Alterations of reproductive hormones and receptors of male rats at the winter and summer solstices and the effects of pinealectomy

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Abstract

BACKGROUND: Photoperiodic changes mediate certain physiological or pathological alterations in organisms. Solstices represent either the longest or shortest photoperiod of a year.
OBJECTIVES: Intact and pinealectomized rats were used to investigate the potential changes of reproductive hormones in the hypothalamus-pituitary-testis (HPT) axis including GnRH, FSH, LH, testosterone and melatonin, and their receptors at summer solstice (SS) and winter solstices (WS).
METHODS: The levels of reproductive hormones in HPT axis and the binding characteristics of their receptors were examined using radioimmunoassay and radioreceptor assay techniques, respectively.
RESULTS: The results indicate that in the intact male rat, GnRH, LH and testosterone are higher at the SS than at the WS. However, FSH exhibited no significant seasonal changes. In the testis, B_{max} and K_d of LH receptors are higher at the WS than at the SS while those of FSH receptors are higher at the SS than at the WS. In addition, the melatonin in HPT axis appeared significant differences between WS and SS. B_{max} and K_d of melatonin receptors in the hypothalamus and pituitary also showed higher at the WS than at the SS. Moreover, reproductive hormone production lost their seasonal rhythms after pinealectomy.
CONCLUSION: The most important discovery in this study is that we first reported that pinealectomy had profound effects on the binding characteristics of melatonin with its subtype receptors. Especially at the hypothalamus, the dominated melatonin receptors shifted from MT1 to MT2 after pinealectomy at the two solstices.

ORIGINAL ARTICLE

Abbreviations:

GnRH	- Gonadotropin-releasing hormone
FSH	- Follicle-stimulating hormone
LH	- Luteinizing Hormone
T	- Testosterone
MT1	- MT1 melatonin receptor
MT2	- MT2 melatonin receptor
K_d	- Equilibrium dissociation constant
B_{max}	- Total binding capacity

INTRODUCTION

The solstice is an astronomical event that happens twice each year when the sun reaches its highest position in the sky as seen from the North or South Pole.The two solstices usually are referred to as the summer solstice (SS) and winter solstice (WS). In the northern hemisphere, the SS is around June 21, when the sun reaches the celestial longitude of 90°. The WS is around December 22, when the sun reaches the celestial longitude of 270°. The photoperiod of the solstices is either the longest or the shortest of the year.

The significance of the two solstices had been recorded in documents of the traditional Chinese medicine (TCM) two thousand years ago. In TCM, the two solstices are considered as yearly turning-points of *Yang Qi* (positive energy charge) and *Yin Qi* (negative energy charge). In the SS, *Yang Qi* is in its highest level and *Yin Qi* is in its lowest level of the year in the human body according to the theory of TCM. In the WS, this relationship is reversed. Accordingly, these changes may associate with certain physiopathological alternations in many organisms, especially in humans (Luo *et al.* 2001; Wang *et al.* 2008; Lu *et al.* 2010; Zhang &Guo, 2010). As a result, the two solstices are considered as the evidence in TCM to initiate prevention or treatment of certain disorders. Some disorders that have association with the seasonal changes, including asthma, allergic rhinitis, rheumatoid arthritis, etc., were found to be more treatable at the periods of the two solstices (Huang *et al.* 2010; Jiao *et al.* 2011; Li *et al.* 2011; Yan, 2012). Therefore, the two solstices are not only represented the special photoperiod of the seasons, but also are very important to human physiology.

It is interesting that even though human beings are non-seasonal breeding creatures, the photoperiod changes also have profound impact on human reproductive physiology. Our previous study has found that the testosterone in the urine of men was significant higher at the SS than at the WS (Luo *et al.* 2001). The mechanism is not completely clear. But study showed that the reproductive activity changes in the solstices were more related with photoperiod rather than temperature in the seasonal breeding animals (Gebbie *et al.* 1999). In additional, significant differences in melatonin levels in the circulation between WS and SS have been reported in the infant (Sivan *et al.* 2001), women (Holdaway *et al.* 1991; 1997), and vertebrates (Reierth *et al.* 1999; Garcia *et al.* 2003; El *et al.* 2005).

The indoleamine melatonin is usually known as the major secretory product of the pineal gland. It is particularly important in a chronobiological context, especially with regard to its effects on the hypothalamic circadian pacemaker. It regulates a variety of physiological functions (Tan *et al.* 2007a; 2011; Luchetti *et al.* 2010; Reiter *et al.* 2010; Cardinali *et al.* 2012), particularly to the seasonal reproduction (Hoffman & Reiter, 1965a; Espino *et al.* 2011; Barrett & Bolborea, 2012). Several decades ago, scientists found that the seasonally breeding mammals used the photoperiodic changes to time their reproductive cycles, and the reproductive system is controlled by the daily rhythm of melatonin production (Reiter & Hester, 1966; Reiter, 1973a; Stetson *et al.* 1975; Turek *et al.* 1976). Recently, scientists have tested melatonin and testosterone in ram seminal plasma and their variations between the breeding and non-breeding seasons. They found melatonin and testosterone both exhibit seasonal variations, with a decrease after the WS and a rise after the SS (Casao *et al.* 2010). Reports also show that exogenously administered melatonin at the solstices can influence reproduction (Fitzgerald *et al.* 2000; Guerin *et al.* 2000; Santiago-Moreno *et al.* 2000).

However, few studies have been conducted on the non-seasonal breeding animals and the mechanism by which melatonin mediates reproductive changes at the two solstices. Laboratory Sprague-Dawley (SD) rats are generally characterized as non-seasonal breeders (Nelson *et al.* 1994; Heideman *et al.* 2001). In this study, we used normal and pinealectomized SD rats to investigate the hormone profiles of the hypothalamus-pituitary-testis axis (HPT axis) and their receptors (FSH receptor and LH receptor) at the WS and the SS. We also compared HPT axis melatonin levels and melatonin receptors at the WS and the SS.

MATERIALS AND METHODS

All procedures performed were approved by the Subcommittee on Research Animal Care of the University.

Experimental animals

Male Sprague-Dawley rats, weighing 180–200 g, were purchased from Vital River Laboratories (VRL), Peking University Health Science Center (PUHSC). The animals were housed at the facility with windows and exposed to natural photoperiod 40 d before the solstices. The periods of animal acclimation were from May 11 to June 21 with the photoperiod from 14 h 17 min to 15 h during the summer and from November 12 to December 22 with the photoperiod from 10h 5min to 9h 20min during the winter, respectively. Temperature was controlled at 22±2 °C. Animals were allowed free access to food and water. At one month before the solstices (SS and WS), rats were randomly separated into 3 groups. One group served as normal controls, the second

group was sham-operated, the third group was pinealectomized. All the animals were kept until the day of solstice (June 21 or December 22). Animals were sacrificed by decapitation at 20:30 h at the solstices under 5w red light. Blood samples were collected from body trunk and serum samples were kept frozen (–80 °C) until analysis. Tissue samples including hypothalamus, pituitary and testis, were removed and weighed immediately after decapitation and were frozen in liquid nitrogen; they were kept frozen (–80 °C) until measurement.

Pinealectomy

Pinealectomy was performed according to the reports (Hoffman & Reiter 1965b**;** Kato *et al.* 1998). Rats (180–200 g) were anesthetized with 3% pentobarbital sodium (0.1ml/100g body weight, i.m.) (Sigma-Aldrich, USA). An incision was made in the mid sagittal plane of the head, and the bones were drilled with a dental bur to allow visualization of the transverse and sagittal sinuses. Dual ligatures were placed on the superior sagittal vein, cut the vein and fold back the end which is near the sagittal sinus, and the pineal gland was removed with an elbow ophthalmic tweezers. In the sham-operated animals, the superior sagittal vein was dual ligatured and cut but without removal of the pineal gland.

Radioimmunoassay of melatonin

Serum and organs levels of melatonin were measured by RIA as reported (Shi *et al.* 1998; Saito *et al.* 2004). Melatonin was extracted from serum, testis, pituitary gland and hypothalamus samples in 4 ml trichloromethane and dried by vacuum evaporation. The organic phase was evaporated and the residue was dissolved in 1.2 ml of PBS containing 0.1% gelatin. Protein concentration was determined with the Bradford protein assay. Aliquots of 500 µl were mixed with 100 µl of PBS, 50 µl of [3H]*o*-methylmelatonin (Amersham International plc, UK; specific activity 83ci/mmol), and 50 µl of a rabbit anti-melatonin antiserum (supplied by Prof. K. Wakabayashi, Gunma University, Japan) at a final dilution of 1 : 40 000. A standard curve (0.3125–40 pg/tube) was constructed by serial two-fold dilutions of synthetic melatonin (Sigma, St. Louis, MO). After incubation overnight at 4 °C, 0.75 ml of saturated ammonium sulfate (pH 7) and 50 µl of normal rabbit serum (1:20) were added. The mixture was incubated at 4 °C for 1 h and centrifuged. The pellet was resuspended in 100 µl of 0.1 N NaOH and 200 µl of distilled water. Radioactivity was determined with liquid scintillation fluid (4 ml) in a β-counter (Beckman, Germany).

HPT axis hormonal measurements

Plasma GnRH levels were assessed by radioimmunoassay using commercial kits (Beijing Sino-uk institute of Biological Technology, China) with intra- and interassay of coefficient of variation (CV) of <8%. Normal ranges for GnRH level were 10–640 pg/mg. Serum FSH, LH and testosterone (T) were assessed by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, China). The intra- and interassay of coefficient of variation (CV) were <10% and <15%, respectively. Normal ranges for FSH, LH and T levels were 2.5–100 mIU/ml, 5–200 mIU/ml, 0.1–20 ng/ml, respectively.

Receptor measurements

Melatonin receptor

Membrane preparations and binding assays were performed according to the procedure described by Iigo (Iigo *et al.* 1994) with some change. [^{125}I] Melatonin (1800 Ci/mmol) was obtained from Atomic Energy Research Institute (Beijing, China). Unlabeled melatonin was obtained from Sigma (St. Louis, MO).

Membrane preparation: Frozen tissues were thawed and homogenized in ten volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with an electric homogenizer, 10s/time, 4 times. The homogenate was centrifuged at 44,000× g for 30 min at 4°C. The pellet was washed, resuspended in Tris-HCl buffer, and centrifuged for a second time under the same conditions. The crude membrane pellet was finally resuspended in Tris-HCl buffer (pH 7.4) to yield a tissue concentration of approximately 1.5 mg protein/ml. Protein concentration was determined with the Bradford protein assay. Membrane preparations were used either immediately or stored at –80 °C till use. Storage of membranes up to 6 months did.

Binding Assays: For binding assays, [^{125}I] melatonin was diluted in 50 mM Tris-HCl buffer (pH 7.4). Melatonin were dissolved in ethanol, then diluted in Tris-HCl buffer to 40 µM, and used for the binding assay. Binding was initiated by the addition of aliquots of membranes (hypothalamus 100 µg, pituitary gland 80 µg). Resuspended in Tris-HCI buffer to tubes containing 50 µl of appropriate concentrations (20000–120000cpm) of [^{125}I]melatonin, and Tris-HCl buffer, the total volume is 300 µl. Non-specific binding was defined as the binding in the presence of 100 µl of 40 µM melatonin, the total volum is also 300 µl. The binding of [^{125}I] melatonin was routinely measured in duplicate after incubation at 37°C shaking bath for 60min. The reaction was terminated by the addition of 3 ml of ice-cold Tris-HCl buffer and immediate vacuum filtration through Whatman GF/B fiber filters (Whatman International Ltd., Maidstone, UK) soaked in 1% BSA. Each filter was washed 4 times with 4 ml of ice-cold Tris-HCl buffer, and placed into test tubes; radioactivity was determined using a gamma counter. Specific binding of [^{125}I] melatonin was calculated by subtracting nonspecific binding from total binding and expressed as fmol/mg protein.

FSH and LH receptors

For this study, [^{125}I] FSH (1839 Ci/mmol) was obtained from Atomic Energy Research Institute (Beijing,

China). Unlabeled FSH was obtained from Calbiochem (4350 IU/mg, USA).

Membrane preparation: Frozen testicular tissue was thawed and tunica albuginea was removed, the tubules were rinsed with physiological saline to remove the mucus. Seminiferous tubules were homogenized in 3 ml ice-cold buffer A (27%(wt/wt) sucrose, 1 mmol/L EDTA, 20 mmol/L Na-HEPES, pH 7.5) with an electric homogenizer, 10 s/time ,4 times. The homogenate was centrifuged at 3 500×g for 10 min at 4 °C, poured and supernatant was saved. The pellet was resuspended in 3 ml ice-cold buffer A, homogenated and centrifuged for a second time under the same conditions. The second supernatant was mixed, centrifuged at 27 000×g for 30 min. The pellet was washed, resuspended in buffer B (1 mmol/L EDTA, 20 mmol/L Na-HEPES, pH 7.5), and centrifuged for a second time under the same conditions. The crude membrane pellet was finally resuspended in buffer B to yield a tissue concentration of approximately 1.5 mg protein/ml. Protein concentration was determined with the Bradford protein assay. Membrane preparations were used either immediately or stored at −80 °C till use. Membranes were saved up to 6 months.

Binding Assays: For binding assays, pure FSH and [^{125}I] FSH was diluted in reaction buffer (10 mM Hepes, 5 mM $MgCl_2$, 0.1 M sucrose, 0.1% ovalbumin, pH 7.4) for the binding assay. Binding was initiated by the addition of aliquots of membranes (1 μg). They were resuspended in reaction buffer to tubes containing 100 μl of appropriate concentrations (20 000–200 000 cpm) of [^{125}I] FSH, and reaction buffer, the total volume is 400 μl. Non-specific binding was defined as the binding in the presence of 100 μl of 7.5 IU FSH, the total volume is also 400 μl. The binding of [^{125}I] FSH was routinely measured in duplicate after incubation at 37 °C in a shaking bath for 90 min. The reaction was terminated by the addition of 3 ml of ice-cold PBS buffer(pH 7.4) and immediate vacuum filtered through Whatman GF/B fiber filters (Whatman International Ltd., Maidstone, UK) soaked in 1% BSA. Each filter was washed 4 times with 4 ml of ice-cold PBS buffer, and placed into test tubes, radioactivity was determined using a gamma counter. Specific binding of [^{125}I] FSH was calculated by subtracting nonspecific binding from total binding and expressed as fmol/mg protein.

LH receptor

[^{125}I] LH (2340 Ci/mmol) was obtained from Atomic Energy Research Institute (Beijing, China). Unlabeled LH was obtained from Calbiochem(6555 IU/mg, USA).

Membrane preparation: This was the same as for the FSH receptor.

Binding Assays: For binding assays, pure LH and [^{125}I] LH was diluted in PBS buffer (including 0.1% Bovine Serum Albumin, pH 7.4) for the binding assay. Binding was initiated by the addition of aliquots of membranes (1 μg). They were resuspended in PBS buffer in tubes containing 100 μl of appropriate concentrations (0.1–10 ng) of [^{125}I]LH, and PBS buffer, the total volume is 300 μl. Non-specific binding was defined as the binding in the presence of 100 μl of 2 ng LH, the total volume is also 300 μl. The binding of [^{125}I] LH was routinely measured in duplicate after incubation at 34 °C shaking bath for 2 h. The reaction was terminated by the addition of 5 ml of 50 mM ice-cold Tris-HCl buffer (pH 7.4) and immediate vacuum filtration through Whatman GF/B fiber filters (Whatman International Ltd., Maidstone, UK) soaked in 1% BSA. Each filter was washed 4 times with 4 ml of ice-cold Tris-HCl buffer, and placed into test tubes, radioactivity was determined using a gamma counter. Specific binding of [^{125}I] LH was calculated by subtracting nonspecific binding from total binding and expressed as fmol/mg protein.

Statistics

Binding curves for K_d and receptor number were analyzed using the Graphpad Prism program (Graphpad Software Inc., CA, USA, v2.01). Results are shown as means ± S.E.M. The data were analyzed by one-way analysis of variance (ANOVA). Differences were considered significant at $p<0.05$.

RESULTS

Seasonal change of HPT axis hormones

Differences were found for hormones of the HPT axis in blood between the SS and the WS. Data showed that the levels of GnRH ($p<0.05$) and testosterone ($p<0.05$) at the SS were higher when compared to those at the WS in the normal control animals. However, the comparisons between the SS and the WS of FSH and LH revealed no statistical differences in the normal control rats (Table 1).

Tab. 1. Comparison of hormones of hypothalamus-pituitary-testis axis at the summer solstice (SS) and the winter solstice (WS).

	GnRH (nmol/L)	FSH (nmol/L)	LH (μmol/L)	T (μmol/L)
SS	0.12±0.004*	13.31±1.20	0.16±0.01	17.41±1.70*
WS	0.10±0.005	13.43±1.13	0.12±0.02	14.29±1.70

Data are expressed by mean ± S.E.M., n=8. *$p<0.05$ versus the WS data.

Seasonal changes in receptor for melatonin, FSH and LH

We found FSH and LH receptors in the testis and melatonin receptors in hypothalamus and pituitary all exhibited significant difference between the SS and the WS. The data showed the B_{max} and K_d of FSH receptors in the testis were higher at the SS than that at the WS in the control rats($p<0.05$) (Figure 1). On the contrary, the B_{max} and K_d of LH receptor were significant higher at the WS than at the SS (Figure 2). As shown in Figure 3, the K_d and B_{max} of melatonin receptor at both hypothalamus and pituitary, were all significantly higher at the WS than at the SS in the normal control rats (Figure 3).

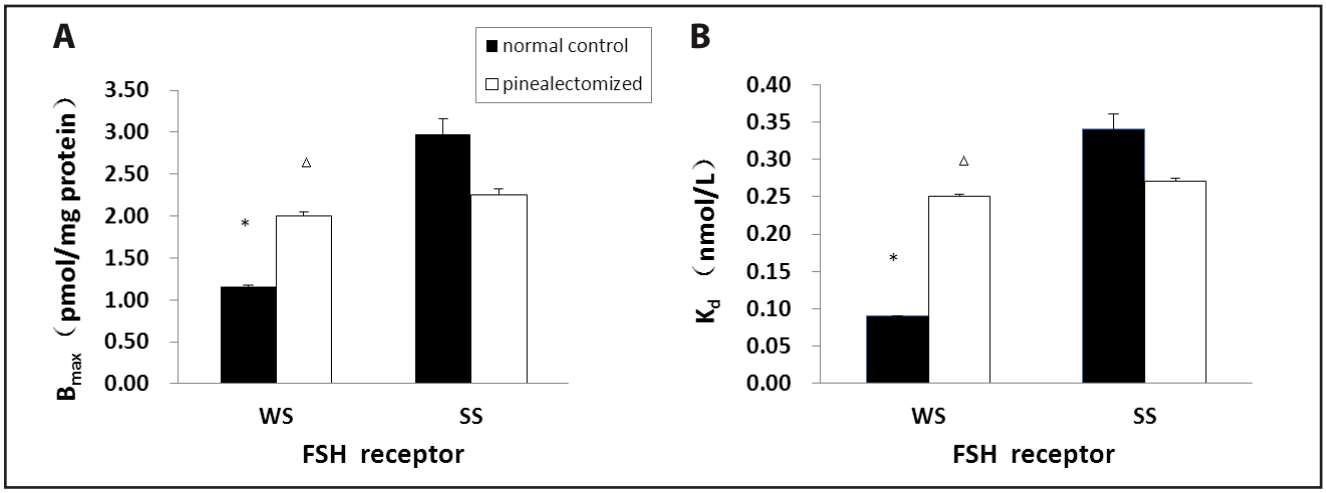

Fig. 1. The difference between the summer solstice (SS) and the winter solstice (WS) on FSH receptor in the testis. (A) B_{max} of FSH receptor. (B) K_d of FSH receptor. Both the affinity (K_d) and the total binding capacity (B_{max}) of FSH receptors were significantly higher at the SS than that at the WS in the control rats ($p<0.05$); in the pinealectomized animals the differences disappeared. N=6. *$p<0.05$ versus the SS data, $^{\Delta}p<0.01$ versus the normal control group data of the same season, respectively.

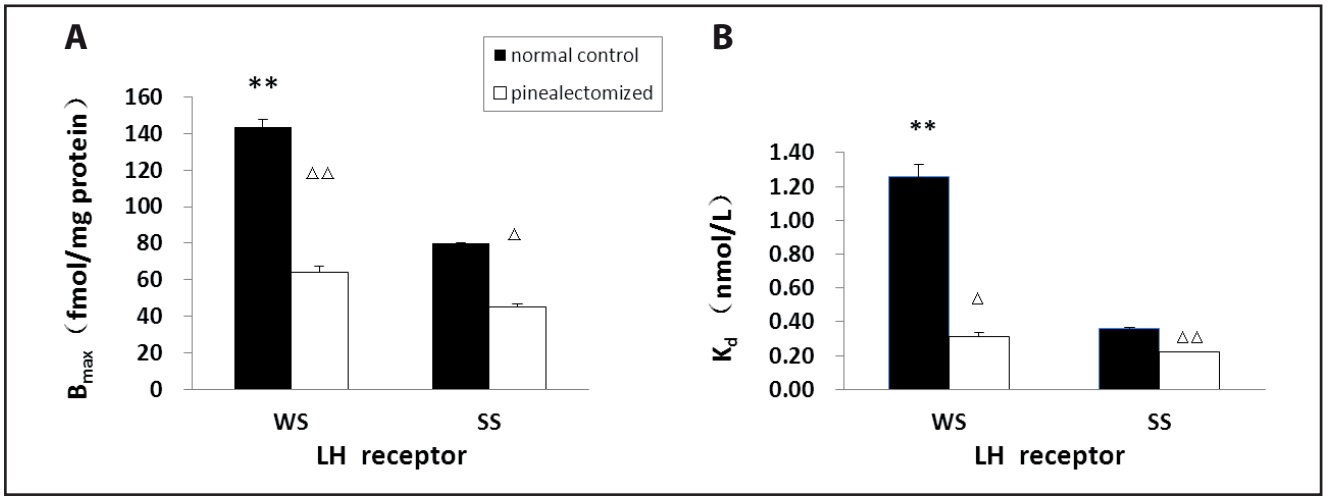

Fig. 2. The difference between the summer solstice (SS) and the winter solstice (WS) on LH receptor in the testis. (A) B_{max} of LH receptor. (B) K_d of LH receptor. The LH receptors in the testis showed higher B_{max} and K_d at the WS than the SS in the control rats ($p<0.01$). After pineal removal, there were no statistical differences between the WS and the SS in the testicular LH receptor. N=6. **$p<0.01$ versus the SS data, $^{\Delta}p<0.01$ versus the normal control group data of the same season, $^{\Delta\Delta}p<0.001$ versus the normal group data of the same season, respectively.

Melatonin in HPT axis differs at the solstices

In rats with the intact pineal gland, the levels of melatonin in pineal and serum were higher at the WS than at the SS ($p<0.05$). Melatonin in HPT axis also exhibited significantly higher values at the WS than at the SS, especially in the hypothalamus ($p<0.001$) (Table 2).

Effect of pinealectomy

Results obtained from the present study with pinealectomy also showed that in SD rats, the reproductive hormones lost their rhythms or even exhibited the reversed rhythm compared to the intact rats after pinealectomy. The melatonin level in the pinealectomized rats in the WS dropped significantly in the hypothalamus ($p<0.001$) and testis ($p<0.01$), when compared to the normal control animals of the same season, and there were no statistical differences between the WS and the SS. However, in pituitary, melatonin levels in the pinealectomized animals were significantly lower at the WS ($p<0.05$) and were elevated at the SS ($p<0.05$) as compared to the normal control rats of the same season; the data showed higher melatonin at the SS than at the WS ($p<0.05$), with completely different seasonal rhythm to the normal control rats (Figure 4). The data showed that GnRH and testosterone (T) different significantly between the WS and the SS in the normal control rats, and had no statistical difference between the WS and the SS after removing the pineal gland. However, LH and

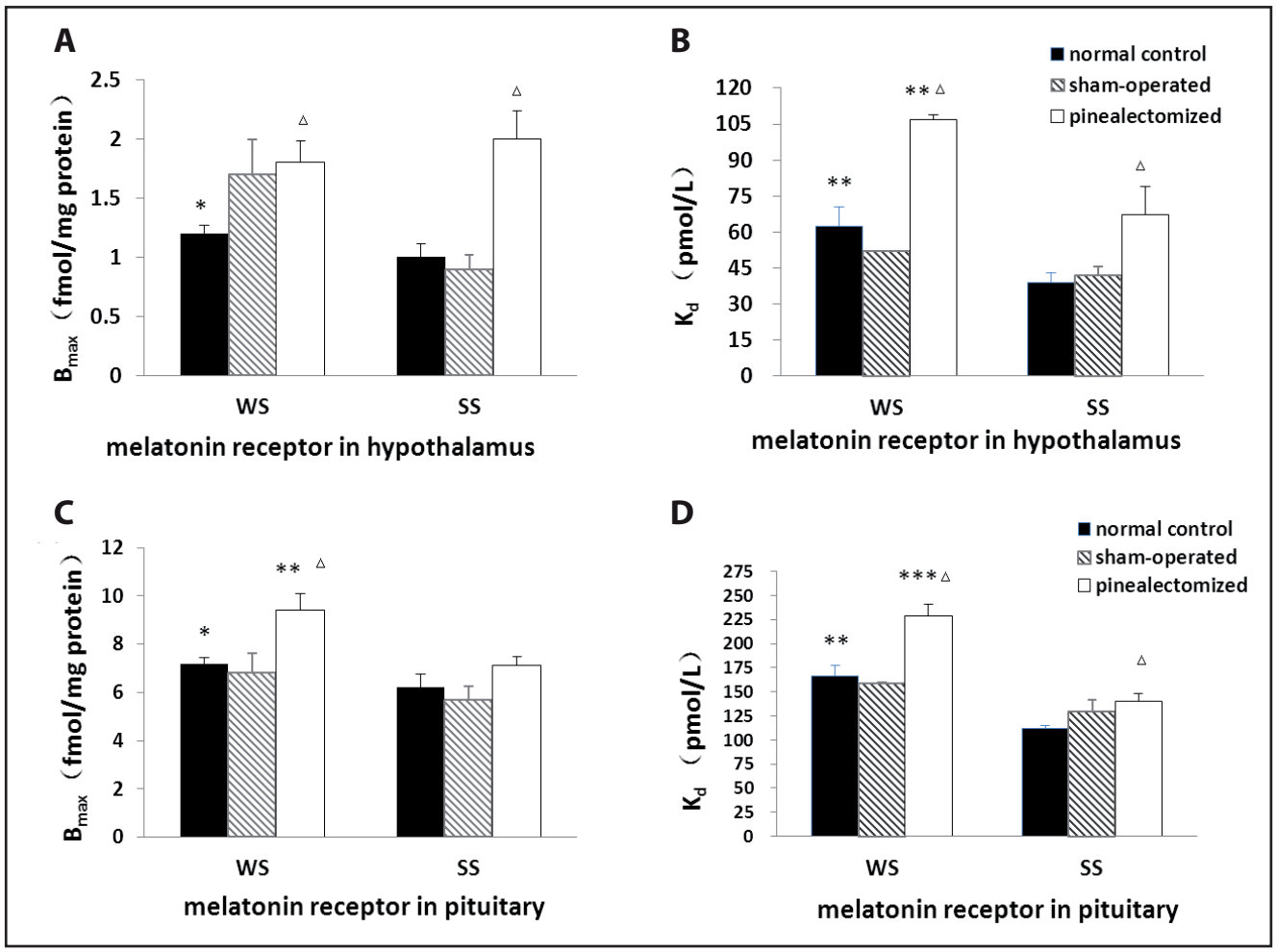

Fig. 3. The difference between the summer solstice (SS) and the winter solstice (WS) on melatonin receptors in the hypothalamus and pituitary. (A) B_{max} of melatonin receptor at hypothalamus. (B) K_d of melatonin receptor at hypothalamus. (C) B_{max} of melatonin receptor at pituitary. (D) K_d of melatonin receptor at pituitary. Melatonin receptor at both hypothalamus (A,B) and pituitary (C,D), were all significantly higher at the WS than at the SS in the normal control rats. However, after pinealectomy the B_{max} and K_d of melatonin receptor were elevated significantly as compared to the normal control rats at both the SS and the WS in both the hypothalamus and pituitary. The differences in the B_{max} of melatonin receptors at hypothalamus between the SS and the WS disappeared after pinealectomy (A). N=6. *p<0.05 versus SS data, **p<0.01 versus SS data, ***p<0.001 versus the SS data; $^{\Delta}p$<0.01 versus normal group data of the same season, respectively.

Tab. 2. Comparison of melatonin in pineal and hypothalamus-pituitary-testis axis between the summer solstice (SS) and the winter solstice(WS) in normal control rats.

	Pineal (pg/mg protein)	serum (pg/ml)	hypothalamus (pg/mg protein)	pituitary (pg/mg protein)	testis (pg/mg protein)
SS	1463±72.7	16.4±1.89	0.7±0.11	3.1±0.49	0.14±0.01
WS	1888±168.3*	22.4±1.47*	4.5±0.29***	5.2±0.53*	0.31±0.06*

Data are expressed by mean± S.E.M., n=6. In normal control animals, melatonin shows significantly higher at the WS than at the SS, *p<0.05 versus SS data, ***p<0.001 versus SS data, respectively.

FSH which had no seasonal rhythm in the normal control rats, had significant differences between the WS and the SS in the pinealectomized groups (Figure 5) .

The pineal also influenced the B_{max} and K_d of the HPT axis receptors. After pineal removal, LH receptors and FSH receptors lost the differences on B_{max} and K_d between the WS and the SS (Figure 2). However, after pinealectomy the K_d of melatonin receptor were elevated significantly as compared to the normal control rats at both the SS and the WS in both hypothalamus and pituitary (Figure 3).

DISCUSSION

Seasonal change of HPT axis hormones

In the present study, we used male Sprague-Dawley (SD) rat to investigate the changes of the reproductive hormones released from HPT axis between the sol-

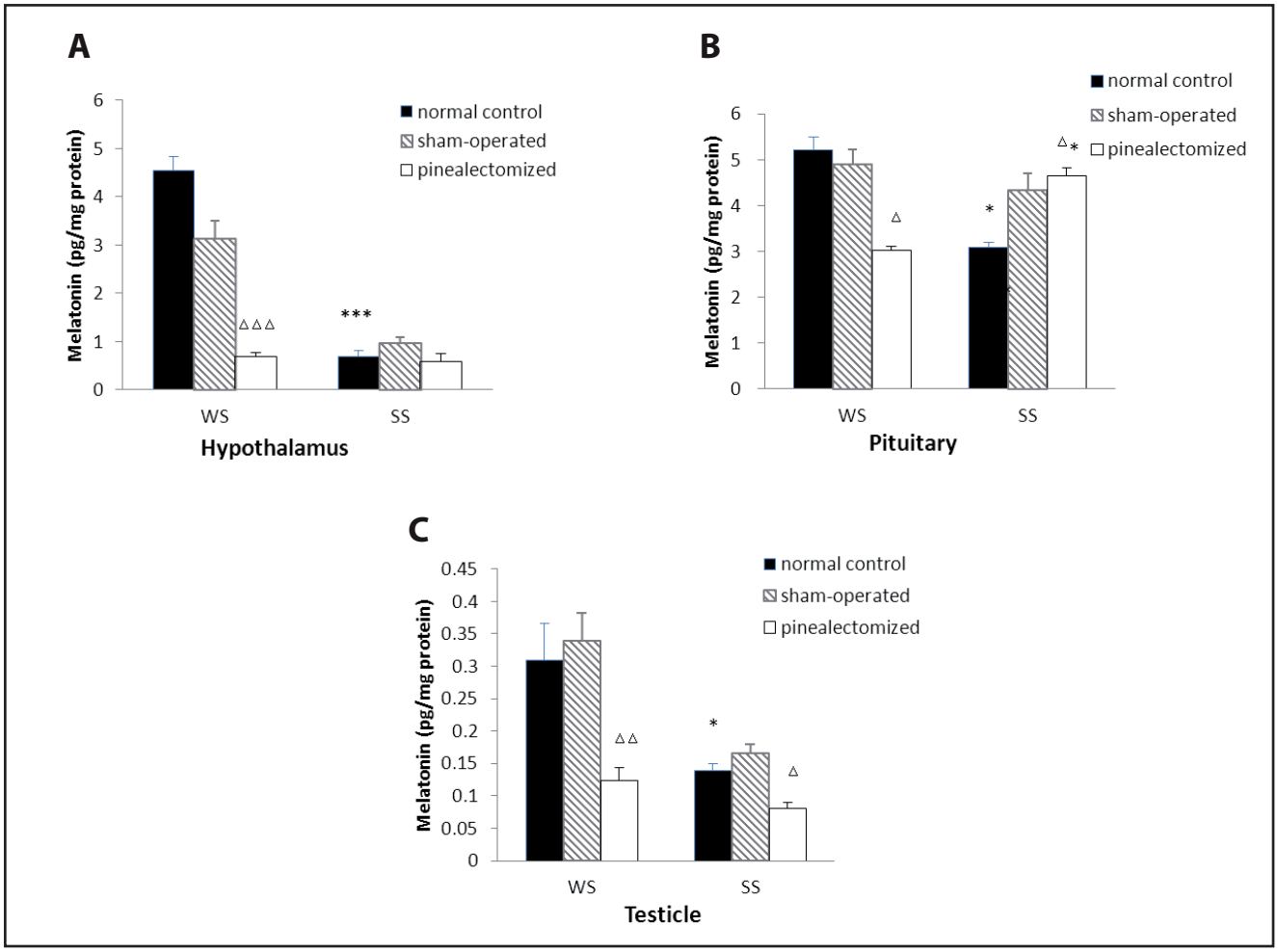

Fig. 4. Effect of pineal gland on the difference in melatonin between the WS and the SS in the hypothalamus-pituitary-testis axis. (A) After removal of the pineal, the significant difference of melatonin between the WS and the SS in hypothalamus disappeared. The same phenomenon was seen in the testicle (C). But in the pituitary (B), pineal removal changed the normal seasonal rhythm of melatonin, and had higher melatonin at the SS than at the WS. No difference between the sham-operated groups and the normal control rats was seen. N=6. Versus the WS data, *$p<0.05$, ***$p<0.001$; Versus normal control group data of the same season, $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$, $^{\Delta\Delta\Delta}p<0.001$.

stices. It is well known that the SD rats have lost the characteristics of seasonal breeding (Nelson *et al.* 1994; Heideman *et al.* 2001), as with the human being. There are numerous publications showing that the reproductive hormones in the seasonal breeding animals have marked annual fluctuations, which lead to the seasonal breeding (Wilson *et al.* 1986; Reiter, 1993; Stenbak *et al.* 2001; Smith *et al.* 2008). But how about the non-seasonal breeding animals? From the data presented in the current study, we found that in the SD rat, the reproductive hormones of the HPT axis and their receptors exhibit significant differences between WS and SS. For the normal male rat, GnRH, LH and testosterone(T) are higher at the SS than at the WS, especially GnRH and T ($p<0.05$), while FSH exhibited no significant seasonal change (Table 1). FSH is the hormone that directly affects the production of spermatozoa (O'Shaughnessy *et al.* 2010; Madani *et al.* 2012). In seasonal breeding animals, FSH usually exhibits a seasonal change (Irby *et al.* 1984), and the size of testis also shows seasonal differences (Reiter, 1973b; Howell-Skalla *et al.* 2002). However, SD rats breed throughout the year and the testicular size does not change as a consequence of the photoperiod (Wallen *et al.* 1987). So we may presume that even though SD rats are not seasonal breeders, their reproductive hormones may still change seasonally. Since the seasonal change does not affect FSH and the production of spermatozoa, this may lead to their non-seasonal character.

Seasonal changes in receptor for melatonin, FSH and LH

Another interesting observation is that we found FSH and LH receptors in the testis of SD rats also exhibited significant difference between the SS and the WS. The B_{max} and K_d of LH receptors in the testis were higher in the WS than in the SS (Figure 1). On the contrary, although there were no seasonal changes of FSH concentration between the WS and the SS, the B_{max} and

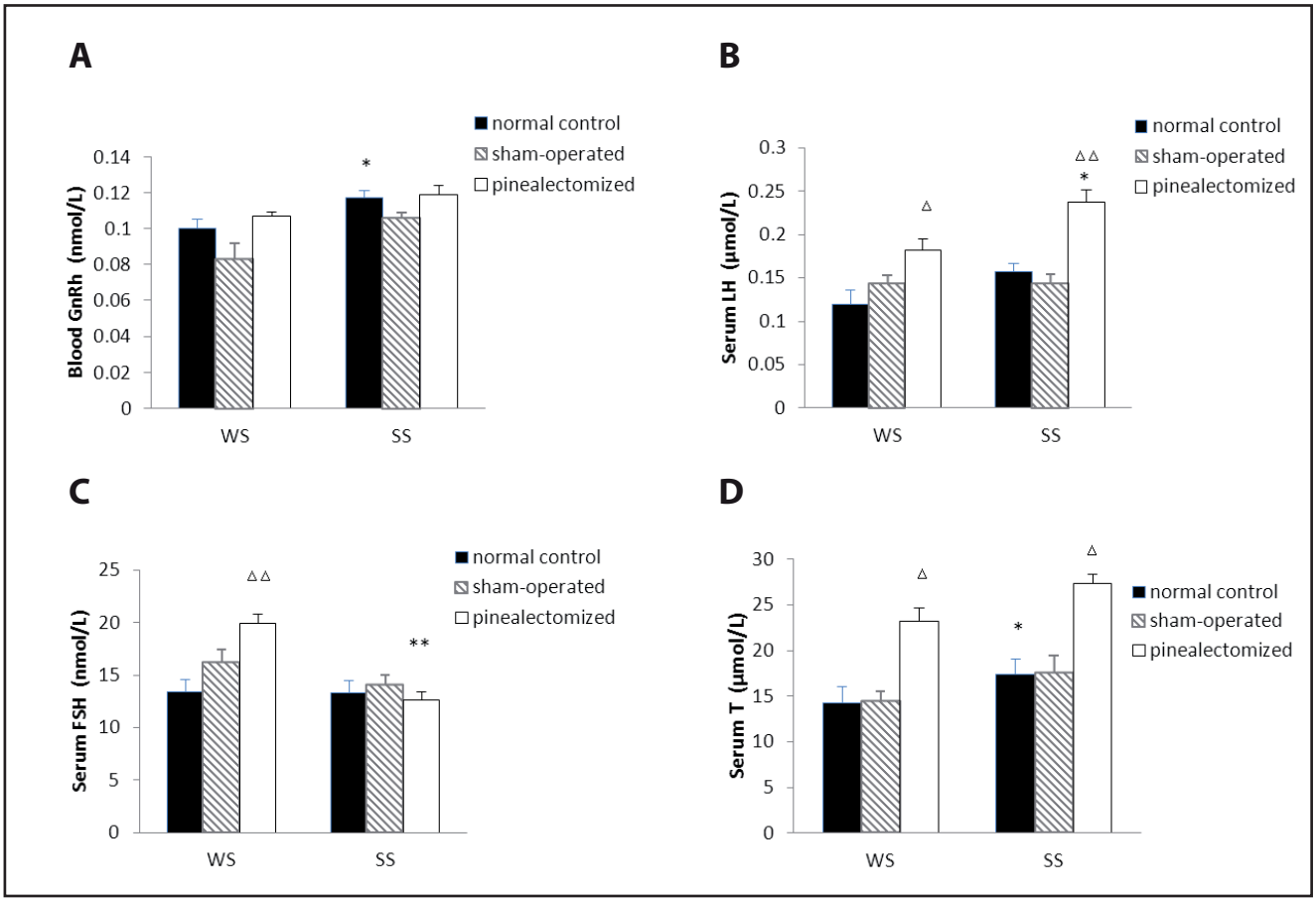

Fig. 5. Effect of pineal gland on the hormones of hypothalamus-pituitary-testis axis between the WS and the SS. (A) After removal of the pineal, the significant difference of GnRH in blood between the WS and the SS disappeared. The same phenomenon was seen in testosterone (D), and the pinealectomized animal had higher levels of testosterone (T) than the normal control rats. (B) (C) Pineal removal made the normal change of LH and FSH in serum more significant between the WS and the SS. No significant difference were seen between the sham-operated groups and the normal control rats in all test, $p>0.05$. N=8. *$p<0.05$ versus the WS data, **$p<0.01$ versus the WS data, $^{\Delta}p<0.05$ versus the normal control group data of the same season $^{\Delta\Delta}p<0.01$ versus the normal control group data of the same season, respectively.

K_d of FSH receptor were significant higher at the SS than at the WS (Figure 2). As we know, the K_d is the equilibrium dissociation constant. By definition, the K_d is the concentration of ligand that will occupy 50% of the receptors (Mather 1996). The B_{max} is the maximal number of receptor binding sites. As a constant, the K_d usually does not change. However, in this study we found that the K_d of FSH and LH receptors both had photoperiodic variations, as did the melatonin receptors (Figure 3). The K_d changes of melatonin receptors in pars tuberalis (Skene *et al.* 1993) and that of FSH and LH receptors in the testis (Bartke *et al.* 1987; Hayashi *et al.* 2002) have been reported. The reasonable explanation of these observations may be related to the different subtype receptors. For example, the MT1 melatonin receptor (MT1) has a higher affinity than the MT2 melatonin receptor (MT2). The K_d of MT1 is 21±3 pM for monkey and 15±3 pM for human; the K_d of MT2 is 107±11 pM for monkey and 328±12 pM for human (Audinot *et al.* 2003; Kato *et al.* 2005). In the rat, the MT1 has the following characteristics: K_d=14±1.2pM; B_{max}=0.9±0.02 fmol/mg protein (Richter *et al.* 2008). In the present study, the K_d of melatonin receptors in SD rat is from 38±4 pM to 228±13 pM, which is consistent with the K_d of MT1 and MT2 as reported previously. Therefore, the seasonal change of the K_d and B_{max} of the melatonin receptor may imply that the various receptors of melatonin show different activities in different seasons, so melatonin may bind different subtype receptors depending on photoperiodic variations. Even though the FSH and LH receptors have no definite subreceptors, our results of the K_d of these two hormones are similar or in the range of the data published previously. The K_d of the FSH receptor is 6.2×10^{-10} M for hamster (Minegishi *et al.* 1994). The K_d of LH receptor is 3.8×10^{-10} M for equine (Saint-Dizier *et al.* 2004) and 0.8×10^{-10} M for rat granulosa cells (Oury & Darbon, 1988). Therefore, there is still the possibility that there are subtypes of FSH receptor and LH receptor. However, these require further investigations.

Melatonin in HPT axis differs at the solstices

Our observations also noted that the seasonal changes of reproductive hormones are associated with the pineal gland function. It is well known that pineal melatonin is a chronobiological factor, which can affect the hypothalamic circadian pacemaker. Many publications have shown that seasonal reproduction is closely related with melatonin (Ancel *et al.* 2012; Lutterschmidt 2012). In the present study, we have observed that melatonin and its receptors in the HPT axis of the SD rats exhibited significant differences between the WS and the SS. In rats with the intact pineal gland, melatonin levels in blood and in HPT axis were significantly higher at the WS than at the SS (Table 2). Illnerova *et al.* have reported consistent with our findings, i.e., under the natural photoperiods the blood melatonin levels and arylalkylamine *N*-acetyltransferase (AANAT) activity in rats are also higher at the WS and lower at the SS (Illnerova & Vanecek 1980; Illnerova *et al.* 2000). Even though our results also showed that the reproductive hormones of rats were lower at the WS than that at the SS, still does not mean the melatonin depressed the reproductive hormones. In fact, in some seasonal breeding animals such as rams, melatonin treatment during non-breeding season actually improves testicular testosterone production, modifies sperm motility parameters and improves the fertilization rate (Faigl *et al.* 2009; Casao *et al.* 2010). Moreover, the study showed that as a seasonal breeding animal, testosterone of the ram at the WS was higher than at the SS (Casao *et al.* 2010). Therefore, no matter if animals are seasonal or non-seasonal breeders, they both need melatonin to provide time of year information. Pineal melatonin is a both necessary and sufficient seasonal chemical signal for the HPT axis.

Effect of pinealectomy

In the current study, we also observed that pinealectomy had profound effects on the binding characteristics of melatonin with its subtype receptors. Judging from the K_d, data we first reported that after pinealectomy, the dominated melatonin receptors in the hypothalamus shifted from MT1 to MT2, i.e., the K_d of the melatonin receptors shifted from 39±4 pM to 67±12 pM in SS and shifted from 62±8 pM to 107±2 pM in WS (Figure 3). It seems that these shifts at the WS are more significant than that at the SS. What is the physiological significance of these changes are currently unknown. However, this definitely is worthy of further research.

Results obtained from the present study with pinealectomy also showed that in SD rats, the reproductive hormones lost their rhythms or even exhibited the reversed rhythm compared to the intact rats after pinealectomy. For example, the levels of GnRH and T in the blood lost their seasonal fluctuations (Figure 5 A,D), while the levels of LH and FSH in serum appeared significantly different (Figure 5 B,C), especially FSH levels were higher at the WS than at the SS. Testicular receptors for LH and FSH in testis also lost their seasonal profiles, such as B_{max} and K_d (Figure 1 and 2). In addition, melatonin levels in the hypothalamus and testis exhibited no seasonal changes after pinealectomy (Figure 4 A,C). However, in the pituitary, after pinealectomy in the summer, the melatonin level was higher than that of the normal control, and in the winter this relationship is reverse (Figure 4 B). As reported, many organs and tissues have the capacity to synthesize melatonin (Iuvone & Besharse, 1983; Huether, 1993; Stefulj *et al.* 2001; Jimenez-Jorge *et al.* 2005; Tan *et al.* 2007b) . One study found that pinealectomy significantly increased the content of melatonin in several subcellular compartments of hepatocytes (Venegas *et al.* 2012). So this phenomenon in our study may suggest the pituitary cells may also produce melatonin, and the pineal melatonin rhythm is an important biological clock mediating photoperiodic melatonin production.

In conclusion, we found that WS and SS which represent the photoperiodic changes have significant impact on the reproductive hormones at the HPT axis and the binding characteristics of their receptors. The changes mentioned above were associated with pineal gland and its secretory product of melatonin, since pinealectomy disturbed their seasonal rhythm and binding characteristics. These observations also support our previous findings in which the reproductive hormones of male testosterone levels also exhibit seasonal changes. The most important discovery in this study is that pinealectomy can shift the dominant melatonin receptor from MT1 to MT2 in the hypothalamus. This has not examined in the previous publications. This shift may be related to seasonal adaptation due to the loss of melatonin rhythm produced by pineal gland. Its significance will be investigated in the future.

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