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RESEARCH ARTICLE

Distribution Patterns and Developmental Changes of GnRH and GnRHR-Immunopositive Cells in the Pituitary of Ji Ning Gray Goats

Liu Xiao[1,2], Wang Shu-Ying[1,*], Huang Li-Bo[1], Hou Yan-Meng[1] and Shi Yun-Zhi[1]

[1]College of Animal Science and Veterinary Medicine, ShanDong Agricultural University, Tai'an 271000, PR China;
[2]Shenzhen Bay Entry-Exit Inspection and Quarantine Bureau, Shenzhen 518000, PR China
*Corresponding author: sywang@sdau.edu.cn

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ABSTRACT

Immunohistochemical Strept Avidin Biotin-Peroxidase Complex (SABC) three-step method was used to investigate the distribution patterns and developmental changes of GnRH and GnRHR immunopositive (GnRH-ip and GnRHR-ip) cells in the pituitary of Ji Ning Gray goats during 0-180 days of age. The results showed that GnRH-ip and GnRHR-ip cells were detected only in the pars distalis of adenohypophysis. There were no positive cells in the pars intermedia and neurohypophysis. GnRH-ip and GnRHR-ip cells were not observed in the anterior pituitary at birth day and 30 days of age. At 60 days, a number of GnRH-ip and GnRHR-ip cells were found in the anterior pituitary. GnRH-ip cells were pale brown which scattered or clustered around the sinusoid capillary; GnRHR-ip cells were brown and the cytomembrane was darker. The size and percentage of GnRH-ip and GnRHR-ip cells increased with the age growth. The numbers of GnRH-ip and GnRHR-ip cells after sexual maturity were significantly bigger than that before sexual maturity. The results above suggested that GnRH and GnRHR in the pituitary of Ji Ning Gray goats play a pivotal role in the sexual development and sexual maturity.

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INTRODUCTION

GnRH, gonadotropin releasing hormone, is central to the sexual development and the process of reproduction through the hypothalamic-pituitary-gonadal axis. The hypothalamic decapeptide GnRH binds to its cognate receptor GnRHR on the gonadotropic cells in the pituitary, it stimulates a cascade of events necessary for the synthesis and release of FSH and LH, and therefore promotes the development and maturation of gonads and regulates the process of reproduction (Ciechanowska *et al.*, 2010). The pituitary which connects the nerve system with the reproductive system plays a pivotal role in the gonadal postnatal development of female animals. Clinically, the removal of pituitary gland leads to gonadal atrophy and the loss of reproductive capacity; the accessory genital organs and mammary gland will atrophy too (Forbes and Dahl, 2010). Recent studies suggested that the number of GnRH cells in the pituitary of ewes (Herman and Tomaszewska-Zaremba, 2010), rat (Kinsey-Jones *et al.*, 2012) differs with different physiological periods. However, the distribution patterns and developmental changes of GnRH-ip and GnRHR-ip cells in the pituitary gland of female Ji Ning Gray goats remain unknown.

Ji Ning Gray goats are known as early sexual maturation and high fecundity mainly located in the southwest of Shandong province in China. In our study, we aim to investigate the mechanism of early sexual maturation of Ji Ning Gray goats by studying the distribution patterns and developmental changes of GnRH-ip and GnRHR-ip cells in the pituitary using immunohistochemistry.

MATERIALS AND METHODS

Animals and tissue preparations: Forty healthy ewes aged 2-3 years old with a weight of 24-26kg were bought from the goat breeding farm of Shan Dong Kelong Co, Ltd. All these ewes were treated with estrus synchronization and then artificial insemination (semen were from male adult Ji Ning Gray goats aged 2-3 years

old with a weight of 30-32kg). All these goat lambs were reared under natural conditions with water and feed available. Healthy and strong female lambs aged 0 (birth), 30, 60 (puberty), 90, 120 (maturation), 150, 180 (after maturation) were randomly sacrificed (n=6 for each age group). All these animals and procedures above were approved by Animal Welfare Protection Committee of Shandong Agriculture University.

Lambs of each month were sacrificed and pituitary tissues were promptly collected. They were fixed in Bouin's solution for more than 24h and then rinsed with flowing water for 12h. After dehydration of gradient ethanol and transparency of xylene, those tissues were then embedded in paraffin wax and serial sections (5μm) were cut on a Leica microtome (RM2235). The sections were mounted on the cover glass coated with 10% polylysine. All these sections were divided into three groups, one group was used for immunohistochemistry, one was used for morphological observation by HE staining and the last one was for negative control.

Antibodies and chemicals: GnRH antibody (bs-0456R) and GnRHR antibody (bs-1464R) were purchased from Beijing Biosynthesis Biotechnology Co. LTD of China. Diaminobenzidine-tetrachloride kit (DAB kit) was purchased from Beijing Zhongshan Goldenbridge Biotechnology Co. LTD of China.

Immunohistochemistry: The pituitary sections were processed through standard protocols of immunohistochemistry (Singh *et al.*, 2011). Sections of negative control group were incubated by normal goat serum instead of GnRH and GnRHR antibody. The other steps were the same as mentioned above.

Morphometric analysis: Using microscope camera system (Olympus BX41 (DP25)), ten different fields of each section were randomly selected to take digital photographs by 40×object lens. The area of positive cells were analyzed by using Image-Pro-Plus software (Version 5.0). The numbers of GnRH-ip cells or GnRHR-ip cells and the cells with discernable nuclei were counted of each field (40×object lens). The percentage of GnRH-ip cells or GnRHR-ip cells of the total was calculated. Five sections of each lamb were chosen randomly to compute by using method as mentioned above, and then the average was calculated. T-test and one-way ANOVA were done by using SPSS 17.0. All the data analysis was reported as mean±SE.

RESULTS

Developmental changes of GnRH-ip cells in pituitary: GnRH-ip cells were not detected from the birth day to 30 days of age (Fig.1A-B). At 60 days, a few number of GnRH-ip cells were observed in part of the adenohypophysis pars distalis. Immunohistochemistry SABC results revealed that the shape of GnRH-ip cells was triangle or irregular and the cytoplasm of GnRH-ip cells was stained brown. GnRH-ip cells were scattered or clustered around the sinusoid capillaries (Fig.1C-G). Thereafter, GnRH-ip cells showed a progressive increase in number (Table 1). The proportion of GnRH positive cells increased significantly during the age of 90-120 days ($P<0.05$), and reached a remarkable level during 120-180 days of age ($P<0.05$). GnRH-ip cells were not observed in the pars intermedia and neurohypophysis. There were no GnRH-ip fibers in the neurohypophysis (Fig.1H). The size of GnRH-ip cells increased with the growth of age. There was a significant increase in size during 90-120 days of age ($P<0.05$). After 120 days of age the size of positive cells reached a plateau and remained steady (Table 1).

Table 1: Distribution of GnRH and GnRHR positive cells in anterior pituitary

Days	Size of positive cell /μm^2	Rate of positive GnRH cells	Rate of positive GnRHR cells
0	----------	----------	----------
30	----------	----------	----------
60	64.7±6.4a	13.4±0.1a	19.9±0.1a
90	73.6±5.3a	13.6±0.1a	21.7±0.1b
120	123.7±3.8b	15.7±0.1b	40.3±0.1c
150	129.6±6.8b	16.4±0.1b	56.2±0.1d
180	131.7±8.7b	21.9±0.1c	60.0±0.1e

Mean values (mean±SE) in each column bearing different alphabets differ significantly ($P<0.05$).

Developmental changes of GnRHR-ip cells in pituitary: GnRHR-ip cells were not observed in the adenohypophysis pars distalis at birth day and 30 days of age. The morphological characteristics and distribution patterns of GnRHR-ip cells were similar to those of GnRH-ip cells. The cytoplasms were brown and the cytomembranes were darker (Fig. 2C-G). The proportion of GnRHR-ip cells increased with age. There was a significant increase in number during 90-150 days of age ($P<0.05$). The proportion of GnRHR-ip cells increased gradually during 150-180 days (Table 1). GnRHR-ip cells were not found in the pars intermedia and neurohypophysis.

DISCUSSION

The onset of puberty might be regulated through alterations of rhythmic hypothalamic GnRH secretion which acts on the pituitary (Clarkson *et al.*, 2010). Recent studies (Kokay *et al.*, 2011) reveal that the activation of GnRH neurons in the hypothalamus is restrained by inhibitory factors such as GABA, opioid peptide and neuropeptide Y at birth, during puberty, the disinhibition of those factors contributes to the activation of GnRH neurons, increased release of GnRH promotes the synthesis and secretion of LH and FSH and therefore initiates the onset of puberty. In our experiment, the clinical symptoms of puberty of Ji Ning Gray goats (60 days) corresponded well with the present of GnRH-ip cells in the adenohypophysis from time, which indicates that the increased secretion of GnRH plays a central role in the initiation of sexual maturity. The percentage of GnRH-ip cells after sexual maturity (180 days) was significantly bigger than that of sexual maturity (120 days), suggesting that the number of GnRH-ip cells does not peak when sexual maturity comes but increases continually with the age growth and the body maturity.

GnRH binds to its cognate receptor on the gonadotropic cells in the pituitary, and stimulates the synthesis and release of LH and FSH according to certain proportion in a dose-dependent manner through alterations

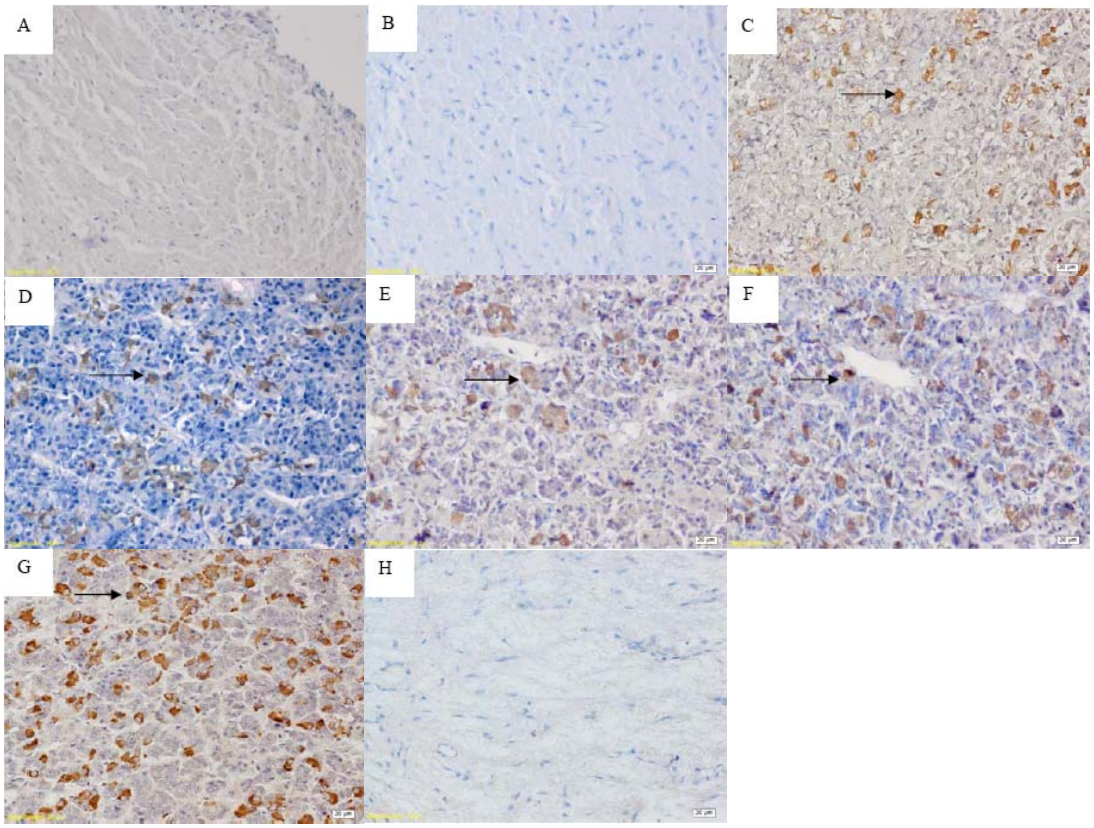

Fig. 1: Anterior pituitary stained by immunohistochemistry (400×). A: day 0; B: days 30; C: day 60; D: days 90; E: day 120; F: days 150; G: day180; H: Fibers of neurohypophysis stained by immunohisto- chemistry. The arrow showed the GnRH positive cells

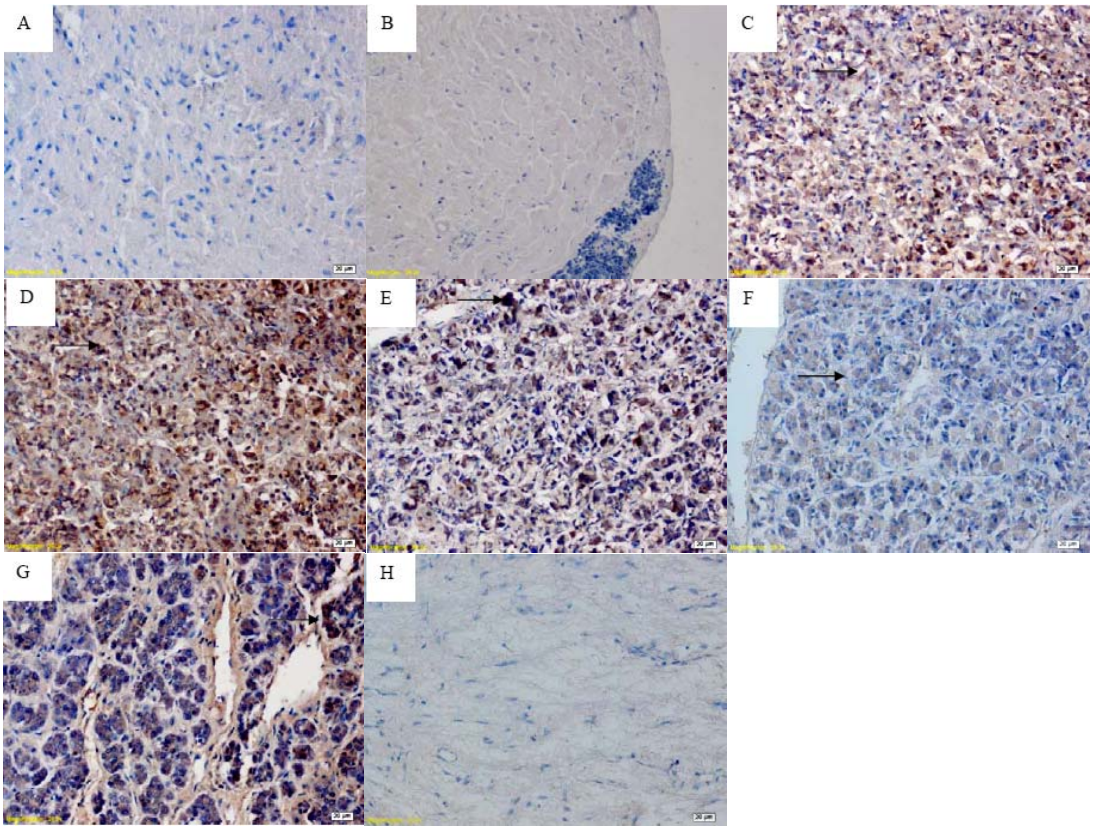

Fig. 2: Anterior pituitary stained by immunohistochemistry (400×). A: day 0; B: days 30; C: day 60; D: days 90; E: day 120; F: days 150; G: day180; H: Fibers of neurohypophysis stained by immunohisto- chemistry. The arrow showed t GnRHR positive cells.

of content and pulse frequency of GnRH, which in turn regulates gonadal steroidogenesis and the development of ovaries (Kalra *et al.*, 1997). Though GnRHR mRNA are reported to express in tissues such as pituitary (Mo *et al.*, 2010), hypothalamus (Maruska and Fernald, 2010), ovary (Wei *et al.*, 2013) and testis (Lirón *et al.*, 2012), the level of GnRHR mRNA is highest in the adenohypophysis. In our study, the percentage of GnRHR-ip cells reached 60% at 180 days of age, confirming the abundance of GnRHR in pituitary morphologically. It is the number of GnRH receptors rather than their affinity which remains constant throughout development that determines binding capacity of GnRH (Rispoli and Nett, *2*005). GnRHR-ip cells showed up at 60 days of age in the adenohypophysis of Ji Ning Gray goats, indicating that GnRH is able to bind to its receptor at 60 days and the binding capacity increases with the growth of age. There was a remarkable increase in the percentage of GnRHR-ip cells during 120-180 days of age, suggesting that the response ability of pituitary gonadotropic cells to GnRH released from hypothalamus increases after sexual maturity and the endocrine function of pituitary is improving with age.

GnRHR-ip cells are found not only in FSH/LH cells, but also in GH- and thyroid-stimulating hormone (TSH) cells reported for rats and human (La Rosa *et al.*, 2000). The present study showed that the percentage of GnRHR-ip cells was bigger than that of GnRH at each days of age in Ji Ning Gray goats. A possible explanation is that GnRHR is expressed on other endocrine cells in anterior pituitary besides gonadotropic cells which remains further study.

Conclusion: In this study, distribution patterns and development changes of GnRH and GnRHR immunopositive cells in the pituitary of Ji Ning Gray goats from birth to the sexual maturation were investigated. The results infer that GnRH and GnRHR in the pituitary play an important role in the postnatal development and early sexual maturation of Ji Ning Gray goats.

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