Melatonin receptor (*MTNR1A* and *MTNR2B*) expression during the breeding season in the yak (*Bos grunniens*)

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ABSTRACT: Melatonin plays key roles in a wide range of mammalian body functions, which are mediated by the melatonin-specific cell surface receptor (*MTNR1A* and *MTNR1B*). To better understand the role of *MTNR* in the yak (*Bos grunniens*), we determined the melatonin receptor mRNA expression level. The analysis showed that the *MTNR* mRNA expression level was higher in the pineal gland tissue than in the hypothalamus, pituitary gland, and ovary ($P < 0.01$) during the breeding season. Immunofluorescence analyses showed that yak *MTNR* was located in the pinealocyte, synaptic ribbon, and synaptic spherules of the pineal gland and that melatonin interacts via nerve fibres. In the hypothalamus, *MTNR* was located in the magnocellular neurons and parvicellular neurons. *MTNR* was localized in acidophilic cells and basophilic cells in the pituitary gland. In the ovary, *MTNR* was present in the ovarian follicle, corpus luteum, and Leydig cells.

Keywords: domestic yak; immunofluorescence; MLTR; real-time PCR

The domestic yak (*Bos grunniens*) is a rare bovine species found at high altitudes in the Qinghai-Tibetan Plateau and adjacent regions. Yaks have unique biological and economic characteristics that make them resistant to cold. They have evolved a unique body structure and physiological adaptation mechanisms. The reproductive mechanism may be the most important requirement for yak survival in this situation. The yak breeding season runs from July to January in China. This period is perfectly synchronized with the long nights and short days between the summer solstice and winter solstice.

The pineal gland is the only endocrine organ that can transform light into endocrine information (Binkley, 1993). Melatonin is a polypeptide hormone, which is synthesized primarily in the pineal gland. The day length is the main factor to initiate the yak breeding season each year via melatonin. Melatonin has an important role in the maturation of oocytes where the melatonin receptor 1A (*MTNR1A*) genes are expressed in the cumulus-oocytes complex, whereas the *MTNR1B* gene is expressed only in the oocytes. Melatonin can enhance the maturation of oocytes *in vitro* (Berlinguer et al., 2009; Kang et al., 2009; El-Raey et al., 2011). There was a positive correlation between *MTNR1A* allele polymorphisms and reproduction in the Sarda sheep breed (Carcangiu et al., 2009, 2011). Concluding data revealed the positive effect of melatonin treatment on the time of the first conception in ewe lambs and highlighted that +/+ genotype is able to influence reproductive response to melatonin treatment. Melatonin-treated animals of +/+ genotype showed a higher number of pregnancies and lambed earlier compared to untreated animals of the same genotype in *MTNR1* (Mura et al., 2010). Melatonin secretion cycle varied under different lighting conditions (Ogino et al., 2013). There is no doubt that melatonin participates in reproduction but the process is not clear. We hypothesized that melatonin may

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regulate yak reproduction via the hypothalamus-pituitary gland-gonad axis.

In this study, we report the expression of *MTNR* in the pineal gland, hypothalamus, pituitary gland, and ovary of the yak. We also examined differences in the tissue-specific mRNA expression level of the *MTNR* gene in the yak during the breeding season.

MATERIAL AND METHODS

Sample collection and preservation. Tissue samples from the pineal gland, hypothalamus, pituitary gland, and ovary were obtained at the time of slaughter and stored immediately in RNAlater (Omega Bio-Tek, Inc., Norcross, USA) and paraformaldehyde until further use. Samples were collected from six yaks (proestrus) on October 4, 2012, which was during the breeding season.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total tissue RNA was extracted using RNAiso Plus (TaKaRa Biotechnology Co., Dalian, P.R. China) and the quality was checked with UV-800 ultraviolet spectrophotometer (Shimadzu Corp., Kyoto, Japan) in A260/280 nm. Reverse transcription was performed using PrimeScript RT Master Mix Perfect Real Time (TaKaRa) in a reaction mixture volume of 10 µl at 37°C for 15 min and 85°C for 5 s.

Real-Time PCR of MTNR mRNA expression. To compare *MTNR* mRNA expression in different yak tissues (pineal gland, hypothalamus, pituitary gland, and ovary) during the breeding season, a real-time PCR assay was developed based on SXBR Premix Ex TaqTM II (Perfect Real Time) (TaKaRa). The *β-actin* was used as a housekeeping gene to correct potential variations in the RNA loading. The primers were designed using Primer Premier 6.0 based on the mRNA sequence of *MTNR1B*. Taurus (NCBI Accession numbers XM614283.3 and XM001254949.3). The primers used for *MTNR1A* were 5'-GGCACTCGTCATCATTCC-3' (forward) and 5'-CGTCCACTCCAGTCTTCT-3' (reverse) while those used for *MTNR1B* were 5'-TCGTCTATGGGCTCCTGAA-3' (forward) and 5'-GCTGCCCTTGGAAGAGTT-3' (reverse). The primers used for the *β-actin* plasmid were 5'-CACAGCCGAGCGGGAAAT-3' (forward) and 5'-CCGTGTTGGCGTAGAGGT-3' (reverse) (Zi et al., 2012).

Real-time PCR conditions: 12.5 µl SXBR Premix Ex TaqTM II (2×), 2.0 µl of template cDNA, 1.0 µl of each primer (10 µmol/l), 8.5 µl deionized H_2O. The PCR parameters were 50°C for 2 min, 95°C for 5 min, 95°C for 10 s, and 60°C for 30 s. The cycle threshold (Ct) was returned to the baseline during each reaction. Based on the melting curve, we determined whether a specific product or a primer dimer was present in the PCR during each reaction.

The standard curves were generated from a standard sample, i.e. a 10-fold serial dilution (from 10^1 to 10^6). The standard curve parameter calculations were used to determine the correlation coefficient value: R^2_{MTNR1A} = 0.997, slope = –3.387, intercept = 28.833; R^2_{MTNR2B} = 0.997, slope = –3.417, intercept = 29.462; $R^2_{\beta\text{-}actin}$ = 0.999, slope = –3.389, intercept = 16.654. Each sample was tested in triplicate.

Immunohistochemical analysis of MTNR protein expression. An immunohistochemical assay was developed to study *MTNR* protein expression in yak tissues (pineal gland, hypothalamus, pituitary gland, and ovary) during the breeding season. The tissues were embedded in paraffin and dehydrated. *MTNR* protein expression was analyzed using an SP immunohistochemistry kit (Maixin, Fuzhou, P.R. China), according to the manufacturer's instructions.

Statistical analysis. The data were expressed as the mean ± SD. The SPSS software package (Version 15.0, 2007) was used to analyze the data. ANOVA was used to test for significant differences between different groups.

RESULTS

Tissue-specific expression of the yak melatonin receptor during the breeding season. The *MTNR1A* and *MTNR1B* mRNA expression levels were higher in pineal gland tissue compared with the hypothalamus, pituitary gland, and ovary ($P < 0.01$) during the yak breeding season (Figure 1).

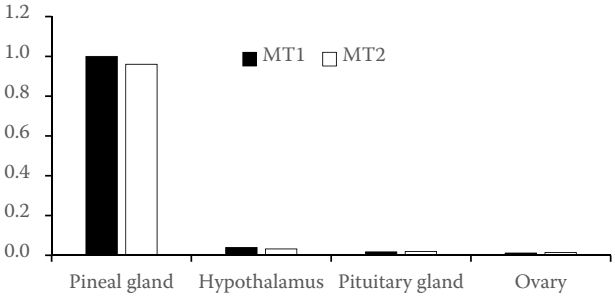

Figure 1. *MTNR1A* and *MTNR2B* mRNA expression in pineal gland, hypothalamus, pituitary gland, and ovary tissue of the yak during breeding season (n = 6) as determined by real-time PCR. Values are normalized with the values for *β-actin*. Each sample was analyzed in triplicate

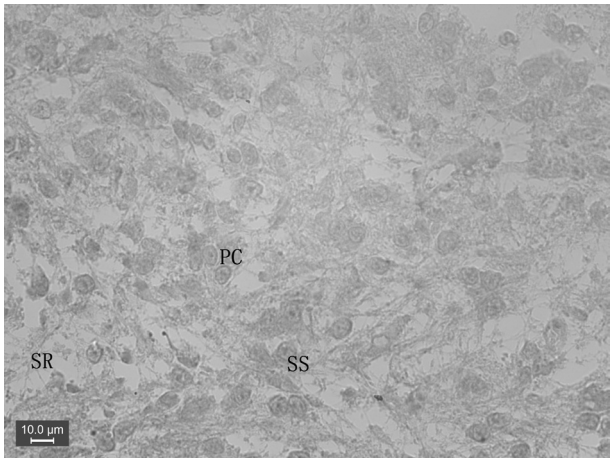

Figure 2. Melatonin receptor protein expressions in pineal gland tissue of the yak during breeding season (n = 6) as determined by immunofluorescence. The pineal gland, pinealocyte (PC), synaptic ribbon (SR), and synaptic spherules (SS) were the receptors of melatonin

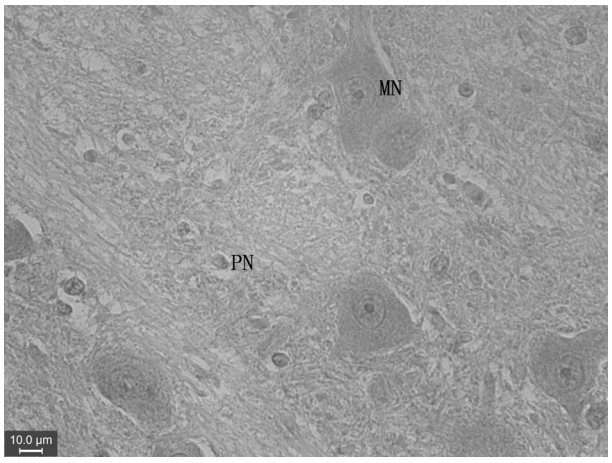

Figure 3. Melatonin receptor protein expression in hypothalamus issue of the yak during breeding season (n = 6) as determined by immunofluorescence. Melatonin receptor was obviously located in magnocellular neurons (MN) and parvicellular neurons (PN)

There were no significant differences between the hypothalamus, pituitary gland, and ovary tissue.

Melatonin receptor protein expression in the pineal gland. The pineal gland is the main tissue where melatonin was produced. The pinealocyte, synaptic ribbon (SR), and synaptic spherules (SS) expressed melatonin in the pineal gland (Figure 2). This suggests that the pinealocytes exchanged information via the SR and SS. Thus, the pinealocytes produced melatonin and they also received it.

Melatonin receptor protein expression in the hypothalamus. High *MTNR* expression was detected in the magnocellular neurons and parvicellular neurons of the hypothalamus tissue during the breeding season (Figure 3).

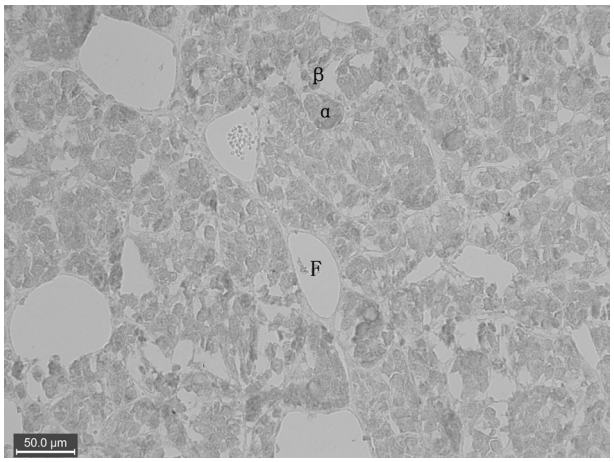

Figure 4. Melatonin receptor protein expression in α-cells (α) and β-cells (β) of pituitary gland tissue during breeding season (n = 6). The colloid lost in the folliculi (F)

Melatonin receptor protein expression in the pituitary gland. Low *MTNR* expression was detected in the pituitary gland tissue α-cells and β-cells during the yak breeding season (Figure 4).

Melatonin receptor protein expression in the ovary. In the ovary, *MTNR* expression was high in the ovarian follicle (Figure 5A) but lower in the corpus luteum (Figure 5B) than in Leydig cells. *MTNR* expression was detected in granular leukocytes and oocytes. This suggests that melatonin was involved with the growth and maturation of ovarian follicles and it also participated in the growth of oocytes. Melatonin also stimulated hormone production by Leydig cells. *MTNR* was expressed by the granulosa cells in the follicle during each stage.

DISCUSSION

This study investigated *MTNR* gene expression in the yak (*B. grunniens*). We examined tissue-specific differences in the *MTNR* mRNA expression levels in the yak during the breeding season. We also studied various characteristics of the tissue-specific *MTNR* protein expression in the yak. To the best of our knowledge, this is the first study to report the tissue-specific *MTNR* mRNA and protein expression levels in the yak.

The yak is a seasonal breeder with a low reproductive rate and milk yield compared with dairy cattle breeds (Zi, 2003; Sarkar et al., 2005; Reiter et al., 2009). Previous studies have shown that

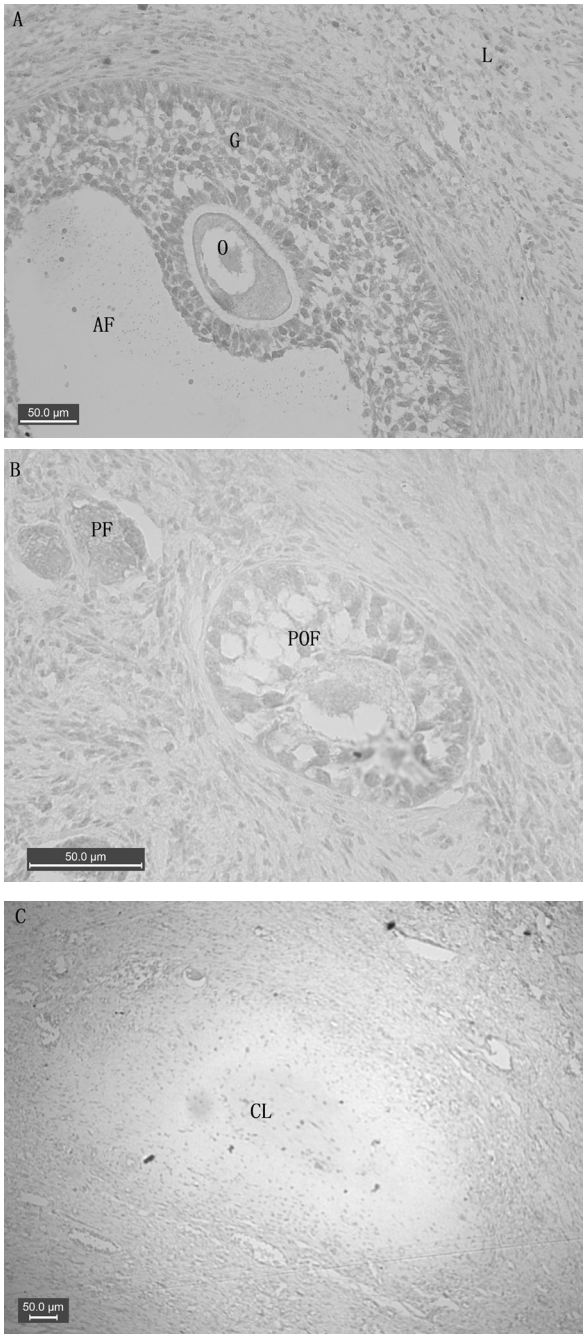

Figure 5. Melatonin receptor protein expression in oocyte (O), granulocyte (G), corpus luteum (CL), and Leydig cells (L) of pituitary gland tissue during breeding season (n = 6)

melatonin receptor protein expression in antral follicle (A), in primordial follicle and primary ovarian follicle (B), and in corpus luteum (C)

AF = autrum of follicle, PF = primordial follicle, POF = primary ovarian follicle

melatonin has a critical role during reproduction as a neural signal in mammals. *MTNR* mRNA was expressed in the hypothalamus, pituitary gland, and ovary during the breeding season in yak, indicating that melatonin may have a critical role in hormonal production during the yak breeding season. Melatonin has direct effects on tissues such as the hypothalamus, pituitary gland, and ovary. We hypothesized that higher melatonin production would stimulate the tissue to produce hormone. However, the melatonin level would have to exceed a threshold amount before hormones would be produced in tissues.

In the first 30 years since the discovery of indole, much of the research related to melatonin and the pineal gland was related to its ability to modulate the reproductive physiology of photoperiod-dependent seasonally breeding mammals (Reiter et al., 1966; Reiter, 1973; Stetson et al., 1975; Turek et al., 1976). Thus, we determined the localization of melatonin and the cells with melatonin receptors. Positive staining for *MTNR* was detected in the pineal gland, pinealocyte, SR, and SS (Figure 2). The pineal gland immunohistochemistry detected a circular pattern in the pineal gland cells. We suggest that the pinealocytes produced melatonin and that melatonin could activate other pinealocytes and the SR and SS in other regions of the pineal gland. This stimulation may have produced more melatonin in pinealocytes. The hypothalamus mainly produces hormones related to reproduction, such as GnRH. GnRH is produced by the hypothalamus and it helps to synchronize ovulation with sexual behaviour (Marshall et al., 1980). Ovulation occurred as early as 15 days postpartum with 1.0 mg GnRH (Pinheiro et al., 2013). Melatonin receptors were observed in magnocellular neurons and parvicellular neurons in the hypothalamus, and mRNA expression was lower than that in the pineal gland ($P < 0.01$). However, this did not show that it was absolutely lower in the hypothalamus. High *MTNR* expression was observed in the magnocellular neurons and parvicellular neurons (Figure 3) but it was unclear whether hormonal production was stimulated or inhibited in these cells. It is clear that melatonin takes part in the generation of GnRH. We suggest that it may increase one type of hormone and decrease another type of hormone in the hypothalamus. *MTNR* protein was observed in the α-cells and β-cells of the pituitary gland (Figure 4). LH and FSH are the main hormones produced in the pituitary gland for reproduction. GnRH could affect LH and FSH via the hypothalamus-pituitary axis to adjust the progress of reproduction (Emanuele

et al., 1987). Melatonin significantly reduced the plasma levels of LH and 17-β-estradiol, while urinary 6-sulfatoximelatonin (STM) was increased at the morning estrus (Chuffa et al., 2011). Immunohistochemical analysis of the pituitary gland showed that the sides of the folliculi had variable levels of *MTNR* expression, which were distinct in the pituitary gland. We suggest that melatonin and GnRH adjust the hormones in the pituitary gland. GnRH operates via the hypothalamus-pituitary axis to participate in precession and melatonin is present. In the ovary, we found that *MTNR* expression was higher in the ovarian follicle (Figure 5A, B) and lower in the corpus luteum (Figure 5C) than in Leydig cells. *MTNR* expression was observed in the granular leukocytes and oocytes. We obtained similar results like Tamura et al. (2009) who showed that melatonin had direct effects on ovarian function. Reactive oxygen species (ROS) and apoptosis are involved with a number of reproductive events, including folliculogenesis, follicular atresia, ovulation, oocyte maturation, and corpus luteum (CL) formation (Tamura et al., 2009). The ROS produced within the follicles, especially during the ovulation process, were scavenged by melatonin and reduced oxidative stress may be involved with oocyte maturation and embryo development (El-Raey et al., 2011; Tamura et al., 2012). We suggest that melatonin was involved with the growth and maturation of ovarian follicles and it also participated in the growth of oocytes. Melatonin also stimulated hormone production by Leydig cells.

Therefore, we can suggest a clear pathway for melatonin. Melatonin production in the pineal gland is affected by the day length, which leads to melatonin accumulation in the pineal gland. Melatonin is released into nearby capillaries until it reaches a threshold level. Next, melatonin enters the hypothalamus-pituitary gland-gonad axis where it adjusts the hormones produced by each tissue. Melatonin also participates in the development of the hypothalamus, pituitary gland, and gonads. Thus, the yak breeding season is initiated between the summer solstice and winter solstice when there are long nights and short days.

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