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—Original Article—

Immunohistochemical characterization of the arcuate kisspeptin/neurokinin B/dynorphin (KNDy) and preoptic kisspeptin neuronal populations in the hypothalamus during the estrous cycle in heifers

Ahmed Saad Ahmed HASSANEEN[1)#], Yousuke NANIWA[1)], Yuta SUETOMI[1)], Shuichi MATSUYAMA[2)], Koji KIMURA[3)], Nahoko IEDA[1)], Naoko INOUE[1)], Yoshihisa UENOYAMA[1)], Hiroko TSUKAMURA[1)], Kei-ichiro MAEDA[4)], Fuko MATSUDA[4)] and Satoshi OHKURA[1)]

[1)]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[2)]*National Agriculture and Food Research Organization (NARO) Institute of Livestock and Grassland Science, Nasushiobara 329-2793, Japan*
[3)]*Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan*
[4)]*Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan*
[#]*Department of Theriogenology, Obstetrics, and Artificial Insemination, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt*

Abstract. Elucidating the physiological mechanisms that control reproduction is an obvious strategy for improving the fertility of cattle and developing new agents to control reproductive functions. The present study aimed to identify kisspeptin neurons in the bovine hypothalamus, clarifying that a central mechanism is also present in the cattle brain, as kisspeptin is known to play an important role in the stimulation of gonadotropin-releasing hormone (GnRH)/gonadotropin secretion in other mammals. To characterize kisspeptin neurons in the bovine hypothalamus, the co-localizations of kisspeptin and neurokinin B (NKB) or kisspeptin and dynorphin A (Dyn) were examined. Hypothalamic tissue was collected from Japanese Black or Japanese Black × Holstein crossbred cows during the follicular and luteal phases. Brain sections, including the arcuate nucleus (ARC) and the preoptic area (POA), were dual immunostained with kisspeptin and either NKB or Dyn. In the ARC, both NKB and Dyn were co-localized in kisspeptin neurons during both the follicular and luteal phases, demonstrating the presence of kisspeptin/NKB/Dyn-containing neurons, referred to as KNDy neurons, in cows. In the POA, no co-localization of kisspeptin with either NKB or Dyn was detected. Kisspeptin expression in the follicular phase was higher than that in the luteal phase, suggesting that kisspeptin expression in the POA is positively controlled by estrogen in cows. The kisspeptin neuronal populations in the ARC and POA likely play important roles in regulating the GnRH pulse and surge, respectively, in cows.
Key words: Estrogen, Estrous cycle, Heifers, Immunohistochemistry, KNDy

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Cattle production is an important part of agriculture in Egypt to meet a growing national demand for beef and milk products. In tropical and subtropical areas, crossbreeding of native cows with pure European breeds, such as Holstein Friesian and Brown Swiss, is used in beef and dairy production, because crossbreeds are much more adaptive to such climates than pure breeds [1–3]. However, crossbred cows still exhibit relatively poor rates of fertility and low productivity in tropical and subtropical climates. To overcome such problems, it is critical to identify the mechanisms controlling reproductive function in cattle, such as steroidogenesis, follicular development, or ovulation. Better understanding of the mechanisms regulating reproduction would contribute to the genetic improvement of fertility or to the development of novel agents that would stimulate the reproductive axis.

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in controlling reproductive functions via two modes of secretion in females. One is pulsatile secretion, which induces gonadotropin pulses, which in turn stimulates follicular development and steroidogenesis. The other is surge secretion, which induces luteinizing hormone (LH) surge followed by ovulation. Accumulating evidence suggests that kisspeptin neurons are closely associated with both types of GnRH release, and therefore, these neurons are considered to be a master regulator of reproduction in many mammalian species [4–10]. Indeed, administration of kisspeptin, or its analogs, stimulates gonadotropin secretion in cattle [11–13], suggesting that kisspeptin neurons are involved in regulating GnRH and subsequent gonadotropin release in cattle.

Kisspeptin neuronal cell bodies are located in two major hypothalamic areas in mammalian species examined to date [4, 14], with one population in the hypothalamic arcuate nucleus (ARC). Because

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Correspondence: S Ohkura (e-mail: saohkura@agr.nagoya-u.ac.jp)

the ARC kisspeptin neurons co-express two other neuropeptides, neurokinin B (NKB) and dynorphin A (Dyn), these neurons are collectively referred to as KNDy neurons [15–20]. The KNDy neurons are implied to be responsible for the control of pulsatile GnRH release. In goats, for instance, periodic increases in multiple unit activity (MUA) volleys recorded in close proximity to the ARC KNDy neurons are closely associated with LH pulses in goats [18, 21]. Central administration of NKB agonist or Dyn antagonist increases the frequency of MUA volleys and LH pulses, whereas Dyn decreases them in goats [18, 22]. Similarly, LH pulses are positively or negatively regulated by NKB or Dyn, respectively, in rodents [23–25]. Recently, kisspeptin immunoreactivity was found in the ARC in cows [26–28], with one study reporting that Dyn immunoreactivity is localized in bovine ARC [26]. These studies imply that the presence of KNDy neurons in the bovine ARC to control pulsatile GnRH/gonadotropin release. However, to our knowledge, no direct evidence has definitively determined the presence of KNDy neurons in cows. The presence of KNDy neurons in cows would be of great importance, because it would improve our understanding of the mechanisms by which these neuropeptides control GnRH/gonadotropin pulses, and consequent follicular development and steroidogenesis in cows.

The other kisspeptin neuronal population is located more rostrally, in the preoptic area (POA) of sheep [29, 30], goats [31], and primates [32, 33]; in the anteroventral periventricular nucleus (AVPV) of rodents [34, 35]; or in the periventricular nucleus of pigs [36]. The anterior hypothalamic population of kisspeptin neurons in the POA/AVPV is thought to be involved in the control of the surge mode of GnRH release and then ovulation. A preovulatory level of estrogen induces an LH surge and increases AVPV *Kiss1* mRNA expression in rodents [35, 37]. *KISS1* mRNA expression in the POA is higher in the late follicular phase than in the luteal phase in sheep [38]. In addition, c-Fos expression in the kisspeptin neurons of the POA is induced by preovulatory levels of estrogen in goats and monkeys [31, 33]. These findings suggest that kisspeptin neurons in the POA play an essential role in generating GnRH/LH surges. Previous studies have demonstrated that little kisspeptin immunoreactivity was found in the anterior hypothalamus, including the POA, in cows [26–28]. Thus, it is unknown whether anterior hypothalamic kisspeptin neurons are involved in the mechanisms regulating GnRH surge and consequent ovulation in cows.

The present study aimed to determine the presence of the ARC KNDy and POA kisspeptin neurons in the cow brain in order to gain a better understanding of the mechanisms controlling ovarian functions. To address these issues, we examined the co-localization of kisspeptin and NKB/Dyn immunoreactivity in the bovine hypothalamus. Because kisspeptin expression is regulated by ovarian steroids, kisspeptin, NKB, and Dyn expressions during the follicular and luteal phases in cows were examined.

Materials and Methods

Animals

Fifteen- to 23-month-old Japanese Black (follicular phase, n = 2; luteal phase, n = 2) or crossbred Japanese Black × Holstein (follicular phase, n = 2; luteal phase, n = 2) heifers weighing between 291 kg and 463 kg were used. All animals were maintained under natural conditions at the National Agriculture and Food Research Organization (NARO) Institute of Livestock and Grassland Science. All procedures involving animals were approved by the Committees of the Care and Use of Experimental Animals at the NARO Institute of Livestock and Grassland Science and the Graduate School of Bioagricultural Sciences, Nagoya University.

Control of estrous cycle

Animals were treated with a progesterone-releasing intravaginal device (PRID; ASKA Pharmaceutical, Tokyo, Japan) for 6 days, and then intramuscularly injected with a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analog (0.75 mg, cloprostenol sodium; Nippon Zenyaku Kogyo, Koriyama, Japan) to synchronize the estrous cycle. Brain samples were collected 2 days and 7 days after the $PGF_{2\alpha}$ injection for the follicular and luteal phases, respectively. Visual inspection of ovaries at the time of slaughter was performed: antral follicles but no corpus luteum were found in the ovaries collected 2 days after the $PGF_{2\alpha}$ injection, whereas corpora lutea and some follicles were found in the ovaries collected 7 days after the injection. Two out of the 4 heifers slaughtered 2 days after the $PGF_{2\alpha}$ injection showed clear estrous behavior, whereas no obvious estrous behavior was observed for the other 2 heifers. Four heifers slaughtered 7 days after the $PGF_{2\alpha}$ injection displayed clear estrous behavior 2 days after the $PGF_{2\alpha}$ injection.

Brain and blood sampling

Blood samples (10 ml) were collected via the jugular vein immediately prior to brain sampling. Plasma samples separated by centrifugation were stored at –30ºC until estradiol and progesterone assays were performed. Heifers were then intravenously injected with 60–260 mg of xylazine (Bayer Yakuhin, Osaka, Japan), 500,000 U of heparin, and an overdose (6,480 mg/heifer) of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan). All heifers were bilaterally perfused through the carotid arteries with 10 l of 10 mM phosphate buffered saline (PBS) containing 13,000 U heparin/liter and 0.7% sodium nitrite, followed by 10–15 l of 4% paraformaldehyde in a 100 mM phosphate buffer (PB; pH 7.4) as a fixative. Brain blocks containing the hypothalamus were collected, with each brain block subsequently divided into two parts: an anterior part containing the POA and a posterior part containing the ARC. Brain blocks were immersed in the same fixative overnight at 4ºC and then placed in a solution of 30% sucrose in 100 mM PB, pH 7.4, at 4ºC, until the block sank. Serial brain sections were cut at 50-µm thickness on a cryostat with reference to a brain atlas of cattle [39]. All sections were stored in a cryoprotectant consisting of 25 mM PBS containing 50% glycerol, 250 mM sucrose, and 3.2 mM magnesium chloride at –20ºC until used for immunohistochemistry.

Dual immunohistochemistry of kisspeptin and NKB/Dyn

Every twelfth section of the hypothalamus was subjected to dual immunostaining of kisspeptin and NKB. Three sections containing either the ARC or POA were selected according to a bovine brain atlas [39]. Sections from the rostral edge of the dorsomedial hypothalamic nucleus (DMH) to the rostral edge of the mammillary bodies were used for the ARC, and those from the rostral end of the organum

vasculosum of the lamina terminalis (OVLT) to the rostral edge of the hypothalamic paraventricular nucleus (PVN) were used for the POA. After rinsing with 0.1 M PB three times for 5 min each time, free-floating sections were subjected to antigen retrieval in 10 mM sodium citrate solution (pH 9.0) for 30 min at 80ºC [40], then allowed to cool to room temperature and rinsed three times with 0.05 M PBS. The sections were then rinsed three more times (15 min each time) with 0.05 M PBS containing 0.3% Triton X-100 (PBST) and incubated with PBST containing 1.5% blocking reagent (Roche Diagnostics, Mannheim, Germany) for 1.5 h. The sections were incubated with the same buffer containing a mouse monoclonal kisspeptin antibody (1:4,000; Takeda No. 254) and a rabbit polyclonal antibody to NKB (1:4,000; Peninsula Laboratories, San Carlos, CA, USA) for 72 h at 4ºC. The sections were then washed four times (15 min each time) with PBST and incubated with Alexa 555-conjugated goat anti-mouse IgG (1:200; Life Technologies, Carlsbad, CA, USA) and Alexa 488-conjugated goat anti-rabbit IgG (1:200; Life Technologies) for 2 h under light shielding at room temperature. After washing, the sections were mounted on silane-coated slides and cover-slipped with a water-soluble anti-fade mounting reagent (Prolong Gold; Invitrogen, Carlsbad, CA, USA). Immunofluorescence was observed under a microscope (BX53; Olympus, Tokyo, Japan) equipped with a CCD camera (DP73; Olympus) and the merged images were obtained with the aid of computer software (Photoshop; Adobe Systems, San Jose, CA, USA). To confirm the specificity of the antibodies, brain sections were incubated with kisspeptin or NKB antibodies, which were preabsorbed overnight with 0.1 nmol/μl of bovine kisspeptin-53 (donated by Dr. S. Oishi, Kyoto University) or 0.1 nmol/μl of NKB (Sigma-Aldrich, St. Louis, MO, USA), respectively. Preincubation of the antibodies with corresponding peptides completely eliminated positive signals (data not shown).

Every twelfth section of the hypothalamus containing the ARC or POA was subjected to dual immunostaining of kisspeptin and Dyn. Immunohistochemical analysis was performed with the same method described above, using rabbit polyclonal antibody to Dyn as the first antibody (1:4,000; Phoenix Pharmaceuticals, Burlingame, CA, USA). To confirm specificity of antibodies, brain sections were incubated with the kisspeptin or Dyn antibodies, which were preabsorbed with 0.1 nmol/μl of bovine kisspeptin-53 or 1 nmol/μl of Dyn (Phoenix Pharmaceuticals), respectively. Preincubation of the antibodies with corresponding peptides eliminated positive signals (data not shown).

Radioimmunoassay

Plasma estradiol and progesterone concentrations were determined using radioimmunoassay. To determine plasma estradiol concentrations, 2-ml plasma samples were extracted with diethyl ether (Dojindo Laboratories, Kamimashiki, Japan), defatted with n-hexane 5000 (Wako Pure Chemical Industries, Osaka, Japan), and then reconstituted in 0.05 M PBS (pH 7.5) containing 0.1% sodium azide and 0.1% gelatin. Synthetic estradiol (Sigma-Aldrich) as standard, a rabbit antiserum against estradiol (1:100,000; GDN244), and tritiated estradiol (PerkinElmer, Waltham, MA, USA) were used. The lowest detectable concentration of estradiol was 0.39 pg/ml and intra-assay coefficients of variation were 9.5% at 1.6 pg/ml and 14.2% at 6.7 pg/ml. To determine plasma progesterone concentrations, unextracted plasma samples (20 μl) were used, along with synthetic progesterone (Sigma-Aldrich) as a standard, a rabbit antiserum for progesterone (1:30,000; OK-1), and tritiated progesterone (PerkinElmer). The lowest detectable concentration of progesterone was 0.625 ng/ml and intra-assay coefficients of variation were 3.9% at 3.0 ng/ml and 5.6% at 13 ng/ml. The radioimmunoassay was performed at the Radioisotope Research Center, Nagoya University.

Statistical analysis

Statistical differences ($P < 0.05$) in plasma estradiol and progesterone concentrations between the follicular and luteal phases were determined using Student's *t*-test.

Results

Plasma estradiol and progesterone concentrations

Plasma estradiol concentrations in the follicular phase were significantly ($P < 0.05$) higher than those in the luteal phase (Fig. 1A). On the other hand, plasma progesterone concentrations in the follicular phase were significantly ($P < 0.05$) lower than in the luteal phase (Fig. 1B).

Dual immunohistochemistry of kisspeptin and NKB/Dyn in the bovine ARC during the follicular and luteal phases

Figure 2 shows the co-localization of kisspeptin and NKB immunoreactivity (Fig. 2A) and kisspeptin and Dyn immunoreactivity (Fig. 2B) in the ARC of representative cows during the follicular or luteal phase. The kisspeptin-immunoreactive cell bodies and fibers were detected throughout the ARC in both the follicular and luteal phases (Fig. 2), and kisspeptin-immunoreactive fibers extended to the median eminence (Supplementary Fig. 1: online only). NKB-immunoreactive cell bodies and fibers were also detected throughout the ARC (Fig. 2A), and immunoreactivity was widely distributed in the ARC beyond the kisspeptin immunoreactivity. It should be noted that almost all kisspeptin-immunoreactive cell bodies co-expressed

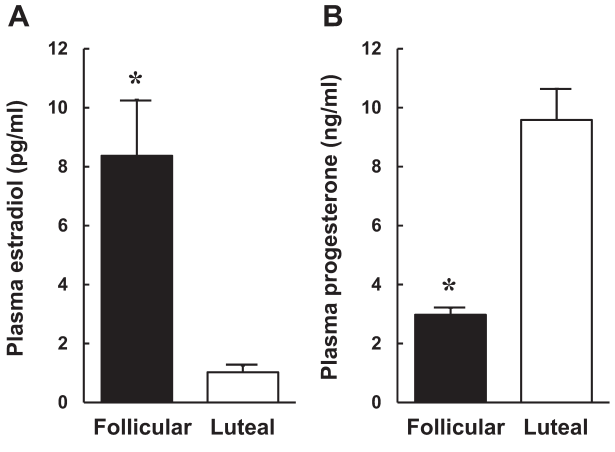
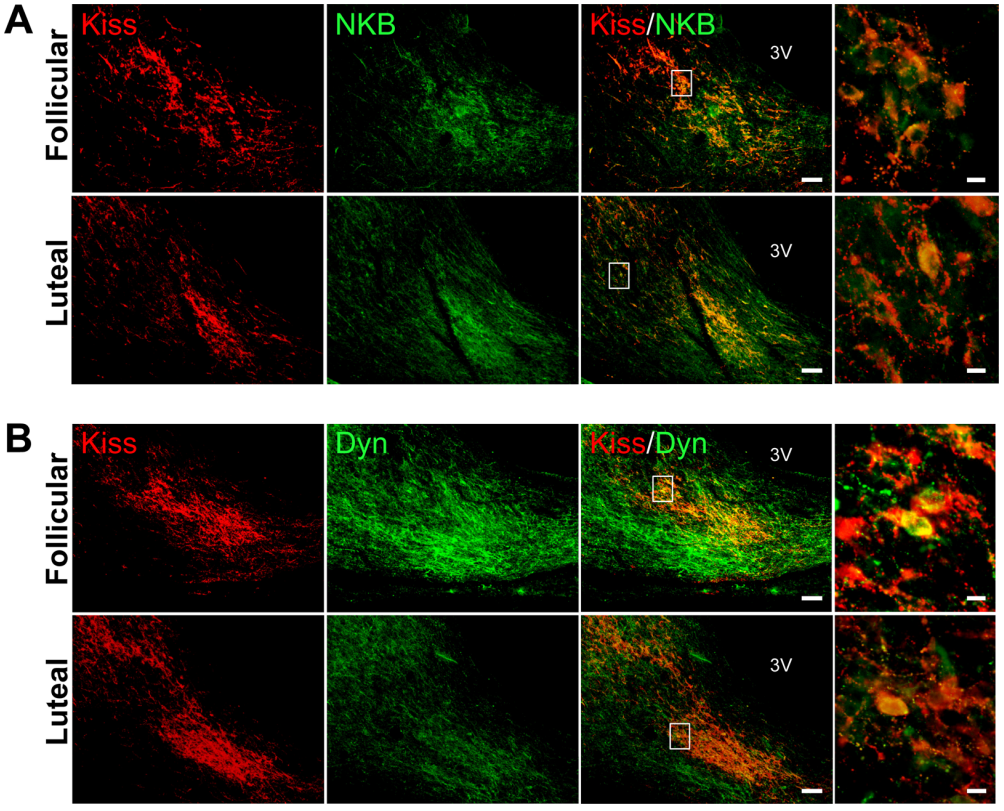


Fig. 1. Plasma estradiol (A) and progesterone (B) concentrations during the follicular (n = 4) and luteal (n = 4) phases in cows. Values are shown as mean ± SEM. * $P < 0.05$ between the follicular and luteal phases.


A
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NKB
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B
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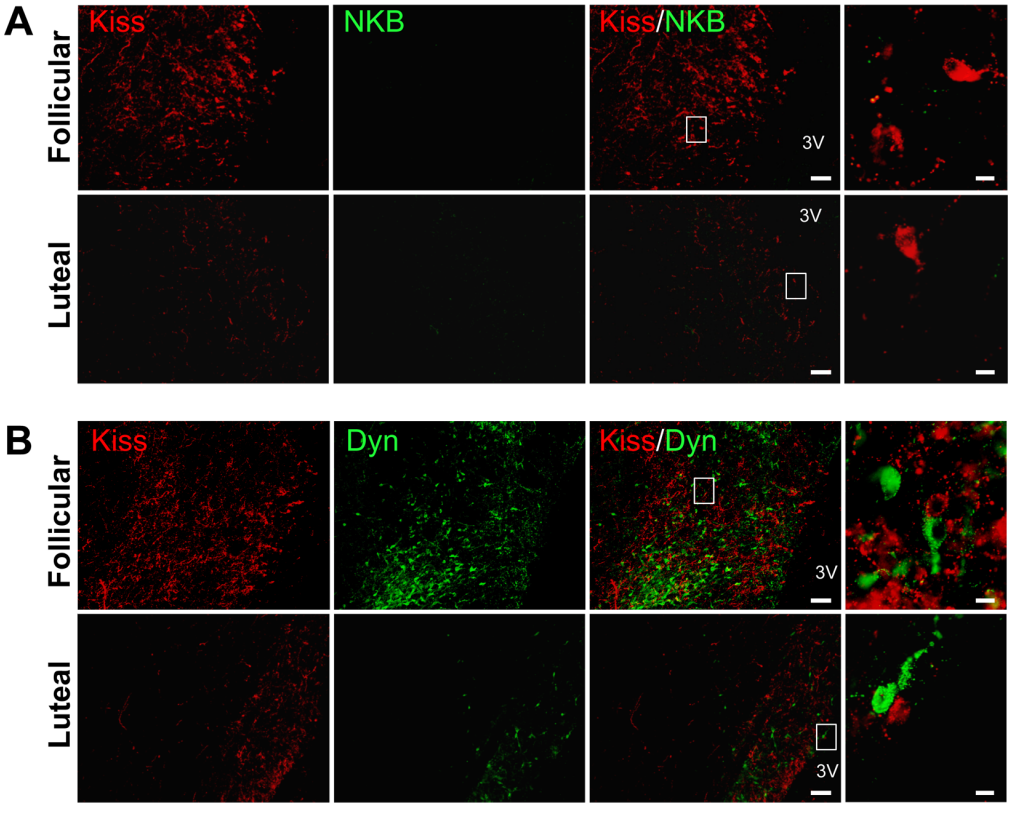
Fig. 2.


A
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NKB
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B
Follicular
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Dyn
Kiss/Dyn
3V
3V

Fig. 3.

NKB immunoreactivity (Fig. 2A, right panel), with similar results observed in all of the cows (n = 4 in each group) we examined.

Dynorphin A-immunoreactive cell bodies and fibers were distributed throughout the ARC in both the follicular and luteal phases (Fig. 2B). In the bovine ARC, more than half of the kisspeptin-immunoreactive cell bodies also exhibited Dyn immunoreactivity (Fig. 2B, right panel). The Dyn-immunoreactive fibers extended ventrolaterally in the ARC in both the follicular and luteal phases when compared with kisspeptin-immunoreactive fibers. Dyn immunoreactivity was detected in a wider area in the bovine ARC in the follicular phase than in the luteal phase (Fig. 2B).

Dual immunohistochemistry of kisspeptin and NKB/Dyn in the bovine POA during the follicular and luteal phases

Figure 3 shows the localization of kisspeptin and NKB immunoreactivity (Fig. 3A), and kisspeptin and Dyn immunoreactivity (Fig. 3B), in the POA of representative cows during the follicular or luteal phases. Numerous kisspeptin-immunoreactive cell bodies were detected in the POA in the follicular phase, whereas only a few were found in the luteal phase (Figs. 3A and 3B). Unlike in the ARC, few NKB-immunoreactive cell bodies and fibers were found in the bovine POA in either the follicular or luteal phase (Fig. 3A). Kisspeptin-immunoreactive fibers were distributed throughout the POA (Figs. 3A and 3B), whereas Dyn-immunoreactive fibers were mainly detected in the medial part of the POA (Fig. 3B). There were more Dyn-immunoreactive cell bodies and fibers in the follicular phase than in the luteal phase (Fig. 3B), and Dyn-immunoreactive cell bodies were in close apposition to kisspeptin neurons (Fig. 3B, right panel). No NKB- or Dyn-immunoreactivity was found in the POA kisspeptin-positive cells. Similar results were observed in all of the cows (n = 4 in each group) we examined.

Distribution of kisspeptin immunoreactivity in other hypothalamic areas

Kisspeptin immunoreactivity was found in other hypothalamic nuclei, including the OVLT, PVN, DMH, ventromedial hypothalamic nucleus (VMH), and lateral hypothalamus (LaH) (Supplementary Fig. 1). Some kisspeptin-immunoreactive cell bodies and fibers were found in the OVLT, whereas only immunoreactive fibers were detected in the PVN, DMH, VMH, and LaH.

Discussion

The present study provides direct evidence of the presence of ARC KNDy neurons as well as POA kisspeptin neurons in the bovine brain. The distribution of kisspeptin neurons was consistent with that observed in other mammalian species [18, 29–31, 34–36]. As the two kisspeptin neuronal populations are known to regulate GnRH release [18, 22, 31, 33, 37, 41], these neurons are also likely to be involved in the regulation of GnRH/gonadotropin release in cows. More specifically, we found a population of kisspeptin neurons co-localized with NKB or Dyn in the ARC, suggesting that the bovine KNDy neurons may have a role in GnRH pulse generation, as has been suggested in goats [18]. A second population of kisspeptin neurons found in abundance in the POA during the follicular phase may be involved in the GnRH/LH surge, as has been suggested in both sheep [29, 30] and goats [31]. Therefore, ARC KNDy and POA kisspeptin neuronal populations might be possible therapeutic targets for improving fertility in cattle raised in tropical and subtropical areas.

The presence of KNDy neurons in the ARC of cows was demonstrated by the co-localization of NKB or Dyn in the ARC kisspeptin neurons. KNDy neurons have been suggested to be the neuronal population that is essential for pulsatile GnRH release in goats and sheep [18, 21, 42]. The dense distribution of KNDy neurons from the middle to the caudal parts of the bovine ARC is consistent with previous findings in sheep [17] and goats [18], suggesting that the ARC KNDy neurons also form a dense network in cows, most likely to facilitate the synchronization of their activity. The dense network is a reminder of electrophysiological results in goats, in which MUA volleys in the ARC reflect the GnRH pulse generator activity [18]. The presence of kisspeptin-immunoreactive fibers in the median eminence further suggests that kisspeptin is released in the median eminence by the KNDy neurons to control GnRH release at GnRH nerve terminals [26]. Previous histological studies have shown that the nerve terminals of kisspeptin neurons make direct contact with GnRH nerve terminals at the median eminence in both goats [43] and rats [44]. The current results show that kisspeptin expression is evident in both the follicular and luteal phases, as previously reported in sheep and goats [38, 45]. This suggests that KNDy neurons are functional throughout the estrous cycle and regulate follicular development and corpus luteum function via the pulsatile release of GnRH/gonadotropins in cows. In addition, the ARC Dyn immunoreactivities were more widely distributed in the follicular phase than in the luteal phase in cows. In mice, estrogen has been reported to suppress Dyn gene expression in the ARC [46]: it is possible then that Dyn peptides accumulate in the ARC neurons because of the suppression of peptide release. Further studies are required to examine the relationship between the Dyn peptide and gene expression in the ARC of cows.

It is likely that kisspeptin expression in the POA is positively

Fig. 2. Expression of kisspeptin, neurokinin B (NKB), and dynorphin A (Dyn) in the hypothalamic arcuate nucleus (ARC) in cows. (A) Representative photomicrographs showing dual immunohistochemistry for kisspeptin (red) and NKB (green) during the follicular and luteal phases. The merged images show the co-localization of kisspeptin and NKB in the ARC. (B) Representative photomicrographs showing dual immunohistochemistry for kisspeptin (red) and Dyn (green) during the follicular and luteal phases. The merged images show the co-localization of kisspeptin and Dyn in the ARC. The white boxes in the merged images show the areas of the magnified images. Scale bars are 100 μm and 10 μm in the merged and magnified images, respectively. 3V, third ventricle.

Fig. 3. Expression of kisspeptin, neurokinin B (NKB), and dynorphin A (Dyn) in the preoptic area (POA) in cows. (A) Representative photomicrographs showing dual immunohistochemistry for kisspeptin (red) and NKB (green) during the follicular and luteal phases. The merged images show the absence of co-localization of kisspeptin and NKB in the POA. (B) Representative photomicrographs showing dual immunohistochemistry for kisspeptin (red) and Dyn (green) during the follicular and luteal phases. The merged images show the absence of co-localization of kisspeptin and Dyn in the POA. The white boxes in the merged images show the areas of the magnified images. Scale bars are 100 μm and 10 μm in the merged and magnified images, respectively. 3V, third ventricle.

controlled by ovarian estrogen in cows. This is supported by the results of the present study, in which the density of POA kisspeptin immunoreactivity was found to be higher in the follicular phase than in the luteal phase. These results are consistent with previous studies demonstrating that estrogen positively regulates *KISS1*/kisspeptin expression in the POA/AVPV of goats, rodents, and monkeys [31, 33, 37]. A high level of circulating estrogen is capable of inducing LH surges in cows [47], probably through estrogen receptor α in bovine kisspeptin neurons in the POA, as has been demonstrated in mice [35, 48]. The POA kisspeptin neuronal population, therefore, would likely be involved in the GnRH/LH surge generation induced by high circulating estrogen in cows, as has been shown in other species [31, 33, 37, 38]. The positive effect of estrogen on kisspeptin expression is supported by previous studies showing few kisspeptin-immunoreactive cell bodies in the bovine POA of juvenile prepubertal heifers [27, 28], in which the circulating level of estrogen remains suppressed. Lower expression of POA kisspeptin during the luteal phase is unlikely to be due to the high progesterone level in this phase compared with that in the follicular phase, because progesterone treatment alone failed to affect *KISS1* expression in the POA in ewes [29]. The notion is supported by a recent study in cows showing that plasma progesterone levels are not associated with POA kisspeptin immunoreactivity [26]. In the present study, kisspeptin immunoreactivity was in close apposition to Dyn immunoreactivity in the POA during the follicular phase in cows, and exhibited no co-localization with either NKB or Dyn immunoreactivity, which is in keeping with the results of a previous study of sheep [17]. The current result that Dyn expression is higher in the follicular phase than in the luteal phase suggests that Dyn is involved in the regulation of the GnRH surge. A kappa opioid receptor, a receptor for Dyn, agonist has been reported to control the activity of kisspeptin neurons in the rostral periventricular preoptic area (equivalent to the POA kisspeptin neuronal population in cows) in high estrogen-treated ovariectomized mice [49]. Further studies are required in order to clarify the role of POA Dyn neurons in controlling kisspeptin/GnRH neuronal activities in cows.

Kisspeptin-immunoreactive cell bodies and fibers found in the OVLT suggest that kisspeptin acts on the OVLT to regulate GnRH release, as a dense distribution of GnRH neuronal cell bodies and fibers are found in the OVLT of cows [26]. Kisspeptin-immunoreactive fibers were found in other hypothalamic areas, including the PVN, DMH, VMH, and LaH, in which few GnRH-immunoreactive cell bodies are found in cows [26], similar to the kisspeptin-immunoreactive fibers found in the OVLT, PVN, DMH, and VMH of rats [50]. The role of kisspeptin located in these areas, however, remains unknown and should be clarified by further studies.

In conclusion, the present study demonstrated that there are two major populations of kisspeptin neurons in the cow hypothalamus, as has been reported in other mammalian species. This finding suggests that the ARC KNDy neurons and POA kisspeptin neurons are involved in GnRH release in cows. These results can be used as the basis for future therapeutic trials solving the common infertility problems of cows experienced in tropical and subtropical areas.

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