Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Kiss-1 mRNA/Kisspeptin Distribution in Preoptic and Arcuate Nuclei of Cycling Buffalo (*Bubalus bubalis*) Hypothalamus

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ARTICLE HISTORY (15-060)

Received: February 02, 2015
Revised: October 12, 2015
Accepted: October 25, 2015
Online available: January 13, 2016

Key words:
Buffalo
Hypothalamus
In situ hybridization
Kiss-1
Kisspeptin

ABSTRACT

In ruminants, the preoptic area (POA) and arcuate (ARC) are the main hypothalamic nuclei through which kisspeptin influences gonadotropin releasing hormone (GnRH) neurons for reproductive functions. The relationship between kisspeptin and GnRH releasing in many species has been studied, but not in buffalo. The aims of this study were to detect the localization of *Kiss-1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of cycling buffalo cows. Brains were collected from 6 buffaloes and processed for paraffin blocks. Four-micron paraffin sections of the POA and ARC hypothalamic nuclei were prepared for (1) chromogenic in situ hybridization using a *Kiss-1*cRNA probe designed from the ovine kisspeptin gene sequence (GenBank accession no. DQ059506) and (2) immunohistochemistry using a rabbit anti-mouse kisspeptin-10 antibody. The signals for *Kiss-1* mRNA and the localization of kisspeptin proteins were detected in the cytoplasm of the POA and ARC neuronal soma and some small neuronal cells. Kisspeptin proteins were also found in the cellular process of the POA and ARC neurons. The population of kisspeptin-immunoreactive neurons distributed in the POA (79.8±2.5%) was greater than in the ARC area (62.5±4.5%) ($P \leq 0.01$). This study provides evidence of *Kiss-1* mRNA and kisspeptin protein in the hypothalamus of buffalo, and it will hopefully help lay the groundwork for a further understanding of the role of kisspeptin in buffalo and its relation to reproduction and the hypothalamic- pituitary- gonadal axis.

To Cite This Article: Chaikhun T, Yanprapasiri C, Sotthibandhu P and Suadsong S, 2016. *Kiss-1* mRNA/kisspeptin distribution in preoptic and arcuate nuclei of cycling buffalo (*Bubalus bubalis*) hypothalamus. Pak Vet J, 36(1): 93-97.

INTRODUCTION

Despite the fact that the buffalo (*Bubalus bubalis*) is a common domestic animal used for meat and milk supply, this species has many reproductive limitations. The long intercalving period of buffalo cows is a main problem affecting their reproductive efficiency (Chaikhun *et al*., 2012). This issue might be related to the hypothalamic-pituitary-ovarian (HPO) axis, in terms of the gonadotropin releasing hormone (GnRH) tonic center and surge center functions in the hypothalamus (Maeda *et al*, 2010). Research in sheep has indicated that, in this species, the preoptic area (POA) functions as a GnRH surge center and that the arcuate (ARC) area functions as a GnRH tonic center (Clarke *et al*., 2009).

In situ hybridization (ISH) and immuno-histochemistry (IHC) are the main techniques used in kisspeptin research. ISH is a hybridization technique that utilizes a labeled complementary DNA or RNA strand to identify, in an area or section of tissue (in situ), the location of a particular DNA or RNA sequence (Jin and Lloyd, 1997). IHC is usually used to localize and detect antigens (e.g., proteins) in tissue sections by taking advantage of the fact that specific antibodies bind to specific antigens in biological tissues (Ramos-Vara, 2005). In our study, therefore, ISH was used to detect kisspeptin gene expression and IHC for kisspeptin protein localization, respectively.

Despite some recent studies there are still many questions regarding the role of kisspeptin in the regulation of buffalo reproduction and basic research on their

neuroanatomy remains to be done. In order to further this basic research, the objectives of this study were to detect the localization of *Kiss-1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of cycling buffalo cows.

MATERIALS AND METHODS

The experiment was approved by Chulalongkorn University Animal Care and Use Committee in accordance with the university regulations and policies governing the care and use of laboratory animals (No.13310007).

Sample: The brains were collected from 6 cycling buffaloes (age 4-7 years old and body condition score 3-4 out of 5) from slaughterhouses. The heads were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) within 15 min of the animal's death. The hypothalami were prepared for paraffin blocks.

The histological location of the POA and ARC areas were determined by hematoxylin and eosin staining on the sample slides (Haines, 2012). The random samples of POA and ARC hypothalamic nuclei of each buffalo, used for ISH and IHC, were serial sample slides, each ISH and IHC slide adjacent to the other, taken from the same tissue paraffin block.

In situ hybridization of Kiss1 mRNA: Plasmid DNA, inserted with a section of ovine *Kiss1* gene (GenBank accession no. DQ059506) was generated by GenScript, NJ, USA. The plasmid DNA was digested for preparation of a sense probe (negative result indicator) and an anti-sense probe (positive result indicator) using a DIG-labeling *in vitro* transcription kit (Roche, Mannheim, Germany).

Four-micron paraffin sections were processed according to the optimal standard procedure. Briefly, the samples were autoclaved for 10 min at 121°C in a citrate buffer (pH 6.0). The samples were blocked for endogenous alkaline phosphatase and post-fixed. Sections were washed in PBS between each step. Prehybridization was conducted with a hybridization cocktail. The hybridization was done at 45°C for 20 hr in hybridizer (S2451-30, Dako, Glostrup, Denmark). Then the sections were stringently washed at 45°C. The sections were equilibrated and unspecific bindings were blocked. The mRNA signals were detected using 1x NBT/BCIP (DIG nucleic acid detection kit, Roche, Mannheim, Germany). The results were reported as "positive" if *Kiss-1* mRNA could be detected by a purple stain reaction. As a positive control for tissue and for the specificity of the probe, the POA and ARC hypothalamic nuclei of ewe were used.

Immunohistochemistry of kisspeptin: Four- microns sections were processed using the optimal standard immunohistochemistry procedure. Briefly, antigen retrieval in a citrate buffer (pH 6.0) was done for 10 min at 70°C. Then 10% normal horse serum (Gibco, NY, USA) was applied. The sections were incubated with a rabbit anti-mouse kisspeptin-10 antibody (1:500 dilution, Millipore catalog number AB9754, MA) (Franceschini *et al*., 2006) at 4°C overnight. A biotinylated antibody and streptavidine horseradish peroxidase (LSAB+System-HRP™, catalog number K0679, Dako, Glostrup, Denmark) were incubated. A chromogen 3, 3'-diaminobenzidine (DAB, Dako, Glostrup, Denmark) was added for 5 min. In each step, the sections were washed in PBS. Positive controls for antibody and tissue specificity were prepared using ewe POA and ARC samples. Negative controls for antibody specificity were conducted by omitting the primary antibody. Negative controls for tissue specificity were the white matter area which is an area known to have no kisspeptin expression. Two observers checked and counted the reaction results (counter- stained by hematoxylin) randomly found in the 100 mm^2 area per slide (serial selected slides from the POA and ARC of each buffalo) under a light microscope.

Statistical analysis: The expression of *Kiss-1* mRNA in the POA and ARC hypothalamic nuclei were explained by descriptive statistics.

The kisspeptin-ir cells from each cow were calculated as a percentage and averaged across animals (mean±SE). The average number of kisspeptin-ir cells between the POA and ARC were analyzed by paired t-test ($P<0.05$). The intensity of kisspeptin reactions were graded into 3 levels; 1= weak, 2= moderate, and 3= intense, and analyzed by paired t-test ($P<0.05$).

RESULTS

Anatomical details of hypothalamus in buffalo: The buffalo hypothalamus was identified approximately 15.00 cm down from the skull bregma. In cross section, optic chiasma (OC) and mammary bodies were the main area for identification of POA and ARC, respectively. The POA was microscopically observed above the OC. The median part of the POA was seen close to the 3rd ventricle (3V) and the lateral part is shown in Fig. 1A. The ARC area was identified above the 3V and in front of the mammary bodies (Fig. 1B). In longitudinal section (Fig. 1C), the medial POA was found 2.5 mm above the OC. The supraoptic area (SON) was detected behind the OC. The ARC was found in a caudal part of the SON at 3.9 mm and was seen as a long area through the caudoventral part.

In situ hybridization of Kiss1 mRNA: The expression of *Kiss-1* mRNA using an antisense *Kiss-1*cRNA probe was detected in the cytoplasm of neuronal soma in the majority of neurons in both the buffalo POA (Fig. 2A and 2C) and ARC hypothalamic nuclei (Fig. 3A and 3C) of all samples. *Kiss-1*mRNA was also found in some small neuronal cells (Fig. 2C) which were distinguished from glia cells by their vesicular nuclei. There was no signal of *Kiss-1* mRNA in the buffalo POA and ARC sections in which the sense *Kiss-1*cRNA probe was applied (Fig. 2B, 2D, 3B and 3D) and these were considered as negative control reactions. Positive control reactions were prepared using the ewe POA (Fig. 2E and 2F) and ARC (Fig. 3E and 3F) hypothalamic nuclei paraffin sections.

Immunohistochemistry of kisspeptin: The results showed kisspeptin reactions located in the cytoplasm of the neuronal soma, the cellular process of the neurons and

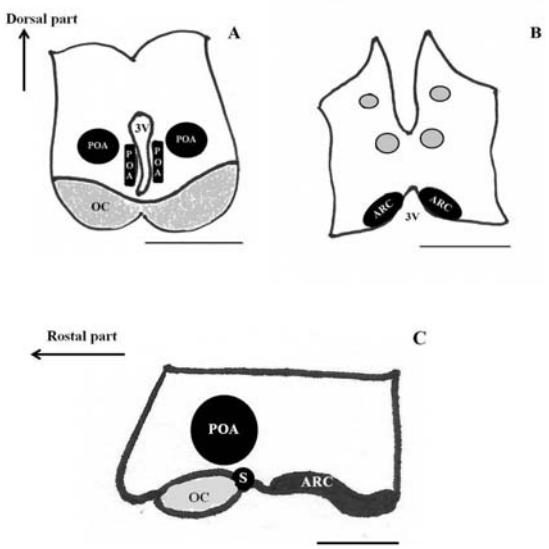

Fig. 1: A drawing of the anatomical and histological location of the POA (Fig. 1A) and ARC (Fig. 1B) hypothalamic nuclei in buffaloes as identified on the sample slides in cross sections. The POA (black areas) is located above the optic chiasma (OC). The black rod shaped areas on either side of the 3rd ventricle (3V) are the median POA and the black oval shaped areas are the lateral POA. The ARC area (black areas) is located above the 3V and in front of the mammary bodies. The longitudinal section of buffalo hypothalamus (Fig.1C) shows the medial POA (black area) located above the OC. The supraoptic area (S) is located next to the OC and has a slightly triangular shape. The ARC (thick curved black line area) is located from S trough the caudal ventral part. The grey areas are nerve tracts. Scale bar is 10 mm in A, B and C.

some small neuronal cells (Fig. 4A and 4B) in both the POA and ARC hypothalamic nuclei. Our study also revealed a larger and denser population of kisspeptin immunoreactive neurons in the POA (79.8±2.5%) than in the ARC (62.5±4.5%) area ($P \leq 0.01$). The distribution pattern of kisspeptin-ir cells in the POA had a more scattered and widespread distribution of these cells throughout this area (Fig. 5A), as opposed to kisspeptin-ir cells in the ARC area which had a clumpy appearance (Fig. 5B). There was no difference in kisspeptin reaction intensity between the POA and ARC hypothalamic nuclei, which were both graded as intense (level 3) ($P>0.5$). The negative control presented no non-specific reactions (Fig. 4C). The positive control for immunohistochemical reactions of kisspeptin proteins in the POA hypothalamic neurons in the ewe are shown in Fig. 4D.

DISCUSSION

The present study's detection and localization of *Kiss-1* mRNA (using in situ hybridization technique with an ovine *Kiss-1*cRNA probe) suggests the ability of buffalo POA and ARC hypothalamic nuclei to synthesize kisspeptin.

In previous studies, *Kiss1* mRNA localization in the POA and ARC was also found in sheep and confirmation of kisspeptin production in the POA and ARC area was later provided by immunohistochemistry tests (Estrada *et al*., 2006). The kisspeptin antibody in this study has been used in mares for kisspeptin-ir cell determination, proving that cross-reactivity can occur in other types of non-ruminants. Interestingly, although there was no reported research on the Kiss-1 sequence in Buffalo at the time of our study, in 2014 three predicted buffalo *Kiss-1* sequences were reported in a gene database (NCBI reference sequences: XM_006062321.1, XM_ 006062322.1 and XM_006062323.1, NCBI GenBank, 2014). In confirmation of our study's assumptions, 94% of the tested sequences showed significant alignments between the predicted buffalo and ovine *Kiss-1* sequences when analyzed by the BLASTN 2.2.30+ program. This may be related to the fact that there is only one difference between the kisspeptin amino acid sequence found in horse and in sheep (Decourt *et al*., 2008). In addition, our study used the POA and ARC of sheep as a positive control for the *Kiss-1* mRNA and kisspeptin-ir cells found in the same areas in the buffalo POA and ARC.

The distribution of *Kiss-1* mRNA in buffalo POA and ARC hypothalamic nuclei is similar, in most regards, to other animals. However, the areas of the hypothalamus which express *Kiss-1* vary among different species of mammals; for example, *Kiss-1* mRNA can be found in the POA and ARC of sheep, as a representative of ruminants (Goodman *et al*., 2007), in the ARC, anterodorsalpreoptic area (ADP) and anteroventral periventricular nucleus (AVPV) of rodents (Gottsch *et al*., 2004; Clarkson *et al*., 2009), in the POA and hypothalamic periventricular nucleus of pigs (Tomikawa *et al*., 2010) and in the POA, ARC and AVPV of primates (Rometo *et al*., 2007). In relation to cattle, the only research that has been done concerns the distribution and localization of *KISS1* and kisspeptin in the hypothalamus. *KISS1* expression was found by qRT-PCR in sulking and weaning Brahman cow hypothalami. The expression within the ventral posterior area (including the ARC) was greater than the expression within the anterior area (including the POA) (Ainu Husna *et al*., 2013). These interspecies variations in *Kiss-1* distribution might be due to factors related to differences between seasonal/ non-seasonal animal species, sex steroid hormone effects, nutritional status, anatomical and physiological variations, and differences in the volume of kisspeptin synthesized from different hypothalamic nuclei (Caraty *et al*, 2007; Colledge, 2008; Overgaard *et al*., 2013; Poling and Kauffman, 2013; Liu *et al*., 2014; Cui *et al*., 2015).

In addition to the finding of *Kiss-1* mRNA in buffalo POA and ARC areas, the results of our study's immunohistochemistry testing found evidence of kisspeptin synthesized from *Kiss-1* mRNA. Kisspeptin neurons in buffalo can also be detected in both the POA and ARC and are similar in their distribution to that of *Kiss-1* mRNA in these areas (the distribution of both in the POA had a more scattered and widespread pattern, as opposed to their distribution in the ARC, which had a clumpy appearance). The variations in kisspeptin neuron distribution in the hypothalamic nuclei have been described in previous studies. The kisspeptin neurons reside in rodent AVPV and ARC (Smith *et al*., 2006; Clarkson *et al*., 2009), in sheep ARC, POA and dorsomedial nucleus (DMN) (Estrada *et al*., 2006; Franceschini *et al*., 2006), in horses ARC and DMN (Decourt *et al*., 2008) and in primates POA and ARC (Rometo *et al*., 2007). This variation in kisspeptin protein distribution might not be only dependant on *Kiss-1* mRNA distribution but also on a possible difference in the role of kisspeptin and its active mode in each species.

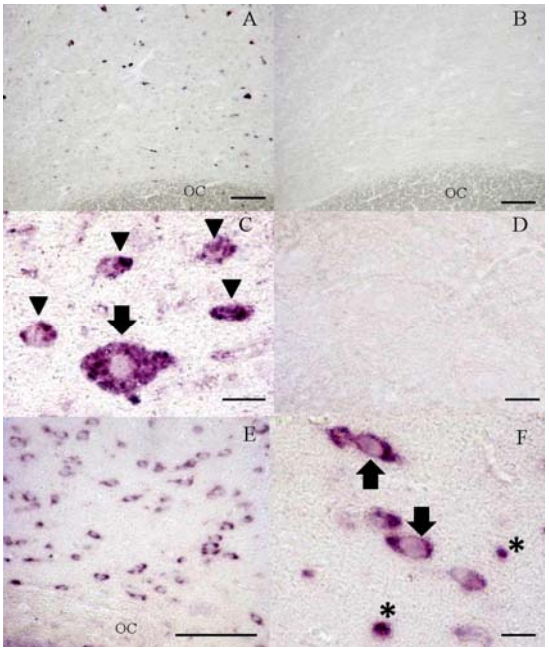

Fig. 2: *Kiss-1* mRNA in the POA, which were taken from the area beside the optic chiasma (OC), is visible in the anti-sense (positive result) of *Kiss-1* mRNA samples (A and C) and is not expressed in the sense (negative result) of *Kiss-1* mRNA samples (B, D). *Kiss-1* mRNA expressions are localized in the cytoplasm of a neuron (full arrow) and some small neuronal cells (arrow heads) in C. In ewe (the positive control), *Kiss-1* mRNA expressions are visible (E and F) in the cytoplasm of neurons (full arrows) and some glias which present dense nuclei (asterisks). Scale bar is 100 µm in A, B and E but it is 10 µm in C, D and F.

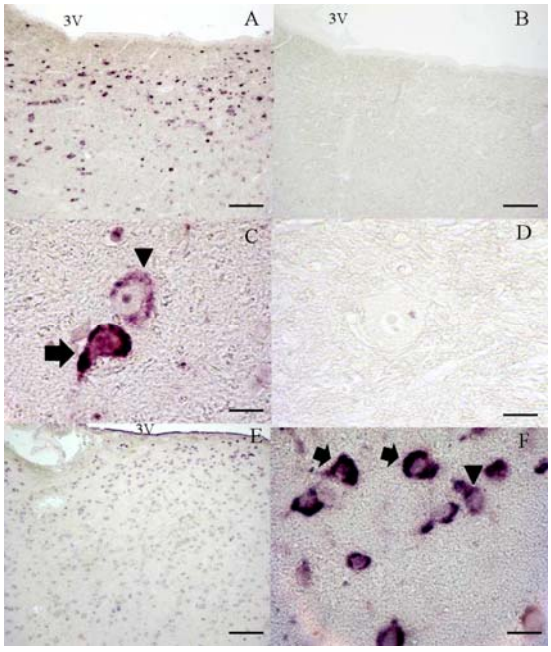

Fig. 3: In the ARC samples, *Kiss-1* mRNA is visible in the anti-sense (positive result) of *Kiss-1* mRNA sample (A, C) but not expressed in the sense (negative result) of *Kiss-1* mRNA sample (B, D). The *Kiss-1* mRNA is localized in the cytoplasm of a neuron with a strong signal (full arrow) but a weak signal in another neuron (arrow head) in C. In ewe as the positive control sample, *Kiss-1* mRNA shows a strongly visible signal in the cytoplasm of neurons (full arrows) and a weak signal in another neuron (arrow head) in E and F. Scale bar is 100 µm in A, B and E but it is 10 µm in C, D and F.

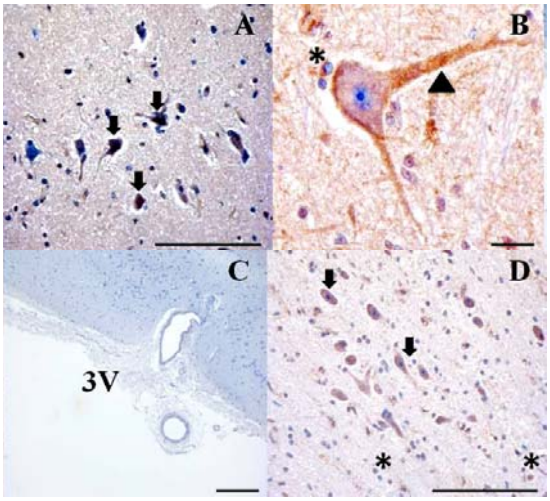

Fig. 4: In counter- stained sections, the kisspeptin immunoreactions (full arrows, A) are located in the neuron cell bodies (in both the cytoplasm and axon) with a dendritic tree when visualized (arrow head, B). Some small neuronal cells present kisspeptin immunoreactions (asterisk, B). There is no reaction in the buffalo POA negative control slide (without primary antibody application, C). In the ewe POA sample (D) the kisspeptin immunoreactions in the neuron cell bodies (full arrows) and some small neuronal cells (asterisks) appear similar to those in the buffalo sample. Scale bar is 100 µm in A, C and D but it is 10 µm in B.

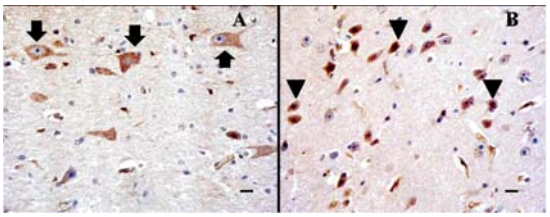

Fig. 5: In the POA (A), the kisspeptin-ir neurons are larger (full arrows) and their distribution is more diffuse than in the ARC (B) hypothalamic nucleus neurons (arrow heads) in our studies' buffalo samples. Scale bar is 10 µm in both A and B.

Interestingly, in contrast to other mammals, the population of kisspeptin-ir cells in buffalo POA is higher in concentration than in the ARC area. In mice (Clarkson *et al*., 2009), rats (Smith *et al*., 2006), pigs (Tomikawa *et al*., 2010), mares (Decourt *et al*., 2008), sheep (Franceschini *et al*., 2006), primates and humans (Rometo *et al*., 2007; George *et al*., 2012), the number of kisspeptin neurons in the ARC is greater than in the POA. It is possible that kisspeptin in the ARC of buffalo may have a different role in its active mode, which may account for this difference in kisspeptin neuron distribution.

Conclusions: In summary, the detection of *Kiss-1* mRNA and kisspeptin protein in the hypothalamus of buffalo in this study provides fundamental data on kisspeptin and its relation to buffalo POA and ARC hypothalamic nuclei which, assuming buffalo is similar to other ruminants such as sheep, might be involved in HPO axis related reproductive functions. *Kiss-1* mRNA was expressed in some neurons of both the POA and the ARC hypothalamic nuclei. Kisspeptin proteins were localized in subpopulations of neurons (in both neuronal soma and cellular processes) and some small neuronal cells. The

kisspeptin-ir cell population in the POA was higher than in the ARC. However, the role of kisspeptin in the HPO axis in buffalo should be further explored.

Acknowledgements: This research was sponsored by Thailand Research Fund (Research and Researchers for Industries), Murrah Dairy Ltd. Company, Faculty of Veterinary Science, Chulalongkorn University Graduate Thesis Grant and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

Author's contributions: Authors declare the contribution that all authors prepared the document for the study funding and designed the experiment. TC and PS performed the experiment and analyzed the results. TC prepared the manuscript. All authors reviewed, revised the manuscript for intellectual contents and approved the final version critically.

REFERENCES

Ainu Husna MSS, Flatscher-Bader T, Lehnert SA, Reverter A, Chan EK *et al*., 2013. Gene expression of GnRH, kisspeptin, neuropeptide Y and receptors for estrogen and leptin in the hypothalamus of suckled and weaned beef cows. J Trop Agric and Fd Sc, 40: 245-255.

Caraty A, Smith JT, Lomet D, SaÏd SB, Morrissey A *et al*., 2007. Kisspeptin synchronizes preovulatiory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. Endocrinology, 148: 5258- 5267.

Chaikhun T, Hengtrakunsin R, De Rensis F, Techakumphu M and Suadsong S, 2012. Reproductive and dairy performances of Thai swamp buffaloes under intensive farm management. Thai J Vet Med, 42: 81-85.

Clarke IJ, Smith JT, Caraty A, Goodman RL and Lehman MN, 2009. Kisspeptin and seasonality in sheep. Peptide, 30: 154-163.

Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A and Herbison AE, 2009. Distribution of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol, 21: 673-682.

Colledge WH, 2008. Kisspeptins and GnRH neuronal signaling. Trends Endocrinol Metab, 20: 115-121.

Cui P, Yang C, Zhang K, Gao X, Luo L *et al*., 2015. Effect of estrogen on the expression of GnRH and kisspeptin in the hypothalamus of rats during puberty. Theriogenology, article in press: http://dx.doi.org/10.1016/j.theriogenology.2015.08.004.

Decourt C, Tillet Y, Caraty A, Franceschini I and Briant C, 2008. Kisspeptin immunoreactive neurons in the equine hypothalamus interactions with GnRH neuronal system. J Chem Neuroanat, 36: 131-137.

Estrada KM, Clay CM, Pompolo S, Smith JT and Clake IJ, 2006. Elevated KiSS-1 expression in arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/luteinizing hormone surge in the ewe suggests a stimulatory role for kisspeptin in estrogen-positive feedback. J Neuroendocrinol, 18: 806-809.

Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y *et al*., 2006. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. Neurosci Lett, 401: 225-230.

George JT, Anderson RA and Millar RP, 2012. Kisspeptin-10 stimulation of gonadotrophin secretion in women is modulated by sex steroid feedback. Hum Reprod, 27: 3552-3559.

Goodman RL, Lehman MN, Smith JT, Coolen LM, De Oliveira CV *et al*., 2007. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology, 148: 5752–5760.

Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV *et al*., 2004. A role for kisspeptin in the regulation of gonadotropin secretion in the mouse. Endocrinology, 145: 4073-4077.

Haines DE, 2012. Neuroanatomy: An atlas of structure, sections and systems, 8th Ed. Wolters Kluwer, China.

Jin L and Lloyd RV, 1997. In situ hybridization: methods and applications. J Clin Lab Anal, 11: 2-9.

Liu R, Li H, Zhang D, Lv Y, Lin X *et al*., 2014. Expression of Kisspeptin/kiss1r System is Down-regulated in the Hypothalamic Arcuate Nucleus of Pubertal Male Rats with High-fat-diet. Reprod Contracept, 25: 1-11.

Maeda K, Ohkura S, Uenoyama Y, Wakabayashi Y, Oka Y *et al*., 2010. Neurobiological mechanisms underlying GnRH pulse generation by the hypothalamus. Brain Res, 1364: 103-115.

NCBI GenBank. NCBI reference sequences: XM_006062321.1, XM_006062322.1 and XM_006062323.1. Available online: www.ncbi.nlm.nih.gov, Retrieval date: December 15, 2014.

Overgaard A, Tena-Sempere M, Franceschini I, Desroziers E, Simonneaux V *et al*., 2013. Comparative analysis of kisspeptin-immunoreacetivity reveals genuine differences in the hypothalamic Kiss1 systems between rats and mice. Peptides, 45: 85-90.

Poling MC and Kauffman AS, 2013. Organizational and activational effects of sex steroids on kisspeptin neuron development. Frontiers Neuroendocrinol, 34: 3-17.

Ramos-Vara JA, 2005. Technical aspects of immunohistochemistry. Vet Pathol, 42: 405-426.

Rometo AM, Krajewski SJ, Voytko ML and Rance NE, 2007. Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab, 92: 2744-2750.

Smith JT, Popa SM, Clifton DK, Hoffman GE and Steiner RA, 2006. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci, 26: 6687-6694.

Tomikawa J, Homma T, Tajima S, Shibata T, Inamoto Y *et al*., 2010. Molecular characterization and estrogen regulation of hypothalamic KISS gene in the pig. Biol Reprod, 82: 313-319.