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Full Length Research Paper

Evidence for a role of KISS-1/GPR54 system in decreased luteinizing hormone (LH) secretion in fasted prepubertal ewes

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Short fasting has been shown to suppress luteinizing hormone (LH) secretion in ewes, however, the molecular mechanism underlying this phenomenon is not clear. Based on recent discovery of the crucial role of KiSS-1/GPR54 system in the central control of the GnRH axis, we hypothesized that KiSS-1/GPR54 system was involved in the inhibitory effect of fasting on LH secretion in ewes. To test this hypothesis, we fed six prepubertal ewes subjected to a control diet or fasting 72 h and determined the expression of KiSS-1/GPR54 system in hypothalamus. To further confirm the effects of fasting on the expression of KiSS-1/GPR54 system in hypothalamus as responsible for the suppression of LH secretion in ewes, we also investigated the effect of kisspeptin-10 on the LH secretion in fasted and standard feeding prepubertal ewes. The results show that fasting for 72 h could significantly suppress hypothalamic expression of KiSS-1 and GPR54 gene and LH secretion in prepubertal ewes. In addition, intravenous injection of 1 mg of kisspeptin-10 could significantly increase LH secretion in fasted and standard ewes. These data suggest that the inhibitory effect of fasting on LH secretion of prepubertal ewes is probably via decreasing the expression of KiSS-1/GPR54 system in hypothalamus.

Key words: Fasting, KiSS-1/GPR54 system, hypothalamus, LH, ewes.

INTRODUCTION

It is well recognized that energy insufficiency can frequently lead to disturbed reproductive maturation and infertility. Fasting or food restriction suppresses pulsatile luteinizing hormone (LH) secretion in rodents, monkeys, humans, cows, sheep and horses (Cameron and Nosbisch, 1991; Cunningham et al., 1999; Laughlin and Yen, 1996). It has been demonstrated that the suppression of LH secretion is caused by decreased release of gonadotropin hormone (GnRH) from the hypothalamus rather than by inhibition of pituitary gonadal axis sensitivity to GnRH (Aloi et al., 1997; I'Anson et al., 2000). However, the exact mechanisms of suppression of LH secretion under fasting or food restriction state are not well understood.

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The GnRH neurons within hypothalamus which receive a plethora of central and peripheral signals, regulate the secretion of pituitary gonadotropin via the pulsatile release of GnRH into the hypophysial portal blood vessels. Although numerous central players in the inhibitory and stimulatory controls of GnRH neuron have been identified during the last decades (Ojeda et al., 2003; Terasawa, 2005; Todman et al., 2005), our knowledge of the hypothalamic GnRH neuron regulating reproduction was recently revolutionized by the identification of KiSS-1/GPR54 system. KiSS-1/GPR54 system is consisted of kisspeptins encoded by KiSS-1 gene and their receptor, G protein-coupled receptor (GPR54) (Roa et al., 2008; Smith et al., 2006). KiSS-1 gene encodes a number of structurally-related peptides (kisspeptin-54, kisspeptin-14, kisspeptin-13 and kisspeptin-10), which are derived from the differential proteolytic processing of a common precursor of 145

Table 1. Real-time fluorescence quantitative PCR probes and primer sequences.

Gene	mRNA primer sequence 5´-3´	Product	Reference
KiSS-1	Forward: CTGGTGCAGCGGGAGAAG Reverse: GCGCAGGCCGAAGGA Probe (FAM labeled): ACGTGTCCGCCTACA	57 bp	Bellingham et al., 2009
GPR54	Forward: TACATCCAGCAGGTCTCGGTG Reverse: ACGTACCAGCGGTCCACACT Probe (FAM labeled): CACGTGTGCCACTCTGACCGCC	71 bp	Bellingham et al., 2009
β-actin	Forward: TGACGTCGACATCCGCAAAG Reverse: GGAGCCGCCAATCCACAC Probe (FAM labeled): CCTCTACGCCAACACGGTGCTGTCC	179 bp	U39357; NCBI2009

amino acids. These kisspeptins all contain an Arg-Phe-NH_2 motif at the C-terminus and have a similar function.

The role of KiSS-1/GPR54 system in regulating mammalian reproduction was first discovered in 2003, when it was independently found that GPR54 is crucial for sexual maturation in both men and mice (De Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). These findings brought about extensive research on the role of KiSS-1/GPR54 system in reproduction. Numerous studies performed in various species have already shown that kisspeptins, mainly produced in hypothalamus, are able to stimulate follicle stimulating hormone (FSH) and LH secretion by regulating GnRH secretion from hypothalamus. This therefore implied that KiSS-1/GPR54 system is a major regulator of reproductive endocrinology (Gianetti and Seminara, 2008; Hashizume et al., 2010; Novaira et al., 2009).

The aim of the present study was to determine whether the hypothalamic expression of the KiSS-1 and GPR54 mRNA decreases during the inhibition of LH secretion induced by fasting in ewes. In addition, we verified if kisspeptin-10 injection could restore the baseline levels of LH changed by fasting.

MATERIALS AND METHODS

Experimental designs

Experiment one

Six female small tail Han sheep (native breed), around 20 kg body weight and three months old, were randomly divided into control (n = 3) and fasted group (n = 3). Ewes were housed by treatment in two separate, adjacent pens. Control ewes were given *ad labium* access to hay and 200 g concentrate formulated according to the NRC (1998) throughout the experiment. Ewes in the fasted group were withheld from feed for 72 h. Ewes in both groups had *ad libitum* access to water. At the end of the fasting period, the animals were killed by decapitation under pentobarbital anesthesia, and the hypothalamus was immediately dissected out and frozen in liquid nitrogen until processing for RNA analysis. Ethical approval for the study was obtained from the Ethical Committee of the Jilin Agricultural University, China.

Experiment two

12 female small tail Han sheep (native breed), around 16.5 kg body weight and three months old, were used for this experiment. 24 h before the start of the experiment, each ewe was fitted with a jugular catheter for intensive blood sampling. The ewes were randomly divided into four groups (n = 3): two groups were fasted for 72 h and the other two were fed with standard diet throughout the experiment. All the groups had free access to water. 72 h later, one fasted and one standard group were given intravenous injection of 5 ml of saline and the remaining fasted and standard group were injected with 1 mg of kisspeptin-10 (amino acid sequence: YNWNSFGLRF-NH_2, synthesized by Shanghai Qiangyao Biotechnology Limited). Blood samples (5 ml) were collected via jugular catheter every 15 min for 210 min (from 30 min before injection to 180 min after injection).

RNA analysis by real-time reverse transcription- polymerase chain reaction

Total RNA was isolated from hypothalamic samples using the Trizol reagent (TAKARA) according to their protocol. Hypothalamic expression of KiSS-1 and GPR54 mRNAs was assessed by real-time reverse transcription- polymerase chain reaction (RT-PCR). Total RNA (1 µg) was then reverse-transcribed using reverse transcription (TAKARA). In order to assess the expression of KiSS-1 and GPR54 in hypothalamus of ewes, β-*actin* genes was selected for reference gene and oligonucleotide primers and TaqMan probes for β-*actin* genes was designed according to ovine GenBank (GeneBank accession NO.U39357). Oligonucleotide primers and TaqMan probes for ovine KiSS-1 and GPR54 genes were obtained from previously published work (Bellingham et al., 2009). The information of oligonucleotide primers and TaqMan probes for β-*actin*, KiSS-1 and GPR54 gene are shown in Table 1.

To verify changes in KiSS-1 and GPR54 gene expression, real-time fluorescence quantitative RT-PCR was performed using StepOne Plus Real Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Each qPCR reaction (25 µL) contained 2 µL of template cDNA, 1 µL of each of specific primers, 0.5 µL of respective probe, 12.5 µL of Premix Ex Taq (TAKARA) and 7.5 µL of dH_2O. PCR amplification protocol was as follows: 95°C for 10 min, followed by 45 cycles of 30 s at 95°C (denaturation step) and 1 min at 60°C (primer annealing and elongation). In order to ensure the amplification efficiencies of the

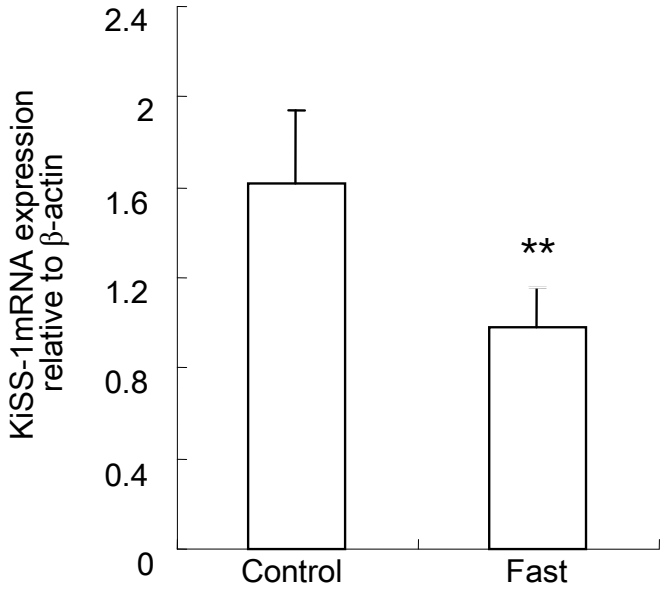

Figure 1. Hypothalamic expression of KiSS-1 gene in prepubertal ewes in control and fasted group (fasting 72 h). **p < 0.01 compared with respective control value.

reference gene, KiSS-1 and GPR54 were equivalent, and relative standard curves for each gene was generated using serial dilutions of cDNA. Expression of *KiSS-1*and GPR54 mRNA was quantified by using the comparative cycle threshold (CT) method (Schmittgen and Livak, 2008), and the results were presented as the ratio of KiSS-1 or GPR54 to reference gene β-actin mRNA expression.

Hormone measurements

Serum LH levels were determined using sheep LH ELISA Kit (Cusabio Biotech CO., Ltd) according to the manufacturer's protocol. The minimum detectable dose of LH is typically less than 0.12 m IU/ml.

Statistical analysis

To calculate the statistical differences among groups, the statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. Student's test was used to determine the significant difference of hypothalamic expression of KiSS-1 and GPR54 gene in ewes between fasted and control groups. One-way analysis of variance (ANOVA) was utilized to determine the significance of differences of LH concentration among groups. Expression of KiSS-1 and GPR54 gene was given as mean ± standard deviation (SD). In general, p values less than 0.05 were considered statistically significant.

RESULTS

The results of hypothalamic expression of KiSS-1and GPR54 gene in ewes between fasted and control groups are shown in Figures 1 and 2. Compared to control group, fasting 72 h significantly suppressed hypothalamic expression of KiSS-1 and GPR54 gene in ewes (*P<0.01*). Effects of intravenous injection of 1 mg of kisspeptin-10 on LH secretion of ewes in fasted and standard groups are shown in Figures 3 to 5. As shown in Figures 3 and 4, intravenous injection of 1 mg of kisspeptin-10 significantly increased pulsatile LH secretion of ewes in fasted and standard groups. As shown in Figure 5, fasting 72 h significantly decreased mean concentration of LH in ewes (*P<0.05*) and injection of kisspeptin-10 also significantly increased mean concentration of LH both in fasted and standard groups (*P<0.01*) .

DISCUSSION

Reproductive maturation and its subsequent functioning in mammalian depend much on the state of energy stores of the body (Schneider, 2004). It has been well established that short fasting can lead to the suppression of LH secretion, which is due to impairment of GnRH secretion (I'Anson et al., 2000; Wójcik-Gładysz et al., 2009). However, the exact mechanism of fasting causing GnRH secretion to decrease is unclear. Recently, studies in various species had already demonstrated that KiSS-1/GPR54 system could increase the secretion of FSH and LH by directly activating GnRH neurons and eliciting GnRH release from hypothalamus (Gianetti and Seminara, 2008; Hashizume et al., 2010; Novaira et al., 2009). Furthermore, studies in rodents have shown that estradiol, photoperiod and alcohol can regulate secretion of GnRH by changing expression of KiSS-1 gene in hypothalamus (Adachi, 2007; Mason et al., 2007; Srivastava et al., 2009). These results t herefore suggest that KiSS-

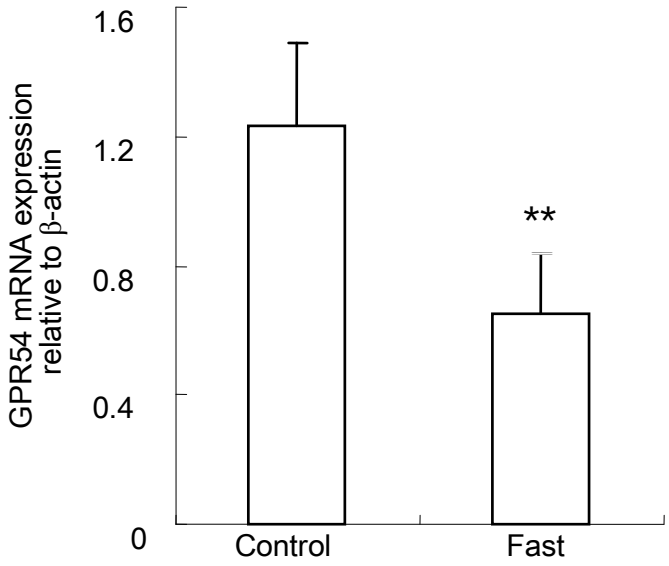

Figure 2. Hypothalamic expression of GPR54 gene in prepubertal ewes in control and fasted group (fasting 72 h). **p < 0.01 compared with respective control value.

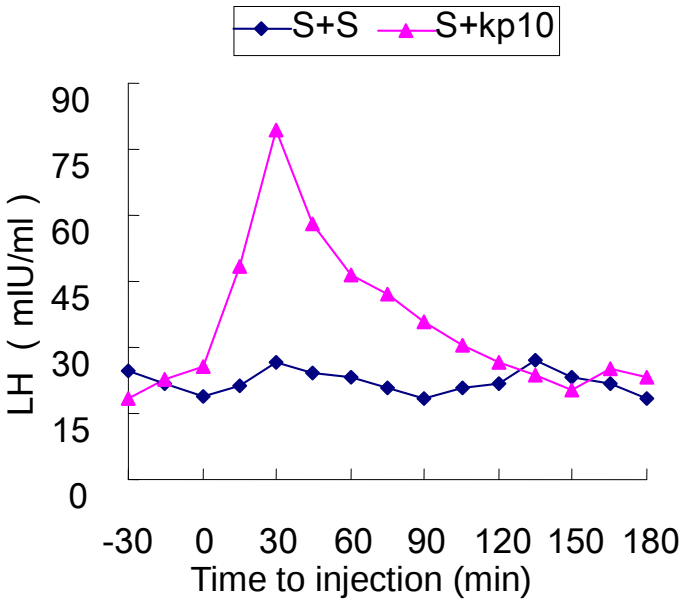

Figure 3. Changes in the blood concentrations of LH over 210 min in individual lamb from standard group (S) that received an intravenous injection of 5 ml of saline (S) or 1 mg kisspeptin-10 dissolved in saline (kp10) at 0 min. LH, Luteinizing hormone.

1/GPR54 system play a critical role in reproductive endocrinology. To our knowledge, however, there is no report on ruminant for the interaction between KiSS-1/GPR54 system and LH secretion under fasting state.

The results of this study show that fasting 72 h can significantly decrease LH secretion and expression of hypothalamic KiSS-1 and GPR54 genes in prepubertal ewes, and kisspeptin-10 encoded by KiSS-1 gene can effectively restore the decreasing of LH secretion in fasted state. It has been demonstrated that fasting 72 h can lead to the suppression of LH secretion in pre-pubertal ewes (Wójcik-Gładysz et al., 2009). The present study showed that fasting for 72 h decreases the expression of KiSS-1 and GPR54 gene of hypothalamus

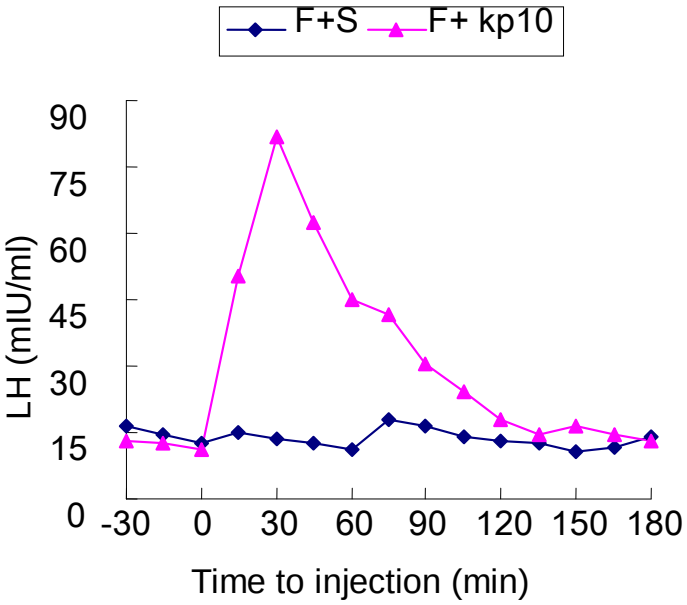

Figure 4. Changes in the blood concentrations of LH over 210 min in individual lamb from fasted group (F) that received an intravenous injection of 5 ml of saline (S) or 1 mg kisspeptin-10 dissolved in saline (kp10) at 0 min. LH, Luteinizing hormone.

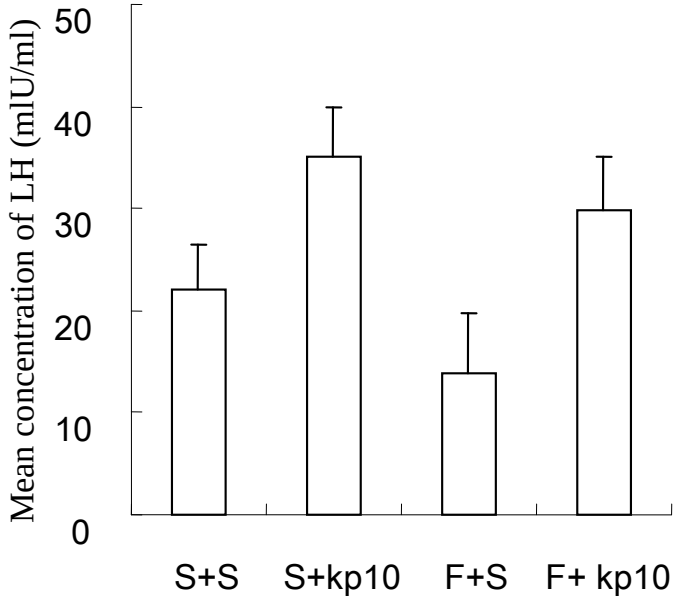

Figure 5. Mean concentration of LH in prepubertal ewes for standard (S) or fasted 72 h (F) and then given intravenous injection of 5 ml of saline (S) or 1 mg kisspeptin-10 dissolved in saline (kp10) at 0 min. *p<0.05, and ** p < 0.01 compared with value of control group (S+S). LH, Luteinizing hormone.

in prepubertal ewes. It is reasonable to speculate that the decreasing expression of hypothalamic KiSS-1 gene may be responsible for the suppression of LH secretion since KiSS-1/GPR54 system can control the GnRH release from hypothalamus. Several studies in rodents have also shown that KiSS-1/GPR54 system is involved in the interaction between negative energy balance and LH secretion. In prepubertal female and male rats, fasting 72

h induced a significant decrease in the expression of hypothalamic KiSS-1 gene which was associated to the reduction of circulating LH (Castellano et al., 2005). Similar result has been observed in adult female rats, where fasting for 18 h decreased the expression of hypothalamic KiSS-1 gene (Brown et al., 2008). Furthermore, adult male mice were used to investigate the time-course for the effects of fasting on expression of hypothalamic KiSS-1 gene, and the results show that fasting for 12, 24 and 48 h can reduce the expression of hypothalamic KiSS-1 gene (Luque et al., 2007).

In addition to fasting, expression of hypothalamic KiSS-1 gene was also determined in other form of negative energy balance. It was reported that expression of hypothalamic KiSS-1 gene in diabetic male rats induced by streptozotocin injection decreased and accompanied by a state of hypogonadotropic hypogonadism, with a significant decrease in LH and testosterone levels (Castellano et al., 2006). To our knowledge, for the expression of hypothalamic GPR54 gene under fasting state, the relevant report is scarce and inconsistent. 72 h fasting increased the expression of hypothalamic GPR54 gene in prepubertal rats (Castellano et al., 2005), however, in adult mice, fasting for 12, 24 and 48 h led to a significant decrease in its expression (Luque et al., 2007). The present study demonstrates that fasting for 72 h could significantly decrease the expression of GPR54 gene of hypothalamus in prepubertal ewes. The inconsistent result is probably caused by differences in species, age and duration of fasting, which were used to conduct the experiment.

In order to confirm the hypothesis that the decreasing expression of hypothalamic KiSS-1 gene is responsible for the suppression of LH secretion in fasted ewes, effect of kisspeptin-10 on LH secretion in standard and fasted prepubertal ewes was analyzed. The result shows that kisspeptin-10 could significantly increase LH secretion of ewes in fasted and standard groups. To our knowledge, although administration of kisspeptin has been shown to elicit the secretion of LH in intact adult sheep (Pompolo et al., 2006) and ovariectomized ewes (Arreguin-Arevalo et al., 2007), there is no data on prepubertal ewes. The present study firstly demonstrated that 1 mg of kisspeptin-10 could significantly promote LH secretion in standard and fasted prepubertal ewe. These results are consistent with data from rodents. Intra-cerebral injection of 1 nmol of kisspeptin-10 could increase LH secretion of female and male rats after fasting for 72 h, and kisspeptin-10 was shown to elicit GnRH release by rat hypothalamic explants from fasting 72 h rats *in vitro* (Castellano et al., 2005). In addition, interrupting puberty onset by a regimen of undernutrition was able to be restored by repeated administration of kisspeptin to immature female rats (Castellano et al., 2005). Similarly, repeated intra-cerebral administration of kisspeptin-10 to uncontrolled diabetic male rats was capable to restore LH secretion (Castellano et al., 2006). These results implied that kisspeptin can restore the low level of LH secretion caused by negative energy balance.

Conclusion

The inhibitory effect of short fasting on LH secretion in prepubertal ewes was probably due to a decrease in the expression of hypothalamic KiSS-1/GPR54 system. These results provide evidence that KiSS-1/GPR54 system is involved in the interaction between negative energy balance caused by short fasting and LH secretion in prepubertal ewes.

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REFERENCES

Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H. & Uenoyama Y (2007). Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J. Reprod. Dev. 53(2): 367-378.

Aloi J, Bergendahl M, Iranmanesh A and Veldhuis J (1997). Pulsatile intravenous gonadotropin-releasing hormone administration averts fasting-induced hypogonadotropism and hypoandrogenemia in healthy, normal weight men. J. Clin. Endocr. Metab. 82(5): 1543-1548.

Arreguin-Arevalo JA, Lents CA, Farmerie TA, Nett TM and Clay CM (2007). KiSS-1 peptide induces release of LH by a direct effect on the hypothalamus of ovariectomized ewes. Anim. Reprod. Sci. 101(3-4): 265-275.

Bellingham M, Fowler PA, Amezaga MR, Rhind SM, Cotinot C, Mandon-Pepin B, Sharpe RM, Evans NP (2009). Exposure to a complex cocktail of environmental endocrine-disrupting compounds disturbs the kisspeptin/GPR54 system in ovine hypothalamus and pituitary gland. Envriron. Health Persp. 117(10): 1556-1562.

Brown R, Imran S, Ur E and Wilkinson M (2008). KiSS-1 mRNA in adipose tissue is regulated by sex hormones and food intake. Mol. Cell Endocrinol. 281(1-2): 64-72.

Cameron JL, Nosbisch C (1991). Suppression of pulsatile luteinizing hormone and testosterone secretion during short term food restriction in the adult male rhesus monkey (*Macaca mulatta*). Endocrinology, 128(3): 1532-1540.

Castellano J, Navarro V, Fernandez-Fernandez R, Roa J, Vigo E, Pineda R, Dieguez C, Aguilar E, Pinilla L and Tena-Sempere M (2006). Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. Diabetes, 55(9):2602-2610.

Castellano J, Navarro V, Fernandez-Fernandez R, Nogueiras R, Tovar S, Roa J, Vazquez M, Vigo E, Casanueva F, Aguilar E (2005). Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. Endocrinology, 146(9): 3917-3925.

Cunningham MJ, Clifton DK, Steiner RA (1999). Leptin's actions on the reproductive axis: perspectives and mechanisms. Biol. Reprod. 60(2): 216-222.

De Roux N, Genin E, Carel J, Matsuda F, Chaussain J, Milgrom E (2003). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc. Natl. Acad. Sci.

USA. 100(19):10972-10976.
Funes S, Hedrick J, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma F, Gustafson E (2003). The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Bioph Res Co. 312(4):1357-1363.
Gianetti E, Seminara S (2008). Kisspeptin and KISS1R: a critical pathway in the reproductive system. Reproduction, 136(3): 295-301.
Hashizume T, Saito H, Sawada T, Yaegashi T, Ezzat A, Sawai K, Yamashita T (2010). Characteristics of stimulation of gonadotropin secretion by kisspeptin-10 in female goats. Anim Reprod. Sci. 118(1): 37-41.
I'Anson H, Manning J, Herbosa C, Pelt J, Friedman C, Wood R, Bucholtz D , Foster D (2000). Central inhibition of gonadotropin-releasing hormone secretion in the growth-restricted hypogonadotropic female sheep. Endocrinology, 141(2): 520-527.
Laughlin G, Yen S (1996). Nutritional and endocrine-metabolic aberrations in amenorrheic athletes. J. Clin. Endocr. Metab. 81(12): 4301-4309.
Luque R, Kineman R, Tena-Sempere M (2007). Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. Endocrinology, 148(10): 4601-4611.
Mason AO, Greives TJ, Scotti MAL, Levine J, Frommeyer S, Ketterson ED, Demas G E and Kriegsfeld LJ (2007). Suppression of kisspeptin expression and gonadotropic axis sensitivity following exposure to inhibitory day lengths in female Siberian hamsters. Horm Behav. 52(4): 492-498.
Novaira H, Ng Y, Wolfe A, Radovick S (2009). Kisspeptin increases GnRH mRNA expression and secretion in GnRH secreting neuronal cell lines. Mol. Cell Endocrinol. 311(1-2): 126-134.
Ojeda S, Prevot V, Heger S, Lomniczi A, Dziedzic B, Mungenast A (2003). Glia-to-neuron signaling and the neuroendocrine control of female puberty. Ann. Med. 35(4): 244-255.
Pompolo S, Pereira A, Estrada K and Clarke I (2006). Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. Endocrinology, 147(2): 804-810.
Roa J, Aguilar E, Dieguez C, Pinilla L , Tena-Sempere M (2008). New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Front Neuroendocrin, 29(1): 48-69.
Schmittgen TD, Livak KJ (2008). Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3(6): 1101-1108.
Schneider JE (2004). Energy balance and reproduction. Physiol. Behav. 81(2): 289-317.
Seminara S, Messager S, Chatzidaki E, Thresher RR, Acierno JS Jr, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Colledge WH (2003). The GPR54 gene as a regulator of puberty. New Engl. J. Med. 349(17): 1614-1627.
Smith J, Clifton D, Steiner R (2006). Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction, 131(4): 623-630.
Srivastava VK, Hiney JK, Dees WL (2009). Short-term alcohol administration alters KiSS-1 gene expression in the reproductive hypothalamus of prepubertal female rats. Alcohol Clin. Express. 33(9): 1605-1614.
Terasawa E (2005). Role of GABA in the mechanism of the onset of puberty in non-human primates. Int Rev Neurobiol. 71:113-129.
Todman M, Han S, Herbison A (2005). Profiling neurotransmitter receptor expression in mouse gonadotropin-releasing hormone neurons using green fluorescent protein-promoter transgenics and microarrays. Neuroscience, 132(3): 703-712.
Wójcik-Gładysz A, Wankowska M, Misztal T, Romanowicz K, Polkowska J (2009). Effect of intracerebroventricular infusion of leptin on the secretory activity of the GnRH/LH axis in fasted prepubertal lambs. Anim. Reprod. Sci. 114(4): 370-383.