IJAS Iranian Journal of Applied Animal Science

# Gonadotropin-Releasing Response to Kisspeptin-10 and Its Modulation by Progesterone in Postpartum Cyclic Cows

**Research Article**

**A. Ezzat Ahmed[1,2*], Y. Goto[1], H. Saito[1], T. Sawada[1], J. Jin[1], T. Hirata[1] and T. Hashizume[1]**

[1] Department of Theriogenology, Faculty of Agriculture, Iwate University, 0208550, Morioka, Japan
[2] Department of Animal Science, Faculty of Veterinary Medicine, South Valley University, 83523, Qena, Egypt

Received on: 7 Feb 2012
Revised on: 5 Oct 2012
Accepted on: 1 Nov 2012
Online Published on: Sep 2013

*Correspondence E-mail: ahmed.abdelrahman@vet.svu.edu.eg

Online version is available on: www.ijas.ir

## ABSTRACT

The present study aimed to evaluate the effect of kisspeptin-10 (Kp10), a shorter variant of kisspeptin retaining full biological activity, on the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in cyclic adult cows, and the effect of plasma progesterone ($P_4$) concentration on the response to Kp10 administration. The experiments were performed using five postpartum cows (4-5 years old) treated with a progesterone-releasing intravaginal device (PRID) for 7 days. The animals received a single intravenous (i.v.) injection of Kp10 (5 µg/kg b.w.: 3.85 nmol/kg b.w.) for three consecutive days after the device's removal. Plasma concentrations of $P_4$ were higher on the day of the PRID's removal (day 0: 7.3±1.1 ng/mL) than 1 (day 1: 0.8±0.1 ng/mL) and 2 (day 2: 0.6±0.1 ng/mL) days later (P<0.05). Kp10 did not alter plasma LH concentrations significantly at day 0. However, it significantly stimulated the release of LH on day 1 and day 2 (P<0.05). Kp10 tended to stimulate the release of FSH at day 2; however, it did not alter the concentrations of FSH in plasma significantly throughout the experiment. The results showed that Kp10 stimulates the release of LH in postpartum cyclic cows, and suggested that high concentrations of $P_4$ in plasma may reduce the effect of kisspeptin on the secretion of gonadotropins.

**KEY WORDS** cow, FSH, kisspeptin-10, LH, progesterone.

## INTRODUCTION

Kisspeptin is a neuropeptide hormone encoded by *KiSS-1* (Gottsch *et al.* 2009) in the hypothalamus. Kisspeptin and its functional ligand, G-protein-coupled receptors (GPR54), play a pivotal role in regulating the secretion of gonadotropins through a mechanism dependent on gonadotropin-releasing hormone (GnRH) (Gottsch *et al.* 2004; Irwig *et al.* 2004; Navarro *et al.* 2005) in both prepubertal and adult animals (Gottsch *et al.* 2004). The effect of kisspeptin on the gonadotropins secretion has been studied in prepubertal female calves (Kadokawa *et al.* 2008; Ezzat *et al.* 2009), male calves (Ezzat *et al.* 2009), and ovariectomized cows (Whitlock *et al.* 2008). However, the gonadotropin-releasing response to kisspeptin in cyclic adult cows remains unclear. Progesterone ($P_4$), an ovarian steroid hormone, is known to be a major regulator of the secretion of gonadotropins (Mahesh and Brann, 1998) via modulation of GnRH. Studies *in vivo* (O'Byrne *et al.* 1991; Skinner *et al.* 1998) and *in vitro* (Genazzani *et al.* 1995; Baulieu, 1998; Mensah-Nyagan *et al.* 1999) demonstrated that high concentrations of $P_4$ inhibit the secretion of GnRH and consequently the release of gonadotropins. However, whether or not the concentration of $P_4$ in cyclic cows affects the secretion of gonadotropins in response to kisspeptin has yet to be examined.

The present study aimed to evaluate the gonadotropin-releasing response to kisspeptin in cyclic adult cows and the sensitivity of the response at high and low concentrations of $P_4$ in plasma. Postpartum cyclic cows were treated with a progesterone-releasing intravaginal device (PRID) and the gonadotropin-releasing response to kisspeptin-10 (Kp10) (a shorter variant of kisspeptin retaining full biological activity) was investigated after removal of the PRID.

## MATERIALS AND METHODS

### Kisspeptin-10 (Kp10)

Human Kp10 amide (amino acid sequence: YNWNSFGLRF-$NH_2$) was synthesized in our laboratory (Ezzat *et al.* 2009; Hashizume *et al.* 2010). The peptide has been confirmed to stimulate the release of gonadotropins in goats (Hashizume *et al.* 2010) and cattle (Ezzat *et al.* 2009) in studies *in vivo*.

### Animals

Five Japanese black cows (age, 4-5 years; mean body weight (b.w) ±SEM, 439±23 kg) were used. They were at 119 ± 16 (Mean±SEM) days postpartum. Their calves were weaned 46 ± 16 days before the experiment. Estrus was detected in all cows by the day of the experiment. The animals were housed in pens, with natural light allowed to enter through windows. They were fed hay and concentrate at 0930 h and 1600 h daily. The residuum of hay was removed at each feeding time. Water was available continuously. The animals were not fed before or during the experiment; they were fed only after the experiment. The experiments were performed from April to June in Morioka, Japan. All animal care and experimental protocols were approved by the Animal Care and Use Committee of Iwate University.

### Experimental design

### Progesterone treatment

Progesterone-releasing intravaginal devices (PRID; each spiral contains 1.55 g of progesterone ($P_4$) and 10 mg of estradiol ($E_2$) benzoate; Aska Pharmaceutical, Tokyo, Japan) were used. The cows were intramuscularly injected with 100 µg of a gonadotropin-releasing hormone (GnRH) analogue (Conceral; Nagase Medicals, Itami, Japan) at the time of the PRID's insertion (1500 h). After seven days, the spirals of the PRID were removed at 1500 h and each animal was intramuscularly injected with 500 µg of a $PGF_{2\alpha}$ analogue.

### Intravenous (i.v.) injection of Kp10

Cows were given a single i.v. injection of Kp10 (5 µg/kg b.w.: 3.85 nmol/kg b.w.) at 1500 h for three consecutive days after PRID withdrawal; day 0 (the day of the PRID's withdrawal), day 1 (1 day after its removal) and day 2 (2 days after its removal). The doses of Kp10 were chosen according to results of preliminary experiments (Ezzat *et al.* 2009; Hashizume *et al.* 2010). Kp10 was injected into freely moving animals via an indwelling catheter previously inserted into one of the external jugular veins.

### Blood sampling

Within a period of 60 minutes before Kp10 injection, blood samples (2.5 mL each) were collected every 20 minutes. At 0 min, the blood samples were collected followed by immediate Kp10 injection. Within a period of 60 minutes after Kp10 injection, blood samples were collected every 10 minutes. Thereafter, blood samples were collected every 20 minutes for another 100 minutes. The blood was collected from 1400 h to 1740 h. It was collected from the indwelling catheter into centrifuge tubes containing heparin and immediately chilled with ice. Individual plasma samples were obtained after centrifugation (2500 xG) and stored at -30 ˚C until assayed for $P_4$, LH, and FSH.

### Hormone assay

Concentrations of $P_4$ in plasma were measured by double-antibody enzyme-immunoassay (EIA) after extraction with diethyl ether. The EIA for $P_4$ was performed as described previously (Prakash *et al.* 1987) but with some modifications. Ninety six-well ELISA plates (Corning Glass Works, Corning, NY) were coated with 50 µg of anti-rabbit IgG (Seikagaku Co., Tokyo, Japan). Twenty five µL of standard or sample was incubated with 100 µL of $P_4$ antibody solution (1:100000; HAC-AAb3-06RBP841) for 24 h at 4 ˚C, the mixture was decanted, 100 µL of horse-radish peroxidase (HRP)-progesterone 3-(o-carboxymethyl) oxime was added (1:10000; $P_4$-HRP), and the incubation was continued a further 2 h at 4 ˚C. The assay's sensitivity was 0.1 ng/mL and the intraassay coefficient of variation (CV) was 8.9 %.

Concentrations of LH and FSH in plasma were measured by a double-antibody radioimmunoassay procedure with slight modifications (Hashizume *et al.* 1999; Hashizume *et al.* 2010). The standard preparation and the hormone for iodination were both USDA-bLH-B-6 for LH, and AFP5346D and AFP5318C, respectively, for FSH. Assay sensitivities for LH and FSH were 0.41 and 0.08 ng/mL, respectively. All samples were assayed in a single run. The intraassay CV was 10.8% for LH and 5.5% for FSH.

### Statistical analysis

All data from the experiments are presented as mean ±SEM. The significant differences in plasma LH and FSH concentrations between sampling times were analyzed with a repeated measure ANOVA, and the differences between sampling times before and after injections were determined using the Newman-Keuls test. The statistical significance of differences in plasma $P_4$ concentrations and the area under the curve (AUC) for LH among the days after removal of the PRID was determined by one-way ANOVA, and the Newman-Keuls procedure was used as a post-hoc test. All data were analyzed using Graph-Pad Prism (GraphPad Software, San Diego, CA, USA). Results were considered significant at the P<0.05 level.

## RESULTS AND DISCUSSION

Plasma $P_4$ profiles in cows after removal of the PRID are shown in Figure 1.

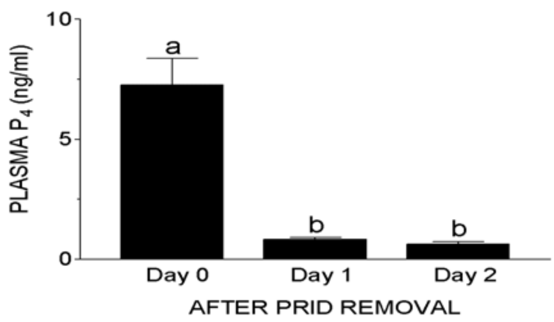


**Figure 1** Plasma concentrations of progesterone ($P_4$) in cows at day 0, day 1, and day 2 after removal of the progesterone-releasing intravaginal device (PRID). Each value represents the mean ±SEM for five animals. Different letters (a, b) on bars denote significant differences at P<0.05

Plasma progesterone concentrations were higher at day 0 than at day 1 or day 2 (7.3±1.1, 0.8±0.1 and 0.6±0.1 ng/mL, respectively. However, there was no significant difference between day 1 and day 2. The plasma concentrations of LH in response to the i.v. injection of 5 µg/kg b.w. of Kp10 after removal of the PRID are shown in Figure 2. Kp10 did not alter the mean concentration significantly at day 0 (Figure 2a). However, it stimulated the release of LH significantly at day 1 and day 2 (Figure 2b and Figure 2c, respectively) (P<0.05). The concentrations at 20 and 30 min (Figure 2b), and 20 to 60 min (Figure 2c) after Kp10 administration were higher than the concentrations before (P<0.05). The AUC of LH for 60 min after the injection of Kp10 was significantly greater at day 1 and day 2 than at day 0 (77.5±3.4, 82.9±3.2 and 54.9±4.3 ng.min.mL$^{-1}$, respectively) (Figure 3). The plasma concentrations of FSH in response to the i.v. injection of 5 µg/kg b.w. of Kp10 after removal of the PRID are shown in Figure 4. After Kp10 administration, the mean plasma FSH concentrations at day 0 (Figure 4a) or day 1 (Figure 4b) was not different from those observed before Kp10 injection. The FSH-releasing response to Kp10 tended to increase at day 2 (Figure 4c).

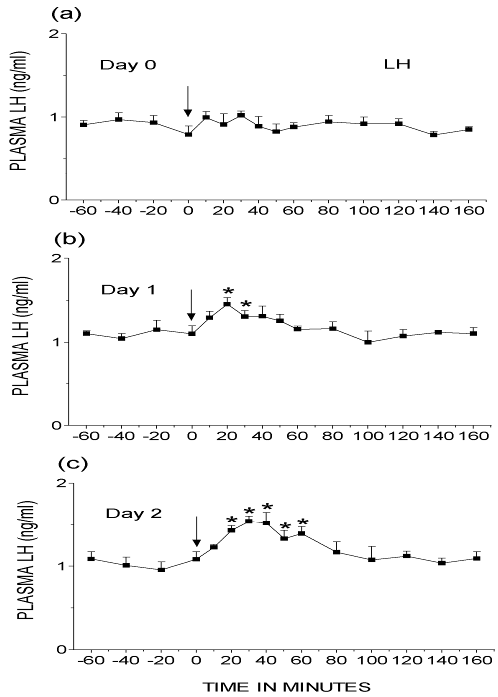


**Figure 2** Plasma concentrations of luteinizing hormone (LH) in response to intravenous (i.v.) injections of 5 µg/kg b.w. (3.85 nmol/kg b.w.) of kisspeptin-10 (Kp10) at day 0 (a), day 1 (b), and day 2 (c) after removal of the PRID in cows. Arrows indicate the time of injection. Each value represents the mean ±SEM for five animals. * P<0.05 indicates a significant difference from the pre-injection values

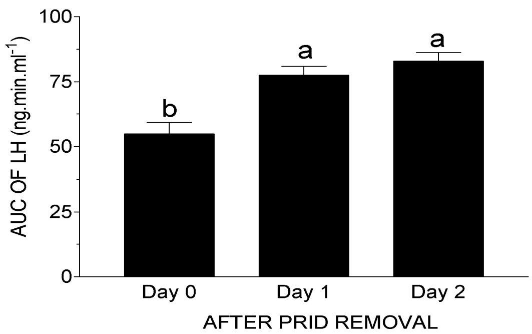


**Figure 3** Area under the LH response curve (AUC) for the 60-min period after the i.v. injection of 5 µg/kg b.w. of Kp10 at day 0, day 1, and day 2 after removal of the PRID in cows. Each value represents the mean ±SEM for five animals. Different letters (a, b) on bars denote significant differences at P<0.05

Kp10 stimulated the release of FSH in 4 of 5 animals; however, there was no significant difference in the mean plasma concentration compared with pre-injection values.

Several studies have reported a stimulatory effect of kisspeptin on the secretion of gonadotropins in cattle. Kisspeptin stimulated the release of gonadotropins in prepubertal female calves (Kadokawa *et al.* 2008; Ezzat *et al.* 2009), male calves (Ezzat *et al.* 2009), and ovariectomized cows (Whitlock *et al.* 2008). However, the gonadotropin-releasing response to kisspeptin in adult cyclic cows remains unclear.

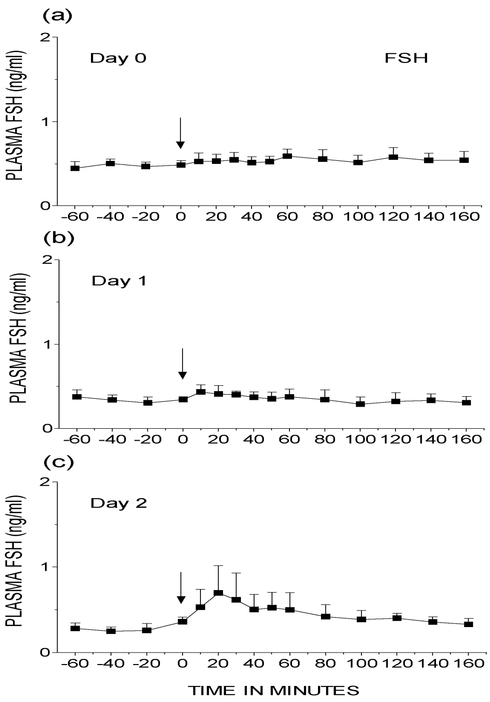


**Figure 4** Plasma concentrations of follicle-stimulating hormone (FSH) in response to the i.v. injection of 5 µg/kg b.w. of Kp10 at day 0 (a), day 1 (b), and day 2 (c) after removal of the PRID in cows. Other explanations are given in Figure 2

The present study is the first to examine this response in postpartum cyclic cows, and compared the characteristics of the LH- and FSH-releasing response to kisspeptin at high and low concentrations of $P_4$ in plasma.

The present study used an injection of GnRH before the insertion of PRID to exclude the effect of $E_2$ from the follicles and maintain the cows in a luteal phase of minimal $E_2$ against maximal $P_4$ concentrations (Ueblinger *et al.* 1995). Moreover, in a recent study (Stevenson, 2008); $PGF_{2\alpha}$ was injected immediately after removal of the PRID to enhance the rapid decline in the $P_4$ concentration. Following these treatments, $P_4$ concentrations remained high up to the day before the PRID's removal, and quickly declined one day after its removal in the present study. Kp10 failed to stimulate the release of LH when plasma $P_4$ concentrations were high, but significantly stimulated it when plasma $P_4$ concentrations decreased to values less than 1 ng/mL.

The present study showed that Kp10 stimulated the release of LH in postpartum cyclic cows. However, the response to Kp10 was less potent than that in our previous study using prepubertal female and male calves (Ezzat *et al.* 2009). The maximum LH concentration after injections of Kp10 in female and male calves was 7.2 and 17.4 ng/mL, respectively, higher than the value on either Day 1 (1.45 ng/mL) or Day 2 (1.54 ng/mL) in the present study. The maximum LH concentration after injections of Kp10 in prepubertal female calves in another study (Kadokawa *et al.* 2008) was also higher (5.0 ng/mL) than that in the present study. The maximum LH concentration in our study was similar to that in ovariectomized parous cows (1-1.4 ng/mL) (Whitlock *et al.* 2008). Therefore, the results in the present study suggest that the effect of kisspeptin on the secretion of LH is lower in adult cows when compared to prepubertal female or male calves.

Higher concentrations of $P_4$ in plasma reduced the response to kisspeptin. The effect of $P_4$ on the secretion of gonadotropins has been investigated both *in vivo* (O'Byrne *et al.* 1991; Skinner *et al.* 1998) and *in vitro* (Sim *et al.* 2001).

A luteal increase in $P_4$ was found to inhibit the secretion of GnRH (Skinner *et al.* 1998; Robertson *et al.* 2009) and subsequently the secretion of both LH and FSH (Robertson *et al.* 2009). $P_4$ receptors were detected on pituitary gonadotropes (Fox *et al.* 1990), and $P_4$ inhibited the release of gonadotropins after 12-h incubation with cultured pituitary cells (Lesoon and Mahesh, 1992). Several studies *in vivo* and *in vitro* have found that the inhibitory effect of $P_4$ on the release of gonadotropins occurs via the hypothalamic GnRH pathway, and that $P_4$ suppresses the secretion of GnRH by binding to its cytoplasmic and nuclear receptors (Calogero *et al.* 1998; Skinner *et al.* 1998; Sleiter *et al.* 2009). However, the precise mechanism through which $P_4$ influences the secretion of gonadotropins is unclear (Sim *et al.* 2001).

Further study will be needed to clarify the mechanism by which $P_4$ influences the kisspeptin-induced release of gonadotropins in cows with regard to the level of gonadotropin-inhibiting factors. Kp10 failed to stimulate significant secretion of FSH regardless of the concentration of $P_4$. This result suggests that less FSH than LH is secreted in response to Kp10 in cows. However, the individual variation of FSH secretion observed among animals may be due to presence of other factors which solely regulate the FSH secretion; i.e. activin and inhibin. The i.v. injection of Kp10 in prepubertal female calves and male calves had a less potent effect on FSH than LH levels (Ezzat *et al.* 2009). The increase in FSH induced by kisspeptin showed a more gradual onset and was less marked than that of LH in pigs (Lents *et al.* 2008). The central administration of kisspeptin-54 caused a slight but not significant increase in FSH levels as opposed to a significant increase in LH levels in mice (Gottsch *et al.* 2004). Our results are, in part, consistent with these reports. However, the secretion of FSH is not fully under the control of GnRH (Kile and Nett, 1994; Phillips, 2005). In conclusion, the present findings show that Kp10 stimulated the release of LH in postpartum cyclic cows, and suggest that high concentrations of $P_4$ in plasma may reduce the effect of kisspeptin on the secretion of gonadotropins.

## ACKNOWLEDGEMENT

The first three authors (A. Ahmed Ezzat, Y. Goto and H. Saito) contributed equally to this work. The authors wish to thank Dr. A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA for providing bFSH (AFP5346D, AFP5318C) and the bFSH antiserum (AFP-C5288113); and Dr. T. Matozaki, Laboratory of Biosignal Science, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan for providing the progesterone antiserum (HAC-AAb3-06RBP841). This research was supported in part by a Grant-in-aid for Scientific Research (No.21380169) provided by the Japan Society for the Promotion of Science (JSPS).

## REFERENCES

Baulieu E.E. (1998). Neurosteroids: a novel function of the brain. *Psychoneuroendocrine*. **23,** 963-987.

Calogero A.E., Palumbo M.A., Bosboom A.M.J., Burrello N., Ferrara E., Palumbo G., Petraglia F. and D'Agata R. (1998). The neuroactive steroid allopregnanolone suppresses hypothalamic gonadotropin releasing hormone release through a mechanism mediated by γ-aminobutyric acid a receptor. *J. Endocrinol*. **158,** 121-125.

Ezzat A.A., Saito H., Sawada T., Yaegashi T., Yamashita T., Hirata T.I., Sawai K. and Hashizume T. (2009). Characteristics of the stimulatory effect of Kisspeptin-10 on the secretion of luteinizing hormone, follicle-stimulating hormone and growth hormone in prepubertal male and female cattle. *J. Reprod. Dev*. **55,** 650-654.

Fox S., Harlan R.E., Shivers B. and Pfaff D.W. (1990). Chemical characterization of neuroendocrine targets for progesterone in the female rat brain and pituitary. *Neuroendocrinology.* **51,** 276-283.

Genazzani A.R., Palumbo M.A., De Micherouz A.A., Artini P.G., Criscuolo M., Ficarra G., Guo A., Benelli A., Bertolini A., Petraglia F. and Purdy R.H. (1995). Evidence for a role for the neurosteroid allopregnanolone in the modulation of reproductive function in female rats. *Eur. J. Endocrinol*. **133,** 375-380.

Gottsch M.L, Clifton D.K. and Steiner R.A. (2009). From KISS1 to kisspeptins: an historical perspective and suggested nomenclature. *Peptides.* **30,** 4-9.

Gottsch M.L., Cunningham M.J., Smith J.T., Popa S.M., Acohido B.V., Crowley W.F., Seminara S., Clifton D.K. and Steiner R.A. (2004). A role of kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology.* **145,** 4073-4077.

Hashizume T., Saito H., Sawada T., Yaegashi T., Ezzat A.A., Sawai K. and Yamashita T. (2010). Characteristics of stimulation of gonadotropin secretion by kisspeptin-10 in female goats. *Anim. Reprod. Sci.* **118,** 37-41.

Hashizume T., Takahashi Y., Numata M., Sasaki K., Ueno K., Ohtsuki K., Kawai M. and Ishi A. (1999). Plasma profiles of growth hormone, prolactin and insulin-like growth factor-I during gestation, lactation and neonatal period in goats. *J. Reprod. Dev*. **45,** 273-281.

Irwig M.S., Fraley G.S., Smith J.T., Acohido B.V., Popa S.M., Cunningham M.J., Gottsch M.L., Clifton D.K. and Steiner R.A. (2004). Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology.* **80,** 264-272.

Kadokawa H., Matsui M., Hayashi K., Matsunaga N., Kawashima C., Shimizu T., Kida K. and Miyamoto A. (2008). Peripheral administration of kisspeptin-10 increases plasma concentrations of GH as well as LH in prepubertal Holstein heifers. *J. Endocrinol*. **196,** 331-334.

Kile J.P. and Nett T.M. (1994). Differential secretion of follicle-stimulating hormone and luteinizing hormone from ovine pituitary cells following activation of protein kinase A, protein kinase C, or increased intracellular calcium. *Biol. Reprod*. **50,** 49-54.

Lents C.A., Heidorn N.I., Barb C.R. and Ford j.j. (2008). Central and peripheral administration of kisspeptin activates gonadotropin but not somatotropin secretion in prepubertal gilts. *Reproduction.* **135,** 879-887.

Lesoon L.A. and Mahesh V.B. (1992). Stimulatory and inhibitory effects of progesterone on FSH secretion by the anterior pituitary. *J. Steroid Biochem. Mol. Biol*. **42,** 479-491.

Mahesh V.B. and Brann D.W. (1998). Regulation of the preovulatory gonadotropin surge by endogenous steroids. *Steroids.* **63,** 616-629.

Mensah Nyagan A.G., Do Rego J.L., Beaujean D., Luu The V., Pelletier G. and Vaudry H. (1999). Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacol. Rev*. **51,** 63-81.

Navarro V.M., Castellano J.M., Fernandez-Fernandez R., Tovar S., Roa J., Mayen A., Barreiro M.L., Casanueva F.F., Aguilar E., Dieguez C., Pinilla L. and Tena-Sempere M. (2005). Effects of kiss-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology.* **146,** 1689-1697.

O'Byrne K.T., Thalabard J.C., Grosser P.M., Wilson R.C., Williams C.L., Chen M.D., Ladendorf D., Hotchkiss J. and Knobil E. (1991). Radiotelemetric monitoring of hypothalamic gonadotropin-releasing hormone pulse generator activity throughout the menstrual cycle of the rhesus monkey. *Endocrinology.* **129,** 1207-1214.

Phillips D.J. (2005). Activins, inhibins, and follistatins in the large domestic species. *Domest. Anim. Endocrinol.* **28,** 1-16.

Prakash B.S., Meyer H.H.D., Schallenberger E. and Van De Wiell D.F.M. (1987). Development of a sensitive enzyme immunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *J. Steroid Biochem*. **28,** 623-627.

Robertson D.M., Hale G.E., Jolley D., Fraser I.S., Hughes C.L. and Burger H.G. (2009). Interrelationships between ovarian and pituitary hormones in ovulatory menstrual cycles across reproductive age. *J. Clin. Endocrinol. Metab.* **94,** 138-144.

Sim J.A., Skynner M.J. and Herbison A.E. (2001). Direct regulation of postnatal GnRH neurons by the progesterone derivative allopregnanolone in the mouse. *Endocrinology.* **142,** 4448-4453.

Skinner D.C., Evans N.P., Delaleu B., Goodman R.L., Bouchard P. and Caraty A. (1998). The negative feedback actions of progesterone on gonadotropin releasing hormone secretion are transduced by the classical progesterone receptor. *Proc. Natl. Acad. Sci. USA.* **95,** 10978-10983.

Sleiter N., Pang Y., Park C., Horton T.H., Dong J., Thomas P. and Levine J.E. (2009). Progesterone receptor A (PRA) and PRB-independent effects of progesterone on gonadotropin-releasing hormone release. *Endocrinology.* **150,** 3833-3844.

Stevenson J.S. (2008). Progesterone, follicular, and estrual responses to progesterone-based estrus and ovulation synchronization protocols at five stages of the estrous cycle. *J. Dairy Sci.* **91,** 4640-4650.

Ueblinger H., Binder H., Hauser B., Rüsch P. and Zerobin K. (1995). Hormonanalytischer vergleich der vaginaleinlagen CIDRTM und PRID bei ovariektomierten kühen. *Schweiz. Arch. Tierheilk.* **137,** 81-86.

Whitlock B.K., Daniel J.A., Wilborn R.R., Rodning S.P., Maxwell H.S., Steele B.P. and Sartin J.L. (2008). Interaction of estrogen and progesterone on kisspeptin-10-stimulated luteinizing hormone and growth hormone in ovariectomized cows. *Neuroendocrinology*. **88,** 212-215.