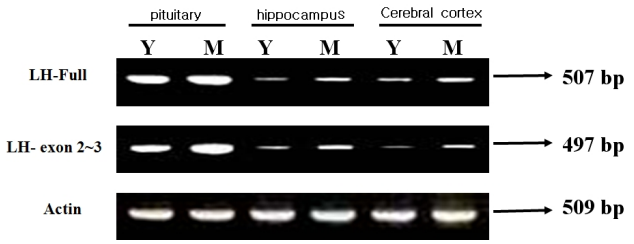

Fig. 5. RT-PCR for LH β subunits and β-actin as internal control. The two primers revealed similar results and the middle- aged rats showed higher abundance of amplified LH β transcripts than young adult rats.

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 7 rats tested (Data not shown).

To verify the detected LH immunoreactivity in the hippocampus and cerebral cortex results from LH mRNA expression, we performed RT-PCR. Two primer pairs revealed similar results in the anterior pituitary, hippocampus and cerebral cortex. Amplified PCR products for 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).

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 & Lee, 1999). However little attention was put on its presence in the brain (Antunes et al., 1979; Gross & Page, 1979; Hostetter 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 has 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 RT-PCR study shows that 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 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 loss of 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 differences between the elderly human and rat in serum concentration 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 through 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 basal forebrain, hippocampus, and cortex. These regions influenced by gonadal hormones are also affected strongly by aging (Geinisamn et al., 1986) and are sites of extensive neural degeneration in Alzheimer's disease (Price et al., 1994). Some epidemiologic study suggest that estrogen and testosterone have a protective effect in Alzheimer's disease (Bowen et al., 2000). For example, long-term consequence of decreased estrogen after menopause increased risk for Alzheimer's disease and this risk can be reduced by estrogen replacement therapy (Kawas et al., 1997). However, little attention has been made on the LH which is regulated by feedback action of estrogen. 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 hippocampus and cerebral cortex. We infer these neuronal LH immunoreactivity might result from lack of negative feedback regulation by steroid hormone and suggest that the decrease in steroids levels in combination with LH in pyramidal neurons of elderly brain could play an important role in aging process or development of Alzheimer's disease.

REFERENCES

Antunes JL, Carmel PW, Zimmerman EA, Ferin M (1979) The pars tuberalis of the rhesus monkey secretes luteinizing hormone. Brain Res 166:49-55.

Anzalone CR, Hong L, Lu JKH, LaPolt PS (2001) Influences of age and follicular reserve on estrous cycle patterns, ovulation, and hormone secretion in the Long-Evans rat. Biol Reprod 64:1056-1062.

Bowen RL, Isley JP, Atkinson RL (2000) Association of elevated serum gonadotropin concentrations and Alzheimer's disease? J Neuroendocri 12:351-354.

Bowen RL, Smith MA, Harris PLR, Kubat Z, Marins RN, Castellani RJ, Perry G, Atwood CS (2002) Elevated luteinizing hormone expression colocalizes with neurons vulnerable to Alzheimer's disease pathology. J Neruosci Res 70:514-518.

Bukovsky A, Indrapichate K, Fujuwara H, Cekanova M, Ayala M, Doninguez R, Caudle MR, Upadhyaya NB (2003) Multiple luteinizing hormone receptor (LHR) protein variants, interspecies reactivity of anti-LHR mAb clone 3B5, subcellular localization of LHR in human placenta, pelvic floor and brain, and possible role for LHR in the development of normal pregnancy, pelvic floor disorders and Alzheimer's disease. Reprod Biol Endocrinol 1:46-54.

Chakravarti SC, Collins WP, Forecast JD, Newton JR, Oram DH, Studd JW (1976) Hormone profiles after menopause. British Medical J 2:784-787.

Chen HF, Jeung EB, Stephenson M, Leung PC (1999) Human peripheral blood mononuclear cells expressing gonadotropin-releasing hormone (GnRH), GnRH receptor, and interleukin-2 receptor gamma-chain messenger ribonucleic acids that are regulated by GnRH *in vitro*. Metab 83:743-750.

Geinisamn Y. de Toledo-Morrel L, Morrell F (1986) Aged

rats need a preserved complement of perforated axospinous synapses per hippocampal neuron to maintain good spatial memory. Brain Res 398:266-275.

Gross DS, Page RB (1979) Luteinizing hormone and follicle stimulating hormone production in the pars tuberalis of hypophysectomized rats. Am J Anat 156:285-291.

Hostetter G, Gallo RV, Brownfield MS (1981) Presence of immunoreactive luteinizing hormone in the rat forebrain. Neuroendocrinol 33:241-245.

Jennes L, Eyigor O, Janovick JA, Conn PM (1997) Brain gonadotropin releasing hormone receptors: Localization and regulation. Recent Prog Hor Res 52:475-491.

Karpas AE, Bremmer WJ, Clifton DK, Steiner RA, Dorsa DM (1983) Diminished luteinizing hormone pulse frequency and amplitude with aging in the male rat. Endocrinology 112:738-792.

Kawas C, Resnick S, Morrison A, Brookmeyer R, Corrada M, Zonderman A (1997) A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: The Baltimore Longitudinal Study of Aging. Neurology 48:1517-1521.

Kim I, Siril Aryyante HB, Chamindrani Mendis-handagama SML (2002) Changes in the testis interstitium of Brown Norway rats with aging and effects of luteinizing and thyroid hormones on the aged testes in enhancing the steroidogenic potential. Biol Reprod 66:1359-1366.

Lee SH, Lee Y (1999) Expression of luteinizing hormone (LH) gene in the rat uterus and epididymis. Kor J Fertil Steril 26(2):157-161.

Lei ZM, Rao CV, Kornyei JL, Light P, Hiatt ES (1993) Novel expression of human chorionic gonadotropin/luteinizing hormone receptor gene in brain. Endocrinology 132:2262-2270.

Lukacs H, Hiatt ES, Lei ZM, Rao CV (1995) Peripheral and intracerebroventricular administration of human chorionic gonadotropin alters several hippocampus-associated behaviors in cycling female rats. Horm Behav 29:42-58.

Markowska AL (1999) Sex dimorphisms in the rate of age-related decline in spatial memory: Relevance to alterations in the estrous cycle. J Neurosci 19:8122-8133.

Neaves WB, Johnson L, Porter JC, Parker CR, Petty CS (1984) Leydig cell numbers, daily sperm production, and sperm gonadotropin levels in aging men. J Clin Endocrinol Metab 59:756-763.

Pacold ST, Kirsteins L, Hojvat S, Lawrence AM, Hagen TC (1978) Biological active pituitary hormones in the rat brain amygdaloid nucleus. Science 199:804-806.

Price DL, Sisoida SS (1994) Cellular and molecular biology of Alzheimer's disease and animal models. Ann Med Rev 45:435-446.2.

Thompson DA, Othman MI, Lei ZM, Li x, Huang ZH, Eadie DM, Rao CV (1998) Localization of receptors for luteinizing hormone/chorionic gonadotropin in neural retina. Life Sci 63:1057-1064.

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