

# Evidence of kisspeptin receptor expression in GnRH neurons in the preoptic area and arcuate hypothalamic nuclei in cycling buffaloes

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## Abstract

Our previous study reported the expression of kisspeptin in the preoptic area (POA) and arcuate nucleus (ARC) of the hypothalamus of cycling buffalo cows. In ruminants, POA and ARC are the main hypothalamic nuclei through which kisspeptin acts on the hypothalamic-pituitary-gonadal axis, due to their stimulation of gonadotropin releasing hormone (GnRH) secretion. In many species, kisspeptin activates GnRH neurons via its receptors (KISS1R or GPR54). The present study was designed to explore the expression of KISS1R by GnRH neurons in the POA and ARC hypothalamic nuclei in buffalo cow brains by double-labeling immunohistochemistry. In both the POA and ARC areas, KISS1R immunoreactivity (ir) was detected in the nucleus and cytoplasm of neuronal soma and some glia. GnRH-ir was found as granular formations in the cytoplasm of neuronal soma. The KISS1R-ir neuron population in the POA was the same as in the ARC (93%), however, the GnRH-ir neuron population in the POA (64%) trended lower than in the ARC (73%) ( $P>0.05$ ). The double-labeling immunohistochemistry showed that all observed GnRH-ir neurons were co-localized with KISS1R-ir. These findings present evidence of kisspeptin receptors in the GnRH neurons in buffalo POA and ARC areas and suggest that kisspeptin has a functional role in GnRH release in buffalo.

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**Keywords:** buffalo, gonadotropin releasing hormone, hypothalamus, kisspeptin receptor, immunohistochemistry

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## Introduction

In the past decade, studies of the function of kisspeptin in animal and human reproduction have shown that it is a potent neuropeptide capable of stimulating GnRH release from the hypothalamus of many mammalian species (Irwig et al., 2004; Han et al., 2005; Messenger et al., 2005; Colledge, 2008; De Tassigny et al., 2008). In female ruminants, the preoptic area (POA) and the arcuate (ARC) hypothalamic nuclei are known as the main regulators in the hypothalamic pituitary ovarian (HPO) axis, due to their involvement in GnRH release (Estrada et al., 2006; Franceschini et al., 2006).

The reproductive performance of ruminant species varies widely. Buffaloes (*Bubalus bubalis*) are common domestic animals used for meat and milk supply. Nevertheless, this species has many unique reproductive limitations. Delays in puberty (and subsequent delay in the age of first conception) as well as a long inter-calving period can cause infertility and represent a major source of economic loss in buffalo, leading to low reproductive performance and lengthening of their non-productive life (Chaikhun et al., 2012). Kisspeptin was shown to stimulate LH release in cattle (Kadokawa et al., 2008; Whitlock et al., 2008) but its role in buffaloes has not been well characterized. A previous study of buffalo reproduction reported on GnRH/gonadotropin releasing levels (Aboul-Ela et al., 1983) but kisspeptin's role in buffalo reproduction, and the mechanism behind this, have not been completely understood.

Interestingly, our previous study found evidence of kisspeptin in the POA and ARC hypothalamic nuclei of cycling buffalo cows (Chaikhun-Marcou et al., 2016). This finding indicates that kisspeptin might be related to the buffalo HPO axis. Kisspeptin activates GnRH neurons mainly via its receptor (KISS1R, GPR54) (Colledge, 2008; Herbison et al., 2010). The present study was designed to explore this possible role of kisspeptin signaling in buffalo cows by studying the expression of KISS1R in GnRH neurons in the POA and ARC hypothalamic nuclei.

## Materials and Methods

The experimental procedures involving animals were 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).

**Animals:** Brains were collected from 6 cycling buffaloes (age between 4-7 years old) and an ewe (5 years old, mixed breed, luteal phase of estrous cycle) from slaughterhouses after perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) into a common carotid artery within 15 min of the animal's death. Three buffaloes were follicular phase cows and the other three were luteal phase cows. Estrous cycle identification of the animals was determined based on observation of the buffalo's estrous signs and rectal palpation before slaughter. Phase confirmation was done by means of postmortem ovarian morphology (Ali et al., 2003).

After removing the brains, the hypothalami were collected and fixed in 4% paraformaldehyde for 24 hr. The samples were embedded in paraffin blocks and stored at room temperature (RT). Since no references to the anatomical structure of the POA and ARC hypothalamic nuclei of buffaloes were available, it was determined in our study by standard cresyl violet staining of adjacent hypothalamic sections, a technique previously used in sheep (anatomical sources: Haines, 2012). Histological location of the POA and ARC areas were determined by hematoxylin and eosin (H&E) staining on the sample slides. The location of the buffalo POA hypothalamic nucleus was identified using the superior optic chiasma and the third (3<sup>rd</sup>) ventricle as landmarks in the anteriodorsal part of the hypothalamus. The ARC hypothalamic nucleus was found in the medioventral part of the hypothalamus using the mammary bodies and the 3<sup>rd</sup> ventricle as guides.

**Double-labeling immunohistochemistry for KISS1R and GnRH:** Paraffin sections of the POA and ARC hypothalamic nuclei were prepared at 4 microns on superfrost-slides (Fisher Brand, Thermo Fisher Scientific, MA, USA) and were processed by the standard double-labeling immunohistochemical method. The paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethyl alcohol. Antigen retrieval in a citrate buffer (pH 6.0) was done for 10 min at 121°C. Non-specific binding was blocked using 1% normal goat serum (Gibco, NY, USA) for 20 min at RT. The sections were incubated with a primary rabbit anti-human KISS1R/GPR54 polyclonal antibody (1:100 dilution) (Bioss, catalog number bs-2501R, MA, USA) overnight (16 hr) at 4°C. A secondary goat anti-rabbit IgG (H+L) antibody (1:500 dilution) (Alexa fluor 488, catalog number A-11008, Life Technologies, CA, USA) was applied to the sections at RT for 2 hr. A mouse anti-mammalian GnRH monoclonal antibody (1:100 dilution) (Chemicon, catalog number MAB5456, CA, USA) was applied to the sections overnight (16 hr) at 4°C. A 1:100 dilution of secondary goat anti-mouse IgG (H+L) (Alexa fluor 568, catalog number A-11004, Life Technologies, CA, USA) was applied to the slides and kept at RT for 2 hr, then washed with PBS. All antibodies were diluted with 10 mM phosphate buffer saline (PBS; Bio-Optica, Milano, Italy) (pH 7.2). In each of the aforementioned washing steps, the sections were washed in 10 mM PBS 3 times for 5 min each. The washing step following the 1<sup>st</sup> secondary antibody application consisted of the sample slides being washed in 10 mM PBS 3 times for 10 min each, by slow shaking in aluminum foil covered Coplin jars. The sections were then mounted and covered by an anti-fade reagent (Molecular probes, catalog number P36930, CA, USA). Double-labeled immunoreactivity was observed under a fluorescent microscope (Axiolab, Zeiss, Oberkochen, Germany). A single observer counted the number of KISS1R-ir cells, GnRH-ir cells and co-localized-ir cells found in 100 mm<sup>2</sup> area slides from each of the buffalo cow's POA and ARC hypothalamic nuclei. Images were captured by Axivision software (Axiolab, Zeiss, Oberkochen, Germany). Layers of the images were combined in Adobe Photoshop.

**Controls and specificity:** Antibody validation and cross-reactivity studies of the rabbit anti-human KISS1R/GPR54 polyclonal antibody used in this study (Bioss, catalog number bs-2501R, MA, USA) have been carried out in humans, rats and mice by the source company (Bioss, MA, USA). Positive controls for antibody and tissue specificity were prepared using brain sections from wild-type mice. Negative controls for antibody specificity were performed using brain sections from GPR54 gene knockout (KO/ GPR54) mice and also by omitting the primary antibody. Negative controls for tissue specificity were performed using the white matter area of the central nervous system of the buffalo and mice, an area known to have no KISS1R expression.

The mouse anti-mammalian GnRH monoclonal antibody (Chemicon, catalog number MAB5456, CA, USA) has been used in buffaloes previously (Zerani et al., 2012) and it has been shown to have cross reactivity in rats, mice, hamsters, sheep and monkeys (El-Majdoubi and Weiner, 2002; Pompolo et al., 2003). Positive controls for antibody and tissue specificity were prepared using the ewe POA and ARC hypothalamic nuclei paraffin sections. Negative controls for antibody specificity were conducted using PBS (instead of a primary antibody application) in combination with 1% normal goat serum, which was applied to reduce the non-specific binding of the primary antibody. Negative controls for tissue specificity were the white matter area of the central nervous system of the ewe and buffalo, which is known to have no GnRH expression.

**Statistical analysis:** The number of each type of immunoreactive cells with co-localization or non-co-localization was calculated as a percentage of the total number and was then averaged across animals to calculate a mean ( $\pm$  SE). Comparison of the average number of each type of immunoreactive cells (with co-localization or non-co-localization) between the POA and ARC was analyzed by a t-test ( $P < 0.05$ ). Characterizations of co-expression and non-co-expression were described.

## Results

The KISS1R antibody in this study was validated by the detection of immunoreactivity in the cytoplasm of some neurons in the POA hypothalamic nucleus (positive control) of the wild-type mice (Fig 1e, 1f) but not in the sections from KISS1R KO mice (Fig 1c, 1d). The positive controls for antibody and tissue specificity in the POA hypothalamic nucleus of the ewe are shown in Fig 2 (b1-b4). The pattern of immunoreactivity for KISS1R and GnRH in the ewe and buffalo was found to be similar (Fig 2, a1-a4, b1-b4). In both the buffalo POA and ARC areas, KISS1R-ir was detected in neuronal soma and some glia (Fig 1a, 1b, 3a, 4a). GnRH-ir appeared to be granular in the cytoplasm of neuronal soma (Fig 3b, 4b). The KISS1R-ir neuron population in the POA was the same as in the ARC ( $93 \pm 2$  vs  $93.2 \pm 2.4\%$ ). Although the GnRH-ir neuron population in the POA ( $64.5 \pm 4.3\%$ ) trended lower than in the ARC ( $72.8 \pm 6.2\%$ ), the difference was

not statistically significant. Double-label results showed that all observed GnRH-ir neurons were co-localized with KISS1R-ir with no difference in population between the follicular phase and the luteal phase cows in both hypothalamic nuclei (Fig 5).

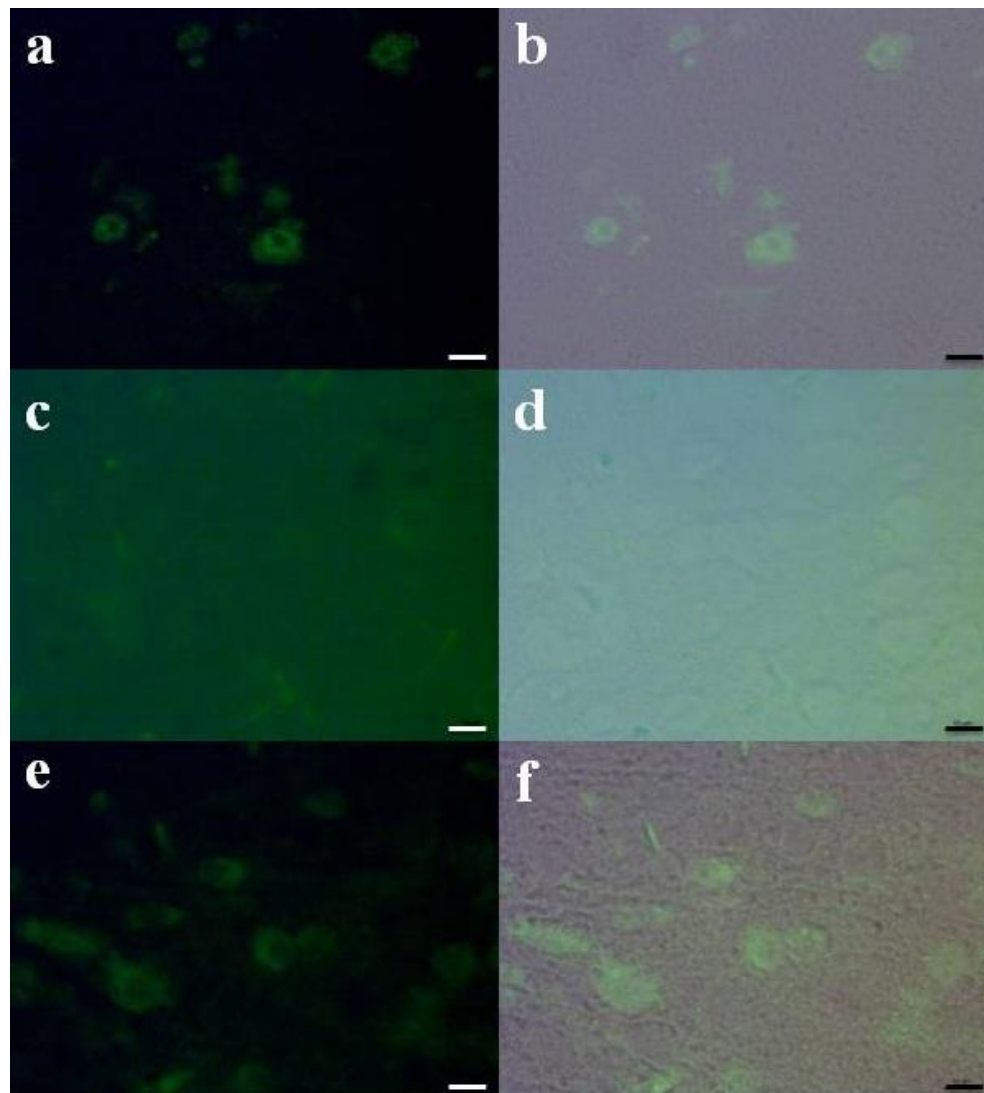
## Discussion

This study provides information about the buffalo HPO axis; as well as the distribution, localization, co-localization, and mode of action of kisspeptin in controlling GnRH release. The co-localization of buffalo kisspeptin receptors (KISS1R) and GnRH neurons in the POA and ARC found in this study is similar to previous findings in other mammals; many hypothalamic GnRH neurons co-express KISS1R mRNA (77% in rat and 55-90% in mouse) (Irwig et al., 2004; Han et al., 2005). In this study, 93% of the GnRH-ir neurons in the buffalo POA and ARC hypothalamic nuclei contained KISS1R in their cells. This finding suggests a pathway of kisspeptin signaling in the buffalo, downstream of kisspeptin-expressing neurons to GnRH neurons as the target cells. Moreover, it suggests that GnRH neurons are influenced by kisspeptin directly, both in terms of controlling prolonged depolarization as well as stimulating their potential firing rate (Han et al., 2005; Quaynor et al., 2007). These effects have also been reported in ruminants such as sheep (Messenger et al., 2005), cattle (Whitlock et al., 2008) and goats (Hashizume et al., 2010).

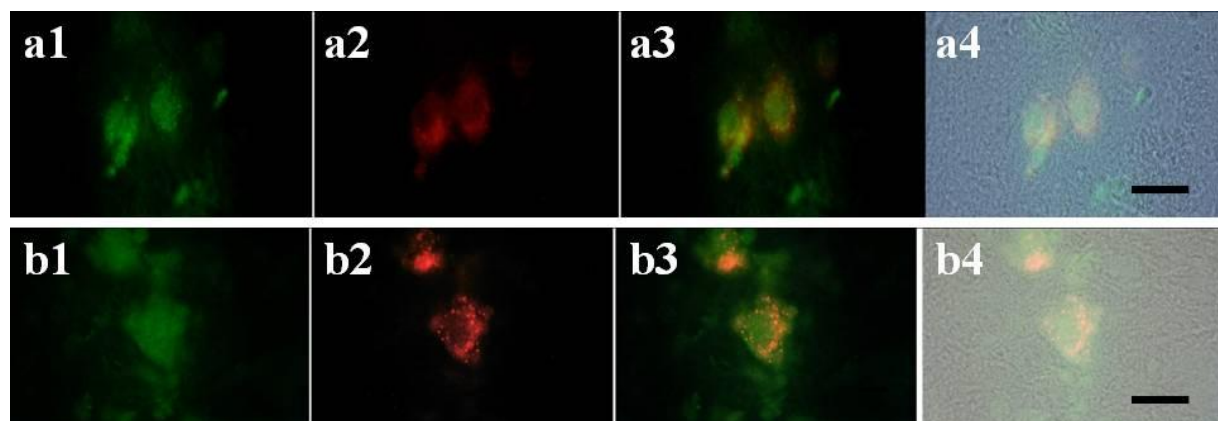
Interestingly, this study found that the GnRH neuron population in the ARC of buffaloes trended greater than in the POA. Similarly, in sheep, GnRH cell bodies are presented both in the POA and ARC. Most GnRH cell bodies in primates are identified lateral to the ARC in the ventral hypothalamic tract of the mediobasal hypothalamus (MBH), but they are rarely found in the POA. In rodents, conversely, GnRH cell bodies are scattered as a continuum from the medial septum and diagonal band of Broca to the medial POA, but they are rarely found in the ARC area (Colledge, 2008). This information suggests the possibility that there are anatomical and functional correlations that depend on the distribution of GnRH-ir neurons and KISS1R-ir cells. A part of ARC called the median eminence (ME) is closely connected to the hypophysis. Our findings could be related to ME and may suggest that this area presents a high sensitivity to GnRH release. This may explain the large amount of co-localized GnRH neurons and KISS1R-ir found in our studies of the ARC area. Previous studies found GnRH cell bodies and kisspeptin-ir cell bodies in close proximity (Clarkson et al., 2006; Decourt et al., 2008). Additionally, kisspeptin-ir fibres were found to extend from the ARC into the external neurosecretory zone of the ME (Franceschini et al., 2006; Pompolo et al., 2006) and it is thought that these terminals might be causative for the kisspeptin identified in ewe hypophyseal portal blood (Smith et al., 2008). This supports the concept that GnRH neurons are controlled directly by kisspeptin at the GnRH nerve terminals through axo-axonal non-synaptic interactions (Decourt et al., 2008; Uenoyama et al., 2011) in this area. A study by Backholer et al. (2010)

supports our current findings. In this study kisspeptin cells in the POA were found to directly stimulate GnRH neurons, but evidence suggested that GnRH

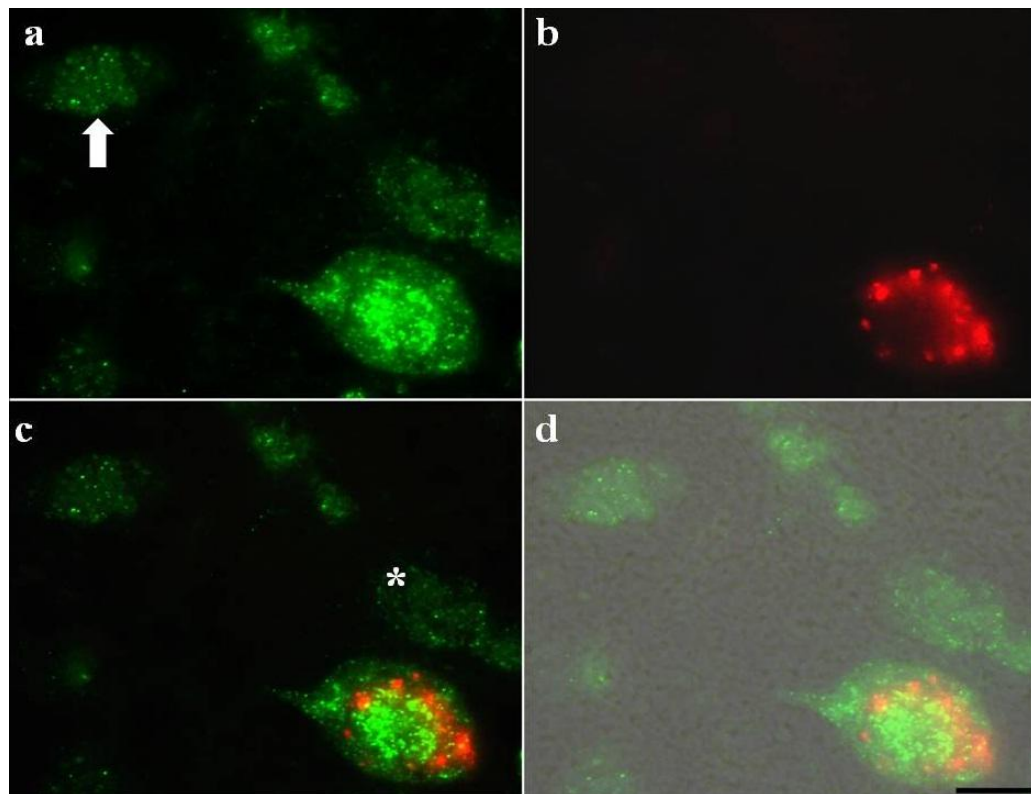
neurons in the ARC might be activated by other cells via poly-transsynaptic regulatory mechanisms (Backholer et al., 2010).



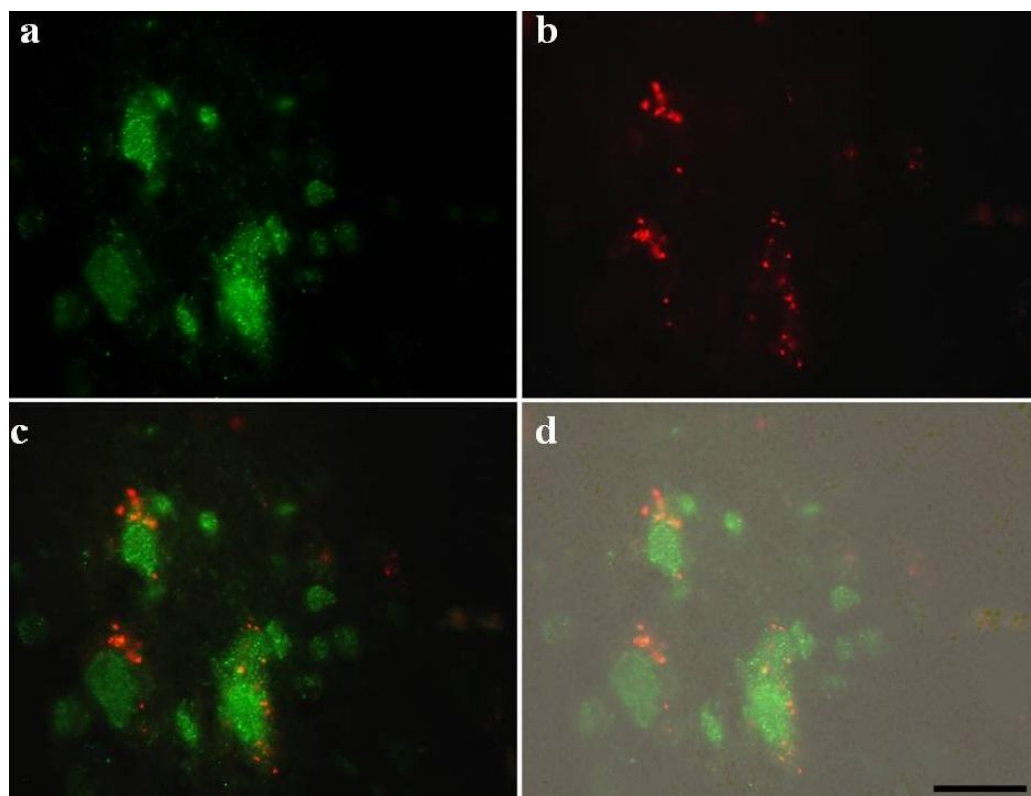
**Figure 1** KISS1R antibody validation in positive and negative control samples: buffalo, wild type and KISS1R KO mice POA hypothalamic nuclei  
KISS1R immunoreactivity was found in the POA of buffalo (a, b), wild type mice (e, f) but not in KISS1R KO mice (c, d). Scale bar = 10  $\mu$ m.



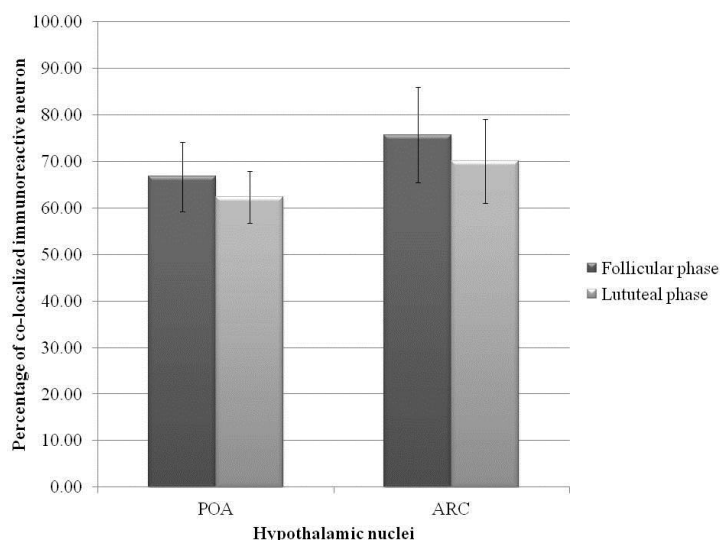
**Figure 2** Immunoreactivity pattern for KISS1R and GnRH in ewe and buffalo  
Both ewe (b1-b4) and buffalo (a1-a4) POA immunoreactivity for KISS1R (green, a1, b1) and GnRH (red, a2, b2) was similar. KISS1R-ir was detected in the neuronal soma and some glia (a1, b1). GnRH-ir appeared to be granular in the cytoplasm of neuronal soma (a2, b2). Co-localizations of KISS1R and GnRH-ir were shown (a3, b3). Combination of the double-label images and differential interference contrast (DIC) images are shown (a4, b4). Scale bar = 10  $\mu$ m.



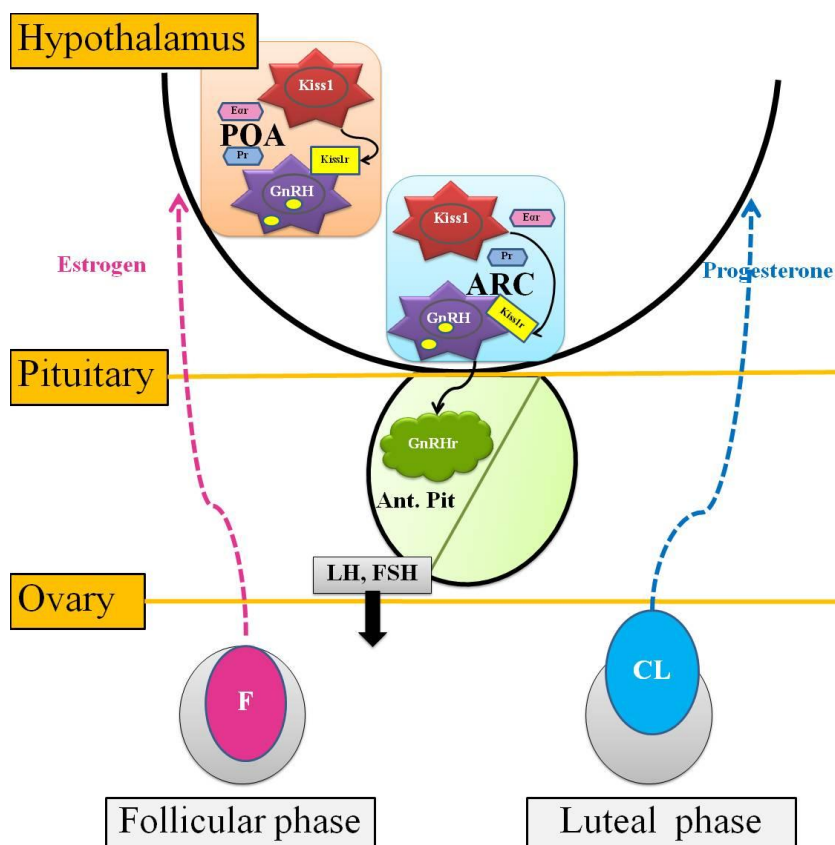
**Figure 3** Double-labeling immunohistochemistry for KISS1R and GnRH in buffalo POA hypothalamic nucleus. KISS1R-ir was detected not only in the neuronal soma but also in glia cells (arrow in a). GnRH-ir appeared as red granular formations in a neuron (b). Combination of the double-label images and differential interference contrast (DIC) images are shown (c, d). Co-localized neurons were found in GnRH-ir neurons but some neurons showed KISS1R-ir without GnRH immunoreaction (asterisk in c). Scale bar = 10  $\mu$ m.



**Figure 4** Double-labeling immunohistochemistry for KISS1R and GnRH in buffalo ARC hypothalamic nucleus. KISS1R-ir was detected in the neuronal soma in green color (a). GnRH-ir appeared as red granular formations in the cytoplasm of neuronal soma (b). Combination of the double-label images and DIC images are shown (c, d). Co-expressed neurons were presented in all GnRH immunoreactive neurons (c, d). Scale bar = 10  $\mu$ m.



**Figure 5** Percentage of double-label KISS1R and GnRH immunoreactive neurons in estrous cycle cows. There was no difference in the co-localized KISS1R and GnRH immunoreactive neuron populations (mean  $\pm$  SE) in both POA and ARC hypothalamic nuclei between buffalo cows in the follicular phase and luteal phase ( $P > 0.05$ ).



**Figure 6** Schematic diagram representing possible role of kisspeptin control in HPO axis in cycling buffaloes. Our previous study of both follicular and luteal phase buffalo cows showed that KISS1 mRNA was detected in the POA and ARC hypothalamic nuclei by *in situ* hybridization. Moreover, kisspeptin-ir cells as a protein was found in the neuron cell bodies (in both the cytoplasm and axon) using the IHC technique. KISS1R-ir was detected in the neuronal soma and some glia in both buffalo POA and ARC areas. GnRH-ir, when present, appeared to be granular in the cytoplasm of neuronal soma but not all neurons presented KISS1R-ir and GnRH-ir together. In the anterior pituitary, distributions of GnRHr were found located in the cytoplasm of subpopulations of cells in the pars distalis and pars intermedia, which may suggest that these cells are gonadotrophs. In both the follicular and luteal phases, the GnRHr positive cells were found intensely accumulated in the proximal part of the pars distalis and pars intermedia. In order to explore the relationship between sex steroid hormones and kisspeptin, estrogen receptors alpha and progesterone receptors were expressed in the nucleus of some neurons in the POA and ARC hypothalamic nuclei (Chaikhun-Marcou et al., 2014a). However, the mechanism by which sex steroid hormones regulate kisspeptin's functions in buffalo reproduction is not yet understood. Our findings highlight some aspects of the role of kisspeptin in controlling GnRH release in the HPO axis, of which the understanding could be important to the enhancement of buffalo reproductive performance.



As for the POA and ARC neurons which presented KISS1R-ir, but no trace of GnRH, this could be explained by a possible un-related GnRH releasing function of kisspeptin. Kisspeptin might have an influence not only on the reproductive system through GnRH neurons but also on other protein-releasing neurons. One of the proteins involved in the metabolic system is the growth hormone (GH), which may be stimulated by kisspeptin in cattle (Whitlock et al., 2008). In terms of reproduction, GnRH neurons may also be stimulated by kisspeptin indirectly. Kisspeptin was found to act on GnRH neurons by way of synaptic interaction from other KISS1R expressed neurons in the hypothalamus (Herbison et al., 2010).

The unexpected pattern of KISS1R localization in the buffalo POA and ARC areas and the presence of KISS1R-ir on some glia cells might suggest a kisspeptin-glia-hypothalamic vascular relationship. It is possible that glia cells might be non-neuronal cells which are related to GnRH release, especially the sub-type of glia cells called astrocytes. The concept of a "Neuro-glia-vascular" relationship (Haydon et al., 2006) might be the key to understanding this possible indirect stimulation of GnRH by kisspeptin through trans-synaptic regulatory mechanisms (Garcia-Segura et al., 2008). Our study found no relationship between the different stages of the estrous cycle and the number of KISS1R-ir in GnRH neurons in the buffalo cows. Research on co-expression, as well as the mechanisms involved in the relationship between sex steroid hormones (estrogen and progesterone) and GnRH and kisspeptin neurons in the POA and ARC areas was done in other species (Gottsch et al., 2004, Franceschini et al., 2006). Similar studies of kisspeptin's role in GnRH release in the follicular and luteal phases in buffalo cows should be conducted in the future. Moreover, although this study used the immunohistochemistry technique for the qualification of localization and distribution of KISS1R-ir in GnRH neurons, confirmation of the KISS1R expression with other molecular techniques such as real-time quantitative polymerase chain reaction (qRT-PCR) and western blot analysis (which have been used in human granulosa cell research (Garcia-Ortega et al., 2014)) should be considered in future studies.

In our current study, along with our previous investigations, kisspeptin's pathway in the buffalo HPO axis was explored. Evidence of mRNA signaling for KISS1 and its protein on kisspeptin-expressing neurons in the POA and ARC of buffalo has been reported recently (Chaikhun-Marcou et al., 2016). In addition, a report on the presence of GnRH receptors (GnRHr) on the par distalis and pars intermediate of buffalo pituitary glands was previously published (Chaikhun et al., 2013b). A diagram, inspired by our research results, of a proposed buffalo kisspeptin pathway in the POA and ARC hypothalamic nuclei via kisspeptin neurons, GnRH neurons, the pituitary, the ovaries a "buffalo kisspeptin HPO axis" is presented in Fig. 6. Our findings indicate that further research into the clinical application of kisspeptin administration in buffalo may offer an alternative way to improve buffalo reproductive performance. Recently, *in vivo* studies have been done on kisspeptin's effect on luteinizing hormone (LH) response in luteal phase

cows (Chaikhun-Marcou et al., 2014b) and ovariectomized cows in both the non-breeding and breeding seasons (Masedo et al., 2014). These studies present preliminary information that kisspeptin might be useful in farm breeding management and infertility treatments due to its ability to influence LH release (Chaikhun et al., 2013a).

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## บทคัดย่อ

### หลักฐานการแสดงออกของตัวรับคิสเปปทีนในเซลล์ประสาทจีเอ็นอาร์เอชบริเวณกลุ่มเซลล์ประสาทไฮโปทาลามัสพรีออปติกและอาร์คูเอทนิวเคลียส ในกระป๋องที่มีวงรอบการเป็นสัด

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การศึกษาของเราก่อนหน้านี้ได้รายงานการแสดงออกของโปรตีนคิสเปปทีนในกลุ่มเซลล์ประสาทไฮโปทาลามัสพรีออปติก (พีโอเอ) และอาร์คูเอท นิวเคลียส (เออาร์ซี) ของแม่กระป๋องที่มีวงรอบการเป็นสัด ในสัตว์เคี้ยวเอื้องพีโอเอและเออาร์ซีเป็นกลุ่มเซลล์ประสาทไฮโปทาลามัสหลักที่คิสเปปทีนส่งผลกระทบต่อการทำงานของไฮโปทาลามัส ต่อมาได้สมอง และอวัยวะเพศ โดยการกระตุ้นให้เกิดการหลั่งของโกนาโดโทรปิน รีลีสซิง ฮอร์โมน (จีเอ็นอาร์เอช) ในสัตว์หลายชนิดพบว่าคิสเปปทีนกระตุ้นเซลล์ประสาทจีเอ็นอาร์เอชผ่านตัวรับคิสเปปทีน (KISS1R หรือ GPR54) การศึกษานี้ได้รับการออกแบบเพื่อสำรวจการแสดงออกของตัวรับคิสเปปทีนในเซลล์ประสาทจีเอ็นอาร์เอชในกลุ่มเซลล์ประสาทไฮโปทาลามัสพรีออปติกและเออาร์ซีของสมองกระป๋องนางด้วยวิธีดับเบิลเบลลิ่งอิมมูโนฮิสโตเคมี ในกลุ่มเซลล์ประสาทไฮโปทาลามัสที่บริเวณพีโอเอและเออาร์ซีพบการแสดงออกของตัวรับคิสเปปทีนทั้งในส่วนนิวเคลียสและไซโตพลาสซึมของตัวเซลล์ประสาทและเซลล์คำจุนบางเซลล์ เซลล์ประสาทที่มีการแสดงออกของจีเอ็นอาร์เอชมีลักษณะเป็นเม็ดเล็กๆในไซโตพลาสซึมและตัวเซลล์ประสาท จำนวนเซลล์ประสาทที่มีการแสดงออกของตัวรับคิสเปปทีนในตำแหน่งพีโอเอมีจำนวนเท่ากับในตำแหน่งเออาร์ซี (93%) อย่างไรก็ตาม จำนวนเซลล์ประสาทที่มีการแสดงออกของจีเอ็นอาร์เอชในตำแหน่งพีโอเอ (64%) มีแนวโน้มน้อยกว่าในตำแหน่งเออาร์ซี (73%) ( $P>0.05$ ) จากการทำดับเบิลเบลลิ่งอิมมูโนฮิสโตเคมีพบว่าทุกเซลล์ประสาทที่มีการแสดงออกของจีเอ็นอาร์เอชมีการแสดงออกร่วมของตัวรับคิสเปปทีน การค้นพบในครั้งนี้แสดงให้เห็นถึงหลักฐานการแสดงออกของตัวรับคิสเปปทีนในเซลล์ประสาทจีเอ็นอาร์เอชบริเวณกลุ่มเซลล์ประสาทไฮโปทาลามัสพีโอเอและเออาร์ซีในกระป๋อง และบ่งชี้ว่าคิสเปปทีนมีบทบาทในการหลั่งของจีเอ็นอาร์เอชในกระป๋อง

**คำสำคัญ:** กระป๋อง โกนาโดโทรปิน รีลีสซิง ฮอร์โมน ไฮโปทาลามัส ตัวรับคิสเปปทีน อิมมูโนฮิสโตเคมี

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