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**Breeding, genetics, and reproduction**

# Protein and mRNA expression of gonadotropin-releasing hormone receptor in yaks during estrus

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**ABSTRACT** - To demonstrate the role of gonadotropin-releasing hormone (GnRH) in yaks (*Bos grunniens*), we characterized the expression of gonadotropin-releasing hormone receptor (GnRHR) mRNA and protein. The level of GnRHR mRNA in the hypothalamus was higher than that in the pineal gland, pituitary gland, and ovary during estrus. Immunofluorescence analysis showed that GnRHR was expressed in the pinealocyte, synaptic ribbon, and synaptic spherules of the pineal gland and that melatonin interacts with GnRHR via nerve fibers. In the hypothalamus, GnRHR was expressed in the magnocellular neurons and parvocellular neurons. In the pituitary gland, GnRHR was expressed in acidophilic cells and basophilic cells. In the ovary, GnRHR was present in the ovarian follicle and Leydig cells. Gonadotropin-releasing hormone receptor is located in the pineal gland, hypothalamus, pituitary, and gonad during estrus of yaks and is mainly expressed in the hypothalamus and ovaries during the estrus period.

Key Words: *Bos grunniens*, gonadotropin-releasing hormone receptor, immunofluorescence, real-time PCR

## Introduction

Yaks are an important domesticated species that have demonstrated outstanding adaptability to the alpine climates of the Qinghai-Tibetan Plateau in China. The number of yaks in China accounts for greater than 94% of the world's total yak population (Wiener et al., 2003). They are seasonally estrous animals. Previous studies have shown that estrus can be induced in yaks (Yu et al., 1993; Yu and Chen, 2000; Huo and Long, 2014).

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that stimulates gonadotropes in the anterior pituitary gland to release gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) by binding to the type I GnRH receptor (GnRHR), a specific G protein-coupled receptor (Sealfon et al., 1997). In the ovary, GnRH accumulates in the median eminence before ovulation. The release of GnRH is controlled by the nervous nuclei of the hypothalamus and the levels of estrogen and progesterone, which are produced by the ovary. Simultaneously, GnRH and neighboring GnRH neurons stimulate the secretion of LH (Conn et al., 1995; Sealfon et al., 1997). However, the secretion of FSH is less dependent upon GnRH and is instead regulated largely by GnRH-independent factors, including gonadal activins, inhibins, and follistatins (Clarke et al., 1983, Pau et al., 1991, Kovacs et al., 1993). Gonadotropin-releasing hormone-III may act as a weak competitor for the mammalian GnRHR, which does not support the hypothesis that it selectively regulates the release of FSH in cattle (Amstalden et al., 2004). Gonadotropin-releasing hormone has been reported to selectively stimulate the release of FSH in rodents (Yu et al., 1997) and in cattle during the luteal phase of the estrous cycle (Dees et al., 2001). In female yaks, GnRH is an important factor for maintaining estrus through the production of FSH and LH.

This is the first study on yaks to see the expression of GnRHR. We evaluated the expression of GnRHR in the pineal gland, hypothalamus, pituitary gland, and ovary of yaks. We also investigated the tissue-specific expression of GnRH mRNA in yaks during estrus.

## Material and Methods

Six female yaks were selected for our study. When the yaks began estrus naturally, they were slaughtered, at which time tissue samples of the pineal gland, hypothalamus,

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pituitary gland, and ovary were obtained. The tissue samples were immediately stored separately in RNAlater solution (Invitrogen, Carlsbad, CA, USA) and paraformaldehyde.

Total RNA was extracted from the tissues using RNAiso Plus (Takara-Bio, Shiga, Japan) and the quality was evaluated based on the ratio of the optical densities at 260 and 280 nm (1.9-2.0). Reverse transcription was performed by AMV reverse transcriptase using the RNA PCR kit (Takara-Bio). First-strand cDNA synthesis was performed using 0.5 μg of RNA, 5mM $MgCl_2$, 1× RT buffer, 1 μL of dNTP mix, 10 U of RNase inhibitor, 2.5 U of AMV-reverse transcriptase, 1.25 pmol of oligo (dT) primer, and 3.75 μL of RNase free dd$H_2O$ in a total volume of 10 μL at 37 °C for 15 min, and the reaction was terminated by heating the samples at 85 °C for 5 s.

To compare the expression of GnRH mRNA in the pineal gland, hypothalamus, pituitary gland, and ovary during estrus, the level of GnRH mRNA in the total RNA isolated from the various tissue samples was quantified using real-time polymerase chain reaction (PCR). The 18s ribosomal RNA was used as a reference RNA for relative quantification. The primers used to amplify GnRHR cDNA were designed using the Primer Premier 6.0 program based on GnRHR mRNA sequence of *Bos taurus* (NCBI accession no. NM_177514). The primers used to amplify GnRH cDNA were 5′-TTCTCATCATGGTGATCTGCAA-3′ (forward) and 5′-GCAAATGCAACCGTCATCTTTA-3′ (reverse). The primers used to amplify the cDNA of the 18s ribosomal RNA were 5′-ACGGACAGGATTGACAGA-3′ (forward) and 5′-TCGCTCCACCAACTAAGA-3′ (reverse).

Real-time PCR was performed using the Perfect Real-Time PCR kit (Takara-Bio), with 12.5 μL of SXBR Premix Ex TaqTM II (2 ×), 2.0 μL of template cDNA, 1.0 μL of each primer (10 μM), and 4.5 μL deionized $H_2O$ in a total volume of 20 μL. Thermal cycling was performed using an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealment and extension at 60 °C for 30 s. Fluorescence was measured at the end of each amplification cycle and the cycle threshold was returned to baseline during each reaction. Primer dimerization was evaluated based on the melting curve for each template. The standard curves were generated from a standard sample using a ten-fold serial dilution (from $1 \times 10^{-1}$ to $1 \times 10^{-6}$). The standard curve parameters were used to calculate the correlation coefficients as follows: $R^2_{GnRR} = 0.996$, with slope = –3.531 and the intercept = 35.821 and $R^2_{18S} = 0.999$, with slope = –3.389 and the intercept = 16.053. Triplicate samples were used in each real-time PCR assay.

Paraffin serial sections of tissue from the cyclic group of gilts (6 mm) were incubated at 56 °C for 40 min, deparaffinized in xylene, and rehydrated in a graded series of ethanol (100–70%, $H_2O$) at 24.5 °C. Next, sections were denatured in citrate buffer (sodium citrate, 0.05% Tween- 20; pH 6.00) at 90 °C for 40 min and cooled down at 24.5 °C, washed (3×10 min) in TBS 0.2% Triton X-100, and incubated in 10% hydrogen peroxide for 20 min. After washing in TBS (3×10 min), sections were incubated in 0.75% glycine for 30 min at 24.5 °C, washed again (TBS; 3×10 min), blocked in buffer for 1 h, and then incubated overnight at 24.5 °C with primary rabbit polyclonal antibodies (GnRHR 1:70; Santa Cruz Biotechnology). The next day, sections were washed in TBS (3×10 min), incubated with biotinylated secondary antibody (goat anti-rabbit IgG, 1:400; Boster Bioengineering Co., Ltd., Wuhan, China) for 1 h, and washed in TBS (3×10 min). Next, sections were incubated with ABC kit solutions (Boster Bioengineering Co), as described by the manufacturer, washed in TBS (3×10 min), incubated in DAB (3,30-diaminobenzidine) solution (Boster Bioengineering Co), and counterstained in Mayer's hematoxylin. A negative control for the primary antibody was made using blocking peptides (Boster Bioengineering Co), whereas a secondary antibody control was performed by replacing the primary antibodies with 10% NGS (Boster Bioengineering Co).

Relative GnRHR mRNA expressions were analyzed in pineal gland, hypothalamus, pituitary gland, and ovary. The significance level was set at 0.05. Data were expressed as mean ± standard deviation. All statistical procedures were performed using the SPSS software, version 15.0 (IBM, Armonk, NY, USA). The following statistical model was used:

$$y_{ij} = \mu + \alpha_i + e_{ij},$$

in which $y_{ij}$ is the expression measured in the *j*-th animal of the *i*-th GnRHR mRNA; μ is the overall mean; $\alpha_i$ is the effect of organ i (pineal gland, hypothalamus, pituitary gland, and ovary); and $e_{ij}$ is the random error associated with each observation. Significant differences were determined using protected Tukey test ($P \leq 0.05$) after the one-way ANOVA.

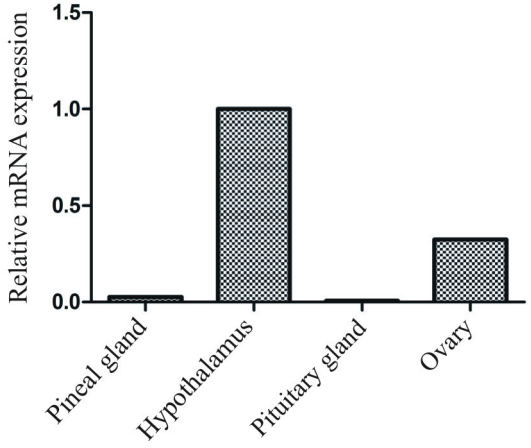
## Results

The level of GnRHR expression in the hypothalamus was significantly higher than that in the pituitary gland, pineal gland, and ovary ($P<0.01$) (Figure 1). The level of GnRHR expression in the ovary ($3.25795 \times 10^{-7}$) was significantly higher than that in the pituitary gland ($8.03846 \times 10^{-9}$) ($P<0.01$). No significant difference in the

level of GnRHR mRNA was observed between the ovary and the pineal gland.

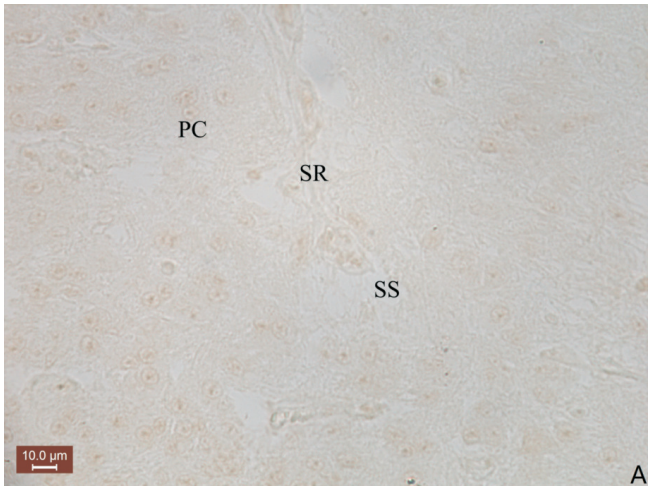
The mammalian melatonin rhythm generating system controls the production of melatonin based on the ambient lighting conditions, with maximum melatonin levels occurring under daytime lighting conditions. The pineal gland is the main tissue where melatonin is produced. The pinealocyte, synaptic ribbon (SR), and synaptic spherules (SS) of the pineal gland expressed GnRHR protein (Figure 2). These results suggest that GnRH regulates the secretion of melatonin from the pinealocyte, SR, and SS of the pineal gland. The immunofluorescence analysis showed that high levels of GnRHR protein were present in the magnocellular neurons (MN) and parvocellular neurons (PN) of the hypothalamus (Figure 3) and that low levels of GnRHR protein were present in α and β cells of the pituitary gland (Figure 4).

In the ovary, GnRHR protein was detected in the ovarian follicle, corpus luteum, and Leydig cells. The level of GnRHR expression was highest in the follicle and lowest



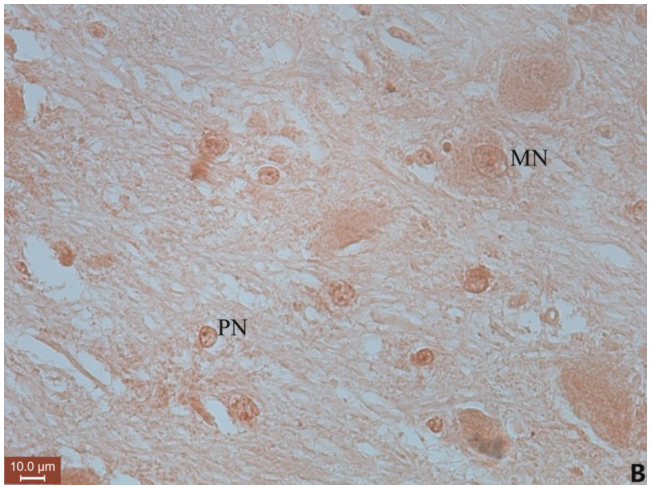

PCR - polymerase chain reaction; GnRHR - gonadotropin-releasing hormone receptor.
Values are normalized relative to the level of the 18s ribosomal RNA.
Each sample was analyzed in triplicate.

Figure 1 - Real-time PCR analysis of GnRHR mRNA expression in the pineal gland, hypothalamus, pituitary gland, and ovary of female yaks during estrus (n = 6).



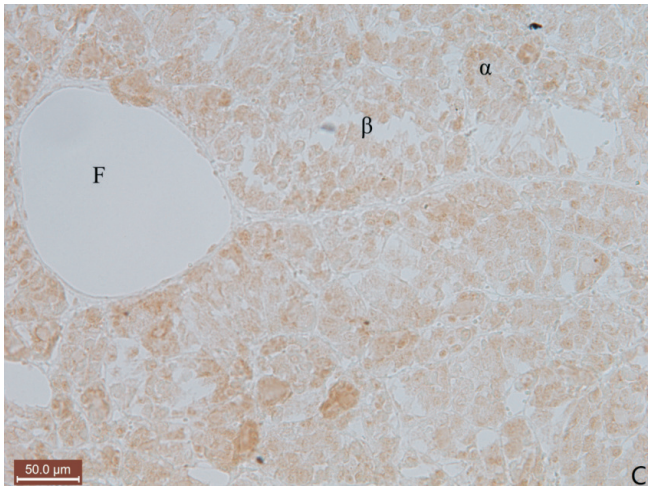

GnRHR - gonadotropin-releasing hormone receptor; GnRH - gonadotropin-releasing hormone.
The pineal gland, pinealocyte (PC), synaptic ribbon (SR), and synaptic spherules (SS) were examined for the presence of the GnRH protein.

Figure 2 - Immunofluorescence analysis of the GnRHR protein expression in the pineal gland of female yaks during estrus (n = 6).




GnRHR - gonadotropin-releasing hormone receptor.
The magnocellular neurons (MN) and parvocellular neurons (PN) were examined for the presence of the GnRHR protein.

Figure 3 - Immunofluorescence analysis of GnRHR protein expression in the hypothalamus of female yaks during estrus (n = 6).




GnRHR - gonadotropin-releasing hormone receptor.
Note the colloid lost in the follicles (F).

Figure 4 - Immunofluorescence analysis of GnRHR protein expression in the α-cells (α) and β-cells (β) of the pituitary gland of female yaks during estrus (n=6).

in the corpus luteum. Gonadotropin-releasing hormone receptor was also expressed in granulosa cells of the follicle during each stage of follicle development.

## Discussion

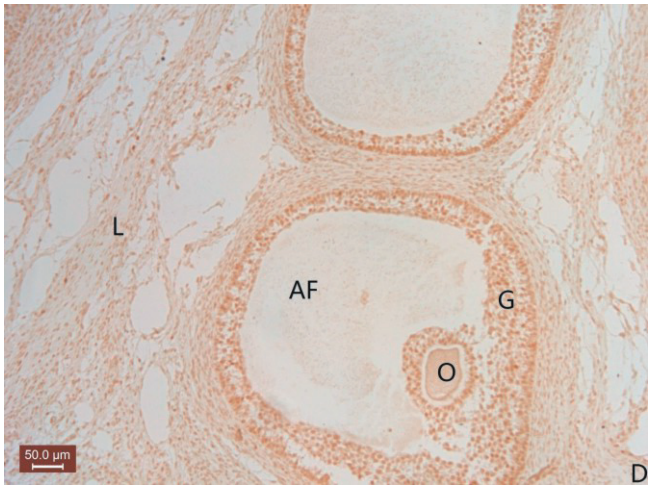
In our current study, we investigated the mechanism through which GnRH regulates reproductive processes in female yaks in estrus. We examined the expression of GnRHR protein and mRNA in the pineal gland, hypothalamus, pituitary gland, and ovary of female yaks in estrus. Secreted by the hypothalamus, GnRH is a key factor in the regulation of mammalian reproduction. The expression of GnRHR was detected in oocytes and granular leukocytes in the ovary. These results suggest that GnRH is involved in the growth and maturation of ovarian follicles and oocytes and that GnRH stimulates hormone production in Leydig cells. We found that the level of GnRHR mRNA was highest in the hypothalamus and lowest in the pineal gland (Figure 1). Gonadotropin-releasing hormone receptor protein was primarily expressed in the pinealocyte (Figure 2) and the GnRHR mRNA expression was relatively low (Figure 1).

Melatonin is secreted by the pineal gland. Melatonin plays an important role in seasonal estrus in yaks (Huo and Long, 2014) and the GnRH secretion is regulated by melatonin. The expression of GnRHR mRNA and the activity of the GnRH promoter have been detected in the pineal gland as early as embryonic day (E) 13.5 (Schang et al., 2013). Melatonin inhibits the GnRH-induced secretion of LH and FSH from the anterior pituitary gland of neonatal rats, but not from that of adult rats. The effects of melatonin are mediated by specific high-affinity membrane-bound receptors that are absent in adult rats (Vanecek, 1999). The neuroendocrine effects of melatonin on reproductive physiology may be mediated by its direct effect on the GnRH neurons of the hypothalamus, through the regulation of the expression of both GnRH and G protein-coupled melatonin receptors (Roy et al., 2001).

Previous studies have found that, although the delivery of melatonin directly into the central nervous system over a period of hours do not affect either the secretion of GnRH by the hypothalamus or the secretion of LH by the pituitary gland in ewes during anestrus, it modifies the pulsatile secretion of LH in ewes deprived of the negative feedback provided by increased levels of estradiol (Romanowicz et al., 2001; Huo et al., 2015). We hypothesized that GnRH mediates melatonin secretion from the pinealocyte, SR, and SS in female yaks in estrus. We detected the expression of GnRH protein in the hypothalamus and the expression of GnRHR protein in MN and PN of the hypothalamus (Figure 3) and the expression of GnRHR mRNA was highest in the hypothalamus (Figure 1).

The expression of GnRH is self-regulated through paracrine and autocrine activation. Gonadotropin-releasing hormone stimulates the pituitary gland to secrete FSH and LH from α- and β-cells (Figure 4). In eutherians, GnRH is released in pulses from the hypothalamus and acts directly on gonadotrophic cells via the portal blood system to stimulate both the biosynthesis and secretion of LH and FSH. The levels of circulating gonadal steroids (Clarke et al., 1988; Gregg and Nett, 1989; Brooks et al., 1993; Sealfon et al., 1990; Yasin et al., 1995), growth factors (Gregg et al., 1991; Braden and Conn, 1992), and GnRH (Turzillo et al., 1995; Turzillo et al., 1998) during estrous modulate the sensitivity of gonadotrophic cells to GnRH by regulating the expression of GnRHR. Changes in GnRH pulse frequencies are capable of differentially regulating LH and FSH expression and secretion throughout the estrous cycle (Dalkin et al., 1989; Burger et al., 2002). Gonadotropin-releasing hormone stimulates the development of ovarian follicles in estrus (Figure 5). A previous study showed that the combination of a GnRH agonist and an antagonist completely prevented the flare-up effect of cisplatin-induced gonadotoxicity and enhanced the protective effects of cisplatin in the ovary of rats (Li et al., 2012).

Therefore, we propose a clear pathway for the effects of GnRH in reproduction. The level of GnRH produced




AF - autrum of follicle; GnRHR - gonadotropin-releasing hormone receptor.

Figure 5 - Immunofluorescence analysis of GnRHR protein expression in oocytes (O), granulocytes (G), and Leydig cells (L) in the ovary of female yaks (n = 6) during estrus.

in the hypothalamus is affected by seasonal estrus, which leads to the accumulation of GnRH in the hypothalamus. Gonadotropin-releasing hormone is secreted into the blood, through which it regulates hormone production by the various tissues of the pineal gland-hypothalamus-pituitary gland-gonad axis and also participates in the development of the pineal gland, hypothalamus, pituitary gland, and gonads. Thus, estrous is initiated and maintained through the secretion of GnRH in yaks during the breeding season.

## Conclusions

The gonadotropin-releasing hormone receptor, located in pineal gland, hypothalamus, pituitary gland, and ovary in the estrus of yaks, and gonadotropin-releasing hormone are mainly expressed in the hypothalamus and ovary by gonadotropin-releasing hormone receptor to regulate the estrous in breeding season.

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