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Original Article

Expression of kisspeptin/kiss1r system in developing hypothalamus of female rat and the possible effects on reproduction development and maintenance

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Abstract

Background: The kisspeptin/kiss1r system, expressed in the hypothalamic arcuate nucleus, has been proclaimed as one of the most powerful factors of the reproduction axis, according to recent researches in the reproductive field. The aim of this study was to ascertain the expression of kisspeptin, its receptor (kiss1r), and gonadotropin-releasing hormone (GnRH), and to explore the role on the development and maintenance of the reproductive function of developing female rats.

Methods: Expressions of the kisspeptin/kiss1r system were examined by immunohistochemistry and Real time Quantitative PCR (qRT-PCR). Expressions of estradiol (E_2), luteinizing hormone, and follicle-stimulating hormone were analyzed by enzyme-linked immunosorbent assay (ELISA).

Results: Expression of the kisspeptin/kiss1r system increased time dependently with aging, and their peak expression was demonstrated in the adult stage. GnRH showed a similar expression pattern to that of the kisspeptin/kiss1r system. ELISA results demonstrated that the E_2, luteinizing hormone, and follicle-stimulating hormone secretion increased time dependently from infancy to prepuberty to puberty. However, E_2 level decreased significantly in adult rats. Morphological changes of ovaries showed that primordial follicles, primary follicles, and growing follicle inhabited the dominant status in infancy, prepuberty, and puberty stages, respectively.

Conclusion: GnRH neurons may play an intermediate role in the activation and maintenance of the reproductive function regulated by the kisspeptin/kiss1r system, which may also indirectly regulate the serum level of luteinizing hormone, follicle-stimulating hormone, and E_2.

Keywords: arcuate nucleus; follicular development; gonadotropin-releasing hormone; kisspeptin; kiss1 receptor

1. Introduction

Kisspeptin, encoded by the *Kiss1* gene, which is a metastasis suppressor, and its receptor kiss1r were first identified for their metastasis-suppressive role in cancers.[1,2] However, Kiss1r knockout and mutations resulted in idiopathic hypogonadotropic hypogonadism, sexual immaturity, and infertility in humans and mice.[3–5] These findings suggest that kisspeptin/kiss1r signaling is pivotal for sexual maturation, and arouses phenomenal interest in the role and functions of such a system on the reproductive axis, especially gonadotropin-releasing hormone (GnRH) secretion and puberty onset.[6] GnRH neurons work as the final common pathway through which the brain regulates gonadotropin secretion from the pituitary.[7,8] Recently, reports have demonstrated that the expression of kiss1r mRNA in GnRH neurons suggests that kisspeptin-expressing neurons directly innervate GnRH

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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neurons, and kisspeptin/kiss1r signaling is critical for the pubertal activation of GnRH neurons. In addition, activation of GnRH neurons by kisspeptin serves as a neuroendocrine switch for the onset of puberty.[9,10] Furthermore, administration of kisspeptin to the brain stimulates an extraordinary release of GnRH and consequent secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Therefore, it is important to detect the expression of the kisspeptin/kiss1r system both in prepuberty and at the onset of puberty. However, neurons that express the kiss1 mRNA have been found in the preoptic area, especially the anteroventral periventricular nucleus, periventricular nucleus, and anterodorsal preoptic nucleus, and in the hypothalamic arcuate nucleus (ARC) in mice.[11] Nonetheless, expressions of kisspeptin and kiss1r in the developmental female rat brain have not been systemically studied.

The hypothalamic–pituitary–gonadal (HPG) axis is responsible for regulating physiological and behavioral reproductive functions. The onset of puberty is pivotal for activation of the reproductive capacity in humans and mammals, and kisspeptin neurons play an essential role in regulating the HPG axis from the perspectives of puberty onset, oscillations of GnRH neuron activity, and preovulatory LH surge. It is important to detect the expression of the kisspeptin/kiss1r system in the developmental female rat brain.

In this study, we detected the expression pattern of the kisspeptin/kiss1r system using immunohistochemistry and Real time Quantitative PCR (qRT-PCR), and the expression of estradiol (E_2), LH, and FSH in the serum of female rats. Expression levels of protein and mRNA of the kisspeptin/kiss1r system increased time dependently with aging, and their peak expression was demonstrated in the adult stage. GnRH showed a similar expression pattern to that of the kisspeptin/kiss1r system. Enzyme-linked immunosorbent assay results demonstrated that E_2, LH, and FSH secretion increased time dependently from infancy to prepuberty to puberty. Nevertheless, compared with puberty, E_2 level was significantly decreased in adult rats. Corresponding to the expression of the kisspeptin/kiss1r system, morphological changes of the ovaries indicated that primordial follicles, primary follicles and growing follicle inhabited the dominant status in infancy, prepuberty, and puberty stages, respectively. Corpora lutea were observed in the adult stage after ovulation. Our study provides an important theoretical basis for future clinical and basic research on infertility.

2. Methods

2.1. Animals

Ten pregnant Wistar rats were purchased from the Experimental Animal Center of Shandong University (EACSU, Jinan, Shandong, China), with 20 of their female offspring in different developmental periods. The day the pups were born was considered Day 1 and the female pups were separated from the male rats on Day 7 ($n = 20$). We divided the female rats into four groups according to their birth time and weight[12]: Day 7, Day 21, Day 35, and Day 63 of the infancy, prepuberty, puberty, and adult stages of postnatal development, respectively. The brain tissues were collected and fixed in every period. This study was approved by the Animal Ethics Committee of Binzhou Medical University (Yantai, China).

2.2. Daily vaginal smears

Estrous periodicity was detected by daily vaginal smears when weaned, and vaginal opening was inspected to assess the onset of puberty.[13] The time point at which 50% of the rats overcame the first estrum was designated as puberty; the point at which 50% of the rats underwent two whole ovarian cycles was recorded as the adult stage. The whole estrous cyclicity included the proestrum, oestrum, metestrus, and diestrum, which lasted approximately 60–70 hours, and follicle development occurred during the diestrum.

2.3. Immunohistochemistry

Rats in the four groups were deeply anesthetized and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.01M phosphate buffer. The brain was removed and postfixed for 6 hours in 4% paraformaldehyde and embedded in paraffin wax. Coronal sections (thickness = 4 μm) were obtained and used to incubate with the primary antibodies: polyclonal rabbit anti-kisspeptin (1:400; Abbiotec, San Diego, CA, USA), polyclonal rabbit anti-kiss1r (1:400; Bioss, Woburn, MA, USA), and polyclonal rabbit anti-GnRH (1:400; Santa Cruz, Dallas, TX, USA) for 18 hours at 4°C in a humidity chamber. Biotinylated secondary antibody antirabbit IgG was applied to the sections for 30 minutes at 37°C, then incubated with ExtrAvidin-peroxidase for 30 minutes at 37°C, and finally developed with diaminobenzidine (CWBIO, Beijing, China) for 2 minutes. Images were captured under 400× magnification using the bright field option of the Nikon Eclipse (E600) fluorescent microscope (Nikon, Melville, New York, USA), and thereafter analyzed using Image-Pro Plus software, version 4.5.1 (Media Cybernetics, Rockville, MD, USA).

2.4. Western blot

Fresh ARC regions were obtained from the four groups and microsected. Tissues were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA). After centrifugation, supernatants were harvested and mixed with 4× protein SDS-PAGE (SDS-polyacrylamide gelelectrophoresis) loading buffer. Protein lysate (80 μg) was resolved on 12% SDS-PAGE followed by transfer to a PVDF (polyvinylidene fluoride) membrane. Subsequently, membranes were probed with primary antibodies: polyclonal anti-kisspeptin (1:200; Abbiotec), polyclonal anti-kiss1r (1:200; Bioss), polyclonal anti-GnRH (1:200, Santa Cruz), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (1:800, Bioss). After being incubated for 12 hours at 4°C, the membranes were then incubated with horseradish peroxidase-

conjugated secondary antibodies (Immunology Consultants Laboratory, Portland, OR, USA). Immunoreactive bands were visualized using ECL (enhanced chemiluminescence) plus Western blot detection system (Thermo Scientific).

2.5. qRT-PCR

Tissues from the ARC region of the hypothalamus were pooled from animals to isolate RNA using the TRIzol reagent (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Expression levels of *Kiss1*-, *Kiss1r*-, and *Gnrh*-mRNA were quantified by qRT-PCR analysis. Briefly, total RNA were used for cDNA synthesis in a reaction volume of 20 μl and the reaction of qRT-PCR, containing SYBR MIX, primers (forward and reverse), 2 μl of total RNA, and ddH_2O were done in this experiment. Cycling conditions comprising initial denaturation of 30 seconds at 95°C, followed by 40 cycles of amplification at 95°C for 5 seconds and at 60°C for 30 seconds, and then the final elongation step at 72°C for 10 minutes. To control the PCR reaction components and the integrity of the RNA, 2 μl of each cDNA sample was amplified separately for β-actin-specific primers. Sequences of all primers are determined as shown in Table 1. qRT-PCR analyses were carried out in duplicate with at least three independent RNA samples of each developmental period group. The amplified product purity was confirmed by melting curves for each PCR reaction. Experimental data were normalized by the β-actin expression value, and the relative expression levels were calculated by the comparative cycle threshold (CT) method.[14] For each developmental period of rats in the experiment, the expression value of 7-day-old rat ARC was considered as the reference sample (Reference Value 1) arbitrarily compared with the other groups. ΔCt is obtained by subtracting each mean Ct value of β-actin (7 days, 21 days, 35 days, 63 days) from the corresponding target specific Ct (*Kiss1*, *Kiss1r* or *Gnrh*), and ΔΔCt is determined by subtracting each ΔCt of the period group from that of the 7-day-old sample. Thus, the fold expression was calculated using the formula $2^{-\Delta\Delta Ct}$ comparing the 7-day group with the 21-day, 35-day, and 63-day groups, respectively.

2.6. Enzyme-linked immunosorbent assay

Serum was obtained from all animals of each treatment group after they were sacrificed. Enzyme-linked immunosorbent assay (ELISA) was performed using Quantikine immunoassay from Amyjet Scientific (Wuhan, China). Briefly, sera were incubated in precoated 96-well plates for 4 hours at room temperature. After three washes, conjugated antibody was added for 2 hours at room temperature and incubated in the substrate solution for 30 minutes, and the reaction was stopped by adding a stop solution. Optical density of each well was determined using a microplate reader at 450 nm.

Table 1
Sequences of PCR primers.

Gene	Primer	Sequence (5′–3′)	Accession no.
Kiss1	Kiss1 F	CACCTGTGGTGAACCCTGAA	NM_181692.1
	Kiss1 R	TTTGCCAGGCATTAACGAGT	
Kiss1r	Kiss1r F	AGCACATGCAGACCGTCACC	NM_023992.2
	Kiss1r R	GACGAATTTGCACATGAAGTCTCC	
Gnrh	Gnrh F	TCCAGCCAGCACTGGTCCTA	NM_012767.2
	Gnrh R	GGGTTCTGCCATTTGATCCTC	
β-actin	β-actin F	GGAGATTACTGCCCTGGCTCCTA	NM_017008.4
	β-actin R	GACTCATCGTACTCCTGCTTGCTG	

PCR = polymerase chain reaction.

2.7. Statistical analysis

All the data of the experiment were analyzed by IBM SPSS Statistics 19.0 software (New York, United States) and presented as the mean ± standard error of means. Data among groups were analyzed using the Dunn–Bonferroni *post hoc* method following a significant Kruskal–Wallis test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Stain smear showing the estrous cycle of pubertal and adult animals

In order to confirm the estrous cycles of every rat in puberty and adult stages, the stain smear was carried out. In contrast to the proestrum, oestrum, and metestrus, the picture showing the amount of leukocytes demonstrated that the rats were in the diestrum phase of estrous cycles.[15] Although the rats underwent two complete estrous cycles (63 days after birth), the reproductive organs and systems attained maturity in the adult periods (Fig. 1). Rats were sacrificed in the diestrum of puberty and adult stages.

3.2. The expression of kisspeptin/kiss1r system and GnRH in ARC of hypothalamus of developing female rats

The expression of kisspeptin/kiss1r system was detected in the cytoplasm of the ARC, which is located in the ventromedial nucleus of the hypothalamus and around the ventral surface of the third ventricle in rats, and the expression showed a time-dependent increase from infancy to prepuberty, puberty, and adult age. GnRH showed a similar expression pattern to that of the kisspeptin/kiss1r system (Fig. 2A). Statistical analysis showed that the expression of both the kisspeptin/kiss1r system and GnRH significantly increased from infancy to prepuberty, puberty, and adult age (Fig. 2B).

In order to precisely detect the expression of the kisspeptin/kiss1r system and GnRH, we obtained the ARC region according to the stereotaxic atlas of rat brain. Western blot results showed a similar expression pattern of the kisspeptin/kiss1r system immunoreactivity. The expression of kisspeptin as well as its receptor kiss1r, and GnRH gradually increased from infancy to prepuberty, puberty, and adult stages (Fig. 3A). Statistical analysis of the bands showed the expression of both the kisspeptin/kiss1r system and GnRH increased in a time-dependent manner (Fig. 3B–D).

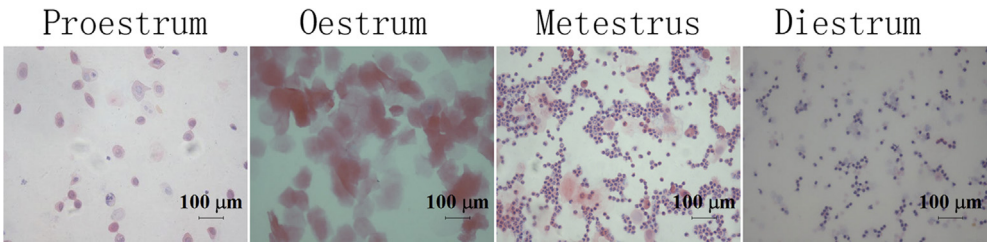

Fig. 1. Morphological changes and cell types displaying estrous cycles of pubertal and adult rats. Cellular characteristics of the proestrum, oestrum, metestrus, and diestrum were observed by a microscope and magnified 400 times to note the distinguishing cellular features. Scale bar: 100 μm.

3.3. Expression of Kiss1-, Kiss1r-, and Gnrh-mRNA levels in ARC region of developing female rats by qRT-PCR

Corresponding to the protein levels of the kisspeptin/kiss1r system and GnRH in the ARC of the hypothalamus, statistical analysis of the *Kiss1*-, *Kiss1r*-, and *Gnrh*-mRNA levels presented a similar expression pattern in that the *Kiss1*-, *Kiss1r*-, *Gnrh*-mRNA levels increased from infancy to prepuberty, puberty, and adult stages (Fig. 4).

3.4. Expression of E_2, LH, and FSH in the serum of developing female rats

The kisspeptin/kiss1r signaling is pivotal for sexual maturation and GnRH secretion.[16] Statistical analysis of the concentration of the serum E_2, LH, and FSH showed that serum E_2, LH, and FSH levels significantly increased from infancy to prepuberty and puberty age. Compared with puberty, E_2 levels of rats were significantly decreased in the adult age. LH and FSH levels were reduced slightly, but were not significantly different in the adult stage (Fig. 5A and 5B).

3.5. Morphological changes of the ovary of developing female rats

Numerous primordial follicles gathered around, and sporadic larger primary follicles were located in, the middle region of the ovary of infancy-aged rats. Then, primary follicles were dominant in the ovary of prepubertal rats. With further development, growth follicles that is primary follicles and secondary follicles were observed and luteal tissues emerged because of estrous cycles in pubertal rats. Finally, corpora

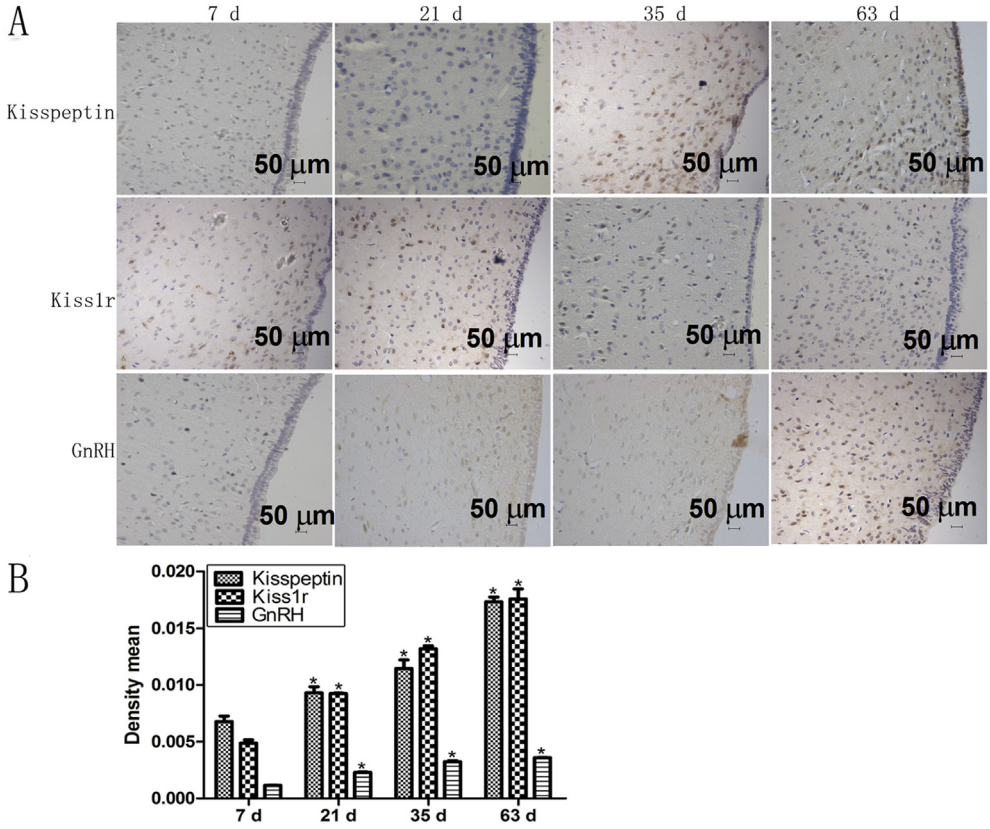

Fig. 2. Representative photomicrographs of kisspeptin, kiss1r, and GnRH expressions in the hypothalamus of developing female rats. Photographs of kisspeptin, kiss1r, and GnRH expressions were (A) reproduced using pathological Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) and (B) analyzed by density mean. Density mean is equivalent to the integrated optical density/area. Results of assays were analyzed using the Dunn–Bonferroni *post hoc* method following a significant Kruskal–Wallis test. $^*P < 0.05$. Scale bar: 50 μm. GnRH = gonadotropin-releasing hormone.

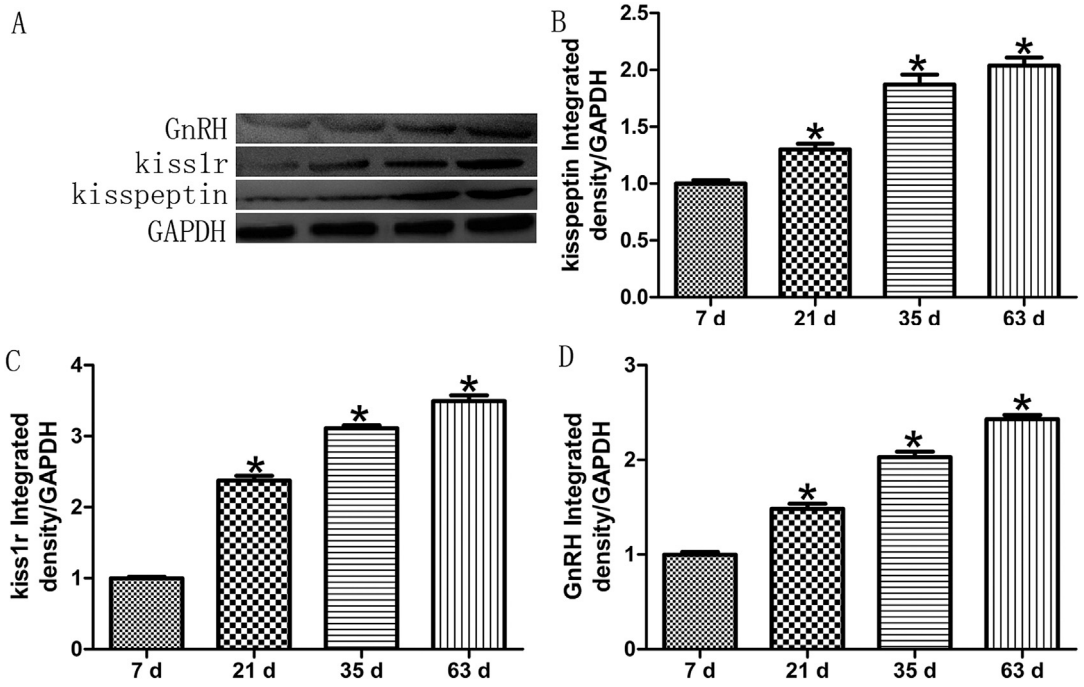

Fig. 3. Immunoblotting analysis of kisspeptin, kiss1r, and GnRH expressions in the arcuate nucleus of developing female rats. (A) The original slot blots of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), kisspeptin, kiss1r, and GnRH are shown. GAPDH was used as the reference protein. (B–D) Kisspeptin density mean/GAPDH, kiss1r density mean/GAPDH, and GnRH density mean/GAPDH were obtained by Image J software (Wayne Rasband National Institutes of Health, USA). Results of assays were analyzed using the Dunn–Bonferroni *post hoc* method following a significant Kruskal–Wallis test. $*P < 0.05$. GnRH = gonadotropin-releasing hormone.

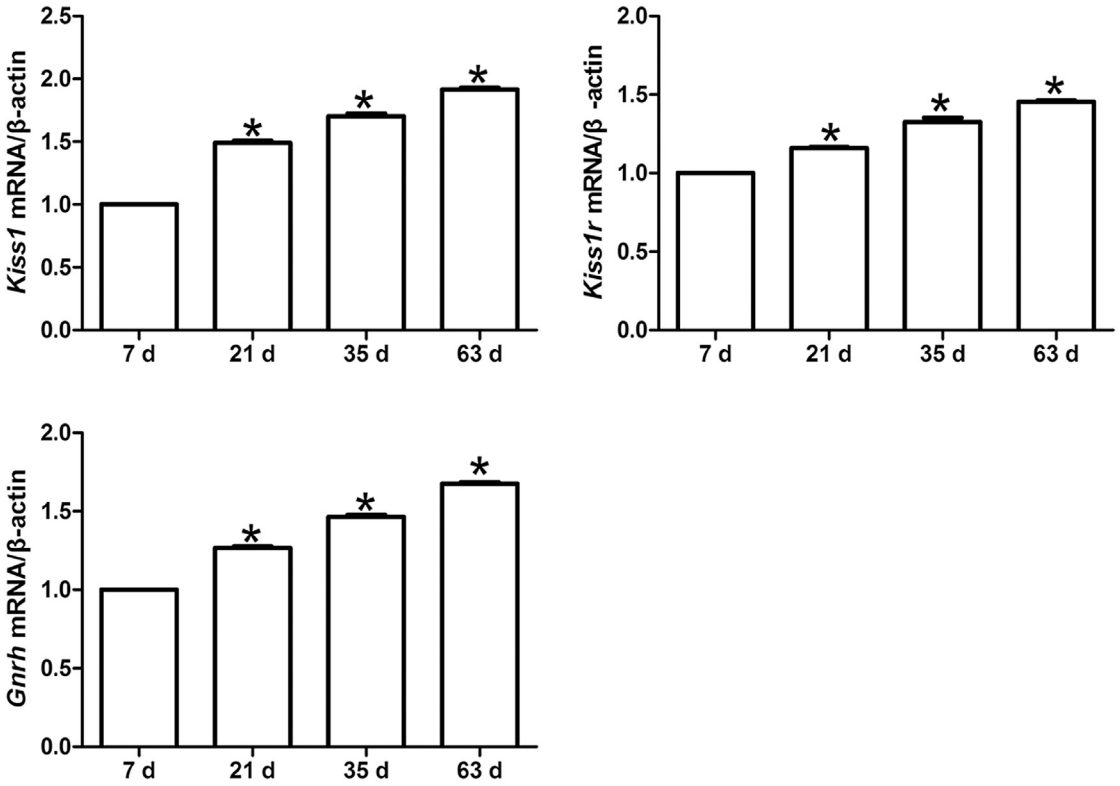

Fig. 4. Real-time quantitative PCR analysis of *kiss1*-, *kiss1r*-, and *Gnrh*-mRNA levels in developing female rats. Histograms were generated to determine the mRNA levels in different groups. The data were expressed as mean ± SE. Results of assays were analyzed using the Dunn–Bonferroni *post hoc* method following a significant Kruskal–Wallis test. $*P < 0.05$. PCR = polymerase chain reaction; SE = standard error.

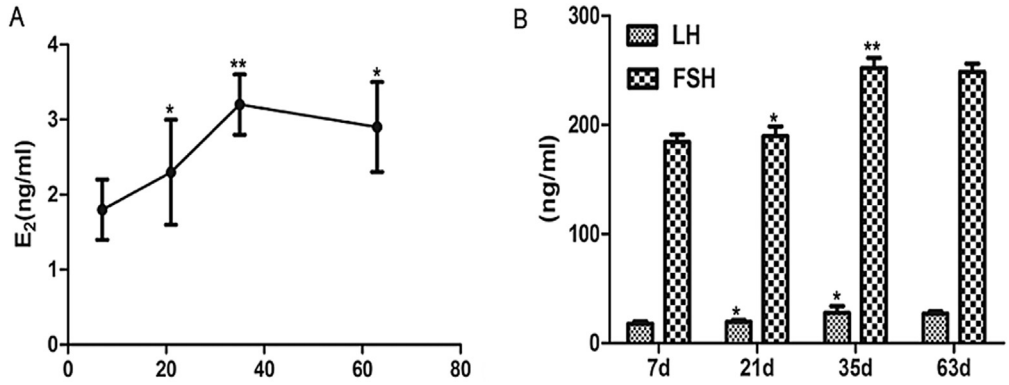

Fig. 5. Hormone levels associated with the reproductive function in developing female rats. (A) E_2 and (B) LH as well as FSH levels in the developmental stages of female Wistar rats were detected. Results of assays were analyzed using the Dunn–Bonferroni *post hoc* method following a significant Kruskal–Wallis test. $*P < 0.05$. $**P < 0.01$. E_2 = estradiol; FSH = follicle-stimulating hormone; LH = luteinizing hormone.

lutea cells were observed after ovulation in adult-aged rats. In addition, the number of atretic follicles and shrinking corpora lutea increased significantly (Fig. 6).

4. Discussion

Numerous studies have reported the importance of kisspeptin signaling for both the onset of puberty and normal adult reproductive function.[17,18]

Although the expression of the kisspeptin/kiss1r system in the human and mouse brain has been studied to some extent, expression of the kisspeptin/kiss1r system and GnRH in the developing female rat brain has not been systemically studied.

Kisspeptin acts as a key neuroendocrine gatekeeper for the activation of the HPG axis,[19] and exogenous administration of kisspeptin can hasten the onset of puberty in rats and induce ovulation in seasonally acyclic ewes.[20,21] These studies showed that a temporal pattern of hypothalamic kisspeptin expression is correlated with reproductive activity. GnRH neurons are considered key hierarchical elements of the HPG axis, and GnRH works as the final output signal downstream of the HPG axis. Adequate pulsatile secretion of GnRH is required for attainment and maintenance of the reproductive function.[22–25] Release of GnRH is the result of a hypothalamic network called GnRH pulse generator that encompasses GnRH neurons. However, GnRH secretion is not only regulated by the intrinsic activity of GnRH neurons, but also requires the contribution of additional hypothalamic afferents.[26,27] Research has shown that approximately 90% of GnRH neurons coexpress kiss1r. These observations suggest that there is a direct synaptic connection between kisspeptin neurons and GnRH neurons.[28] In addition, GnRH neurons expressing kiss1r is a momentous key point for the regulation of fertility.[10] In the present study, we demonstrated that the protein and mRNA levels of both the kisspeptin/kissr1 system and GnRH increased time dependently from infantile to adult rats. According to the relationship between kiss1 neurons and GnRH neurons, we deduce that GnRH neurons may play an intermediate role in the activation and maintenance of the reproductive function regulated by the kisspeptin/kiss1r system. Furthermore, the kisspeptin/kiss1r system was demonstrated to be present in the female genital tract,[29] which suggests that kisspeptin/kiss1r system may directly affect the peripheral reproductive system. In summary, the kisspeptin/kiss1r system may regulate the reproductive function in both a direct and an indirect manner.

The development and maturation of functional follicles rely on LH and FSH that are secreted from the pituitary, and the secretion of LH and FSH from the pituitary is directly regulated by GnRH. Activation of GnRH neurons by kisspeptin serves as a neuroendocrine switch for the onset of puberty.[9,10] With the onset of puberty, GnRH neurons will secrete GnRH pulsingly and transfer it to the pituitary, to promote the secretion of LH and FSH; these hormones work on the follicle

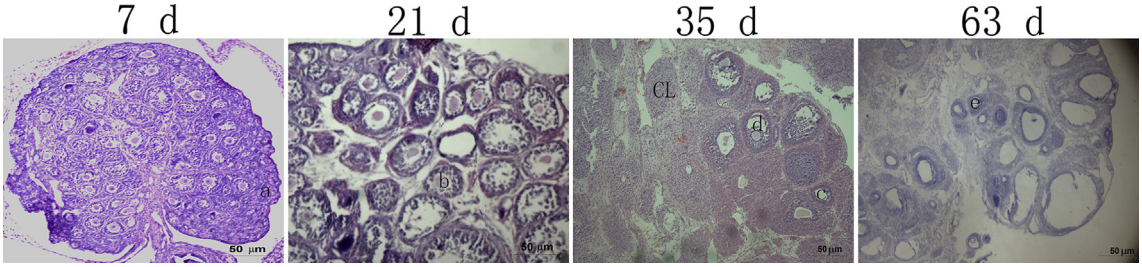

Fig. 6. Morphological changes of ovary in different developmental periods of female rats. The main cell types in the ovaries were observed and analyzed by microscopic observation (100 ×). Ovaries were dissected from infancy, prepuberty, puberty, and adult stages. Scale bar: 50 μm. a = primordial follicle; b = primary follicle; c = primary follicle; CL = corpus luteum; d = secondary follicle; e = atretic follicle.

and result in follicle development and ovulation and further produce E_2 and progestin, as shown in our results (Fig. 6). In addition, it is demonstrated that kisspeptin promotes GnRH neurons, capable of secreting GnRH, depolarizing and increasing the GnRH secretion directly in an *in vitro* experiment.[30] These are consistent with our results, which showed that the serum LH and FSH levels increased significantly from infancy to puberty, with a slight decrease in the adult age. Our results also showed that the protein and mRNA levels of the kisspeptin/kiss1r system and GnRH presented a time-dependent increase from infancy to adult stage. Therefore, the expression of hormones levels and the kisspeptin/kiss1r system in the adult stage showed an opposite pattern. Other research also showed that sex steroids inhibit the expression of Kiss1 in ARC.[31−33] It is unclear why LH and FSH levels in the adult stage were slightly lower than those in puberty; further studies are required to explore this phenomenon. Therefore, the kisspeptin/kiss1r system may regulate follicle development, consequently affecting the reproductive function by regulating the secretion of GnRH. A recent study showed that the kisspeptin/kiss1r system was expressed in ovarian granulosa cells and cumulus cells,[34] which suggests that the kisspeptin/kiss1r system may directly regulate follicular development. Taken together, the kisspeptin/kiss1r system possibly not only had an effect on the hypothalamic−pituitary−ovarian axis with aging by indirectly regulating the serum level of LH, FSH and E_2, but also could directly regulate follicular development.

In this study, we demonstrated the protein and mRNA levels of the kisspeptin/kiss1r system and GnRH in the developing female rat brain. Our results showed the expression of both of them increased time dependently from infancy to adult stage. Furthermore, we revealed the serum levels of LH, FSH, and E_2 together with follicle development in developing female rats. Our data suggest that GnRH neurons may play an intermediate role in the activation and maintenance of the reproductive function regulated by the kisspeptin/kiss1r system, and the kisspeptin/kiss1r system possibly exerts an effect on the hypothalamic−pituitary−ovarian axis with aging by indirectly regulating the serum levels of LH, FSH, and E_2. Our study provides an important theoretical basis for future clinical and basic research on infertility.

Acknowledgments

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