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—Original Article—

Mapping of KNDy neurons and immunohistochemical analysis of the interaction between KNDy and substance P neural systems in goat

Hiroaki OKAMURA[1)], Takashi YAMAMURA[1)] and Yoshihiro WAKABAYASHI[1)]

[1)]*Division of Animal Breeding and Reproduction Research, Institute of Livestock and Grassland Science, NARO, Ibaraki 305-0901, Japan*

Abstract. A population of neurons in the arcuate nucleus (ARC) coexpresses kisspeptin, neurokinin B (NKB), and dynorphin, and therefore they are referred to as KNDy neurons. It has been suggested that KNDy neurons participate in several brain functions, including the control of reproduction. The present study aimed to advance our understanding of the anatomy of the KNDy neural system. We first produced an antiserum against goat kisspeptin. After confirming its specificity, the antiserum was used to histochemically detect kisspeptin-positive signals. Using the colocalization of kisspeptin and NKB immunoreactivity as a marker for KNDy neurons, we mapped distributions of their cell somata and fibers in the whole brain (except the cerebellum) of ovariectomized (OVX) goats. KNDy neuronal somata were distributed throughout the ARC, and were particularly abundant in its caudal aspect. KNDy neuronal fibers projected into several areas within the septo-preoptic-hypothalamic continuum, such as the ARC, median eminence, medial preoptic nucleus, and bed nucleus of the stria terminalis. Kisspeptin immunoreactivity was not found outside of the continuum. We then addressed to the hypothesis that substance P (SP) is also involved in the KNDy neural system. Double-labeling immunohistochemistry for kisspeptin and SP revealed that KNDy neurons did not coexpress SP, but nearly all of the KNDy neuronal somata were surrounded by fibers containing SP in the OVX goats. The present results demonstrate anatomical evidence for a robust association between the KNDy and SP neural systems.

Key words: Anti-kisspeptin antibody, Goat, Kisspeptin, KNDy neuron, Substance P

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Kisspeptin is a neuropeptide that regulates reproduction by stimulating gonadotropin-releasing hormone (GnRH) release [1, 2]. In general, it is considered that kisspeptin is expressed in two major populations of hypothalamic neurons located rostrally in the anteroventral periventricular nucleus (AVPV) of rodents, or the preoptic area (POA) of other species, and caudally in the arcuate nucleus (ARC) [3, 4]. The latter, but not the former, population of kisspeptin neurons coexpresses two other neuropeptides, neurokinin B (NKB) and dynorphin (Dyn); therefore, they are referred to as KNDy (Kisspeptin, Neurokinin B, and Dynorphin) neurons [5]. By histochemically detecting the colocalization of two (in either combination) or three of the three peptides or their mRNAs, KNDy neurons have been identified in various animals, such as mice [6], rats [7], sheep [8], goats [9], heifers [10], monkeys [11], and humans [12].

The hypothalamic GnRH pulse generator drives pulsatile discharges of the hormone into the hypophysial portal vessels that triggers episodic gonadotropin release from the pituitary, which is a key determinant of gonadal function [13]. Although its neural identity has yet to be fully described, several groups have hypothesized that KNDy neurons consist, at least in part, of the GnRH pulse generator and play a pivotal role in the control of pulsatile GnRH/luteinizing hormone (LH) release [6, 14–16]. Recent findings have suggested that KNDy neurons are also involved in other functions, such as thermoregulation [17], body weight control [18], and energy homeostasis [19]. Therefore, KNDy neurons appear to play more roles in a variety of biological functions than initially thought. For our better understanding of the physiological functions of KNDy neurons, the elucidation of the anatomy of the KNDy neural system is of fundamental importance.

Detailed maps of kisspeptin-immunoreactive (ir) cell somata and fibers have been reported for the whole brains of mice [20] and rats [21]. However, because other neurons synthesize kisspeptin, not only KNDy neurons, it is unclear which parts of those maps belong to the KNDy neuronal population. KNDy neuronal cell somata and fibers have been identified in several species by double-labeling immunohistochemistry [5, 8–12]; however, these studies have only elucidated parts of the KNDy neural system, and a complete analysis of the whole brain has yet to be performed.

Substance P (SP), together with neurokinin A (NKA) and NKB, comprise the tachykinin peptide family [22]. As with KNDy peptides, SP has also been suggested to be involved in the neuroendocrine control of reproduction [23]. In humans, this peptide is coexpressed in 30% of kisspeptin-ir and 25% of NKB-ir cell somata in the ARC [24], suggesting that SP may also participate in the KNDy neural system as a fourth player [25]. However, although *Tac1* (encoding SP)-positive cells are concentrated in the ARC, they do not overlap with cells expressing *Kiss1* (encoding kisspeptin) in mice [26]. In monkeys [25] and ewes [27], SP-ir cell somata are only infrequently

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Correspondence: Y Wakabayashi (e-mail: yoswaka@affrc.go.jp)

distributed in the ARC, and while SP-ir fibers surround kisspeptin cell somata, SP immunoreactivity does not appear to colocalize with kisspeptin-ir neurons. Therefore, whether SP is involved in the KNDy neural system remains to be clarified.

The present study had two aims: 1) to map KNDy cell somata and fibers in the whole brain (except the cerebellum); and 2) to elucidate the anatomical interactions between the KNDy and SP neural systems. We used ovariectomized (OVX) goats, in which the effects of tachykinins on GnRH pulse generation have been examined previously [9, 28]. Firstly, we produced a polyclonal antiserum against goat kisspeptin. After confirming its specificity, kisspeptin single-labeling immunohistochemistry, double-labeling fluorescence immunohistochemistry (kisspeptin/NKB or Dyn), and double-labeling fluorescence or diaminobenzidine (DAB)-nickel immunohistochemistry (kisspeptin/SP) were conducted using the antiserum.

Materials and Methods

Animals

Nine adult female Shiba goats (*Capra hircus*) aged 3 to 7 years old were used. They were OVX at least 6 months prior to this study to enhance kisspeptin expression in ARC kisspeptin neurons [29]. The goats were housed in an animal facility and maintained under natural daylight, with a standard pelleted diet and dry hay. Water and supplemental minerals were always available. All of the experiments were approved by the Committee for the Care and Use of Experimental Animals of Institute of Livestock and Grassland Science, NARO, Japan.

Tissue preparation

Because Shiba goats are nonseasonal breeders under natural daylight [30], brain tissues were randomly collected over a period of three years without taking into account the season. The goats were killed with an overdose of sodium pentobarbital (25 mg/kg body weight), and the heads were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, as described previously [9, 31]. Three goats were used to examine the distribution of kisspeptin-ir cell bodies and fibers. Brain blocks containing the diencephalon (limited anteriorly at a level slightly rostral to the vascular organ of the lamina terminalis (OVLT), posteriorly at the rostral edge of the mammillary body (MM), and dorsally at a level slightly ventral to the dorsal edge of the lateral ventricle), the olfactory bulb (OB), the area between the OB and the diencephalon, the amygdaloid complex, the hippocampus, the brain stem, and the cerebral cortex were taken. The cerebellum was not examined in this study. Sagittal sections of the OB and coronal sections of the other brain blocks were serially cut on a freezing microtome to a 50-µm thickness. For examination of the diencephalon and OB, all of the serial sections were collected, whereas every 4th section was taken from the other brain blocks. In the other five goats, only the diencephalon (between the OVLT and MM) was taken, and coronal floating sections were serially cut to a 50-µm thickness. They were then used for double-labeling immunohistochemistry.

In all of the section preparations for each goat, every 6th section was subjected to Nissl staining using cresyl violet. All of the sections were placed in a cryoprotectant solution [32] at –20°C until use.

A brain block containing the ARC of one goat was cut at 12 µm on a cryostat. The sections were mounted on coated slides and kept at –20°C until use for *in situ* hybridization.

Production of an anti-kisspeptin polyclonal antibody

A full-length cDNA for goat *Kiss1* (798 bp) has been cloned previously (DDBJ/EMBL/GenBank, accession number AB433789) [31], which codes for a putative goat kisspeptin of 53 amino acids. A peptide corresponding to amino acid residue 42-53 (C-SAYNWNSFGLRY) was coupled with keyhole limpet hemocyanin at the N-terminal additional cysteine residue, and the conjugate was immunized to rabbits using the standard procedure. The specificity of the anti-serum obtained (gC2) was confirmed as described below.

Other antibodies used for immunohistochemistry

A monoclonal antibody against SP (Creative Diagnostics, Shirley, NY, USA) and polyclonal antibodies against Dyn (Phoenix Pharmaceuticals, Burlingame, CA, USA) and NKB (Peninsula Laboratories, San Carlos, CA, USA) were used. The specificity of the anti-SP antibody in the goat tissues was examined by an absorption test as described below, and specificities of the anti-NKB and anti-Dyn antibodies have been confirmed previously [9].

Single-labeling immunohistochemistry for kisspeptin

To examine the distribution of cell bodies containing kisspeptin, every 6th section of the diencephalons of three goats was used. After being washed with 50 mM phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBST), the sections were treated with 3% H_2O_2 in methanol for 30 min. They were subsequently incubated with 10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in PBST containing 1% bovine serum albumin (BSA) and 0.05% sodium azide (PBST-BSA) for 1 h, gC2 (1:30,000 in PBST-BSA containing 2% NGS) at room temperature (RT) for 1 h and at 4°C for 48 h, biotinylated goat anti-rabbit IgG (1:400 in PBST-BSA containing 2% NGS, Vector Laboratories) for 3 h, and an avidin-biotin complex solution (15 µl/ml PBST, ABC elite kit, Vector Laboratories) for 1 h. Each step, except for incubation with NGS, was followed by washing with PBST (15 min × 3). After immersing in 50 mM Tris-HCl, pH 7.6, the immunoreactive products were visualized with a chromogen solution consisting of 0.04% 3,3′-DAB and 0.0026% H_2O_2 in Tris buffer for 4 min. All of the reactions were performed at RT unless otherwise stated, and the sections were mounted on slides as previously described [31].

To examine the projections of kisspeptin-ir fibers, every 6th section of the diencephalon (separated by 300 µm), OB (separated by 300 µm), and other brain areas (separated by 1200 µm) was immunostained by the same protocol as described above, except that 0.08% ammonium nickel sulfate hexahydrate was included in the chromogen solution to enhance the immunoreactive signals. Some immunostained sections were subjected to a brief Nissl counter-staining. Because markedly enhanced immunoreactivity sometimes made it difficult to distinguish positive cell somata in the caudal ARC, the DAB-nickel-stained sections were only used to examine kisspeptin-ir fibers.

Double-labeling fluorescence immunohistochemistry for kisspeptin/NKB or Dyn

Every 6th section of the diencephalon was processed for kisspeptin/NKB double-labeling fluorescence immunohistochemistry using the tyramide signal amplification (TSA) method [8, 33]. Sections were sequentially incubated with 10% NGS in PBST-BSA for 1 h, anti-NKB polyclonal antibody (1:6,000) in PBST-BSA containing 2% NGS at 4°C for 72 h, biotinylated goat anti-rabbit IgG (1:200 in PBST-BSA containing 2% NGS) for 3 h, and avidin-biotin complex (2 µl/ml PBST) for 1 h. After being soaked in the TSA blocking solution for 30 min, the sections were reacted with biotinyl tyramide solution (1:200, PerkinElmer, Waltham, MA, USA) for 10 min, and streptavidin-Alexa 488 (1:200, Invitrogen, Carlsbad, CA, USA) in PBST for 1 h. They were then incubated with gC2 (1:2,000) for 48 h at 4°C and Alexa-555 conjugated anti-rabbit IgG (1:200, Invitrogen) for 3 h. Kisspeptin/Dyn double-labeling immunohistochemistry was conducted using the same protocol and the anti-Dyn polyclonal antibody (1:20,000). Sections were mounted on gelatin-coated slides and cover-slipped with a water-soluble mounting medium (Vector Laboratories). The sections were observed under a microscope (ECLIPSE E800M, Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (AxioCam HRc, Carl Zeiss, Jena, Germany). The two fluorescent images were merged using computer software (AxioVision, Carl Zeiss).

Double-labeling fluorescence immunohistochemistry for kisspeptin/SP

Sections containing the caudal part of the diencephalon (between the optic chiasm and MM) were used. They were treated with 10% fetal bovine serum (FBS; HyClone Laboratories, UT, USA) in BSA-PBST for 1 h, and then incubated with a mixture of gC2 (1:2,000) and the anti-SP (1:30,000) monoclonal antibody in PBST-BSA containing 2% FBS at 4°C for 72 h, a mixture of biotinylated horse anti-mouse IgG (1:200, Vector Laboratories) and Alexa-555 conjugated anti-rabbit IgG (1:200) in PBST-BSA containing 2% FBS at 4°C overnight, and streptavidin-Alexa 488 (1:200) in PBST at RT for 3 h. After being mounted on gelatin-coated slides and cover-slipped, the sections were observed under the microscope, and the two fluorescent images were merged using computer software.

Some sections were further analyzed using a confocal microscope (LSM700, Carl Zeiss) with sequential imaging of the two channels. Photomicrographs were taken at the same focal plane of 1-µm thickness, and cell somata and fibers were considered double-labeled for kisspeptin/SP when positive signals overlapped in the same focal plane.

Double-labeling DAB-nickel immunohistochemistry for kisspeptin/SP

After treatment with hydrogen peroxide and 10% FBS, sections containing the caudal part of the diencephalon were reacted with a mixture of gC2 (1:80,000) and the anti-SP (1:4,000,000) monoclonal antibody in PBST-BSA containing 2% FBS (PBST-BSA-FBS) at 4°C for 48 h. Firstly, SP-ir materials were visualized by sequentially incubating sections with biotinylated horse anti-mouse IgG (1:600 in PBST-BSA containing 2% FBS, Vector Laboratories), avidin-biotin complex (7.5 µl/ml PBST), and a DAB plus nickel salt (0.08%) solution. Positive signals for SP were identified as black-purple products.

After extensive washing with PBST, gC2-ir materials were visualized by sequentially incubating sections with biotinylated goat anti-rabbit IgG (1:600, in PBST-BSA containing 2% FBS) at 4°C overnight, avidin-biotin complex (7.5 µl/ml PBST), and the DAB solution. Positive signals for kisspeptin were identified as brown products.

Double-labeling of kisspeptin and its mRNA (Kiss1)

Cryostat sections from one goat were subjected to *in situ* hybridization. Digoxigenin (DIG)-labeled *Kiss1* riboprobes (640 bp) were synthesized with templates containing a partial sequence of the 5′ untranslated region and *Kiss1* coding region of the goat. Hybridization was performed as previously described [31]. To detect the DIG-labeled *Kiss1* probe after hybridization, a peroxidase-conjugated anti-DIG Fab fragment (1:250, Roche Diagnostics, IN, USA) and a TSA-Cy3 amplification system (1:100, PerkinElmer) were used. The sections were then incubated with gC2 (1: 2,000) for 18 h at 4°C, and immunoreactive materials were visualized using Alexa 488-conjugated anti-rabbit IgG antibody (1:400).

Absorption test

The antiserum obtained (gC2 (1:30,000)) was incubated with 10 mmol of several hypothalamic peptides dissolved in 100 or 200 µl distilled water, or an equal volume of distilled water (control), for 1 h at RT and overnight at 4°C. The pre-treated serum was then used for kisspeptin single-labeling immunohistochemistry as described above. The following peptides were used: rat- or human-type kisspeptin-10 and GnRH (Peptide Institute, Minoh, Osaka, Japan), NKB, β-endorphin (ovine), Neuropeptide Y (NPY, ovine), α-melanocyte-stimulating hormone (Sigma-Aldrich, St Louis, MO, USA), Dyn (porcine), prolactin-releasing peptide-31 (bovine), prepro-RF-amide-related peptide (RFRP) (rat, 103-125), pyroglutamylated-RF-amide peptide (rat, 13-26), and SP (human, 2-11) (Phoenix Pharmaceuticals Inc).

The anti-SP monoclonal antibody (1:2,000,000) was incubated with either SP (10 nmol), NKB (10 nmol), or 100 µl distilled water for 1 h at RT and overnight at 4°C, and subjected to SP immunohistochemistry using a similar protocol as the kisspeptin single-labeling immunohistochemistry, except that the normal serum and the second antibody were substituted with normal horse serum and anti-mouse IgG, respectively.

Mapping kisspeptin-ir cell somata and fibers

A set of kisspeptin-labeled DAB-stained, DAB/nickel-stained, and Nissl-stained sections was chosen from one representative goat. Brain structures and nuclear boundaries from Nissl-stained sections were drawn with reference to the Shiba goat brain atlas [34]. Kisspeptin-positive cell somata and fibers were mapped onto corresponding drawings using a microscope (ECLIPSE E400, Nikon) equipped with a camera lucida (Y-IDT, Nikon).

Data analysis

Quantitative analyses of the colocalization of NKB- or Dyn-ir materials in kisspeptin-ir cell somata were conducted using sections from three goats. Two sections containing the middle part of the ARC were chosen from each goat, and the number of kisspeptin single-

labeled and kisspeptin/NKB or Dyn double-labeled cells was counted on the unilateral side of the ARC. The total numbers of kisspeptin single-labeled cells examined were 676 and 666, respectively, for kisspeptin/NKB and Dyn double-labeling.

The colocalization of SP-ir materials in kisspeptin-ir neurons (either cell bodies or fibers) was analyzed by double-labeling fluorescence immunohistochemistry using sections from three goats. From the rostral, middle, and caudal parts of the ARC, three sections were obtained from each individual (nine sections per goat), and the bilateral sides of the ARC were examined under the fluorescence microscope.

The number of kisspeptin-positive cells with appositions of SP-ir fibers was quantitatively analyzed in DAB-nickel double-labeled sections from three goats. Three sections from the rostral to the middle parts of the ARC were chosen from each goat, and the numbers of kisspeptin-positive cells and those having appositions of SP-ir fibers were determined on the unilateral side of the ARC under a high magnification (× 400). The total number of kisspeptin-positive cells examined was 1,030 in nine sections.

The number of SP-positive cells was counted on the bilateral sides of the same nine sections mentioned above. In addition, three sections from the caudal part of the ARC of each goat were also examined. Therefore, 18 sections were used in total for this analysis.

The middle to caudal parts of the ARC contained more kisspeptin-positive cells than the others. However, they were excluded from the quantitative analyses, because it was difficult in some cases to accurately count the number of positive cells due to densely packed immunoreactivities, as described below.

Results

Assessment of gC2 specificity

When gC2 immunohistochemistry was combined with kisspeptin mRNA (*Kiss1*) *in situ* hybridization, the cell bodies containing gC2-ir products perfectly overlapped with *Kiss1*-positive signals in the ARC (Supplementary Fig. 1: online only). Although the densities of the two signals in a single cell were not always in parallel, no cell was only stained by immunohistochemistry, and *vice versa*. In the absorption test, the control treatment had no effect on gC2 immunoreactivity. Positive signals in the control sections (Supplementary Fig. 2A: online only) were equivalent to those in the gC2-stained sections without the pre-absorption step. Pre-incubation of gC2 with rat- (Supplementary Fig. 2B) or human-type Kp-10 (Supplementary Fig. 2C) resulted in a complete elimination of immunopositive signals. In contrast, pre-incubation with other peptides had no effect on gC2 immunohistochemistry (data not shown). Double-fluorescence labeling of gC2- and NKB-ir materials revealed that nearly all (97.9 ± 0.51%, n = 3) of gC2-positive cell somata coexpressed NKB-ir materials in the ARC of OVX goats (Supplementary Fig. 3: online only). Dyn-ir materials colocalized with 61.1 ± 2.1% (n = 3) of gC2-positive cell somata (Supplementary Fig. 3). These colocalization rates are comparable with those from a previous study that used an anti-kisspeptin monoclonal antibody (Takeda, #254) and the same anti-NKB and anti-Dyn polyclonal antibodies in OVX goats [9]. These results, together with the characteristic distribution of gC2-positive cells in the brain as described below, indicate that gC2 specifically interacts with kisspeptin molecules, and gC2-ir signals represent kisspeptin immunoreactivity.

Distribution of kisspeptin-ir cell somata and fibers

Figure 1 shows representative profiles obtained by gC2 immunohistochemistry in the OVX goat. Kisspeptin-ir signals were identified in a subpopulation of cells and neural fibers (Fig. 1A). A characteristic anatomical feature of the caudal part of the ARC was a densely packed immunopositive structure consisting of numerous immunopositive somata and fibers at the center of the nucleus (Fig. 1A, asterisk). Kisspeptin-ir cell bodies were round, oval, or fusiform in shape, with a diameter of 10−20 μm and extended thick neural processes (Fig. 1B). Their nuclei did not contain any immunopositive products. There were no regional differences in the size or appearance of the immunoreactive cells. Kisspeptin-ir fibers were characterized as thin and frequently beaded structures (Fig. 1C), with *en passant* and terminal buttons (Fig. 1C, arrowheads) in the hypothalamic nuclei and median eminence (ME; Fig. 1D). Almost all of the kisspeptin-ir cells had numerous appositions of kisspeptin-ir fibers (Fig. 1E).

Figure 2 shows the distribution of kisspeptin-ir cell bodies in the OVX goat. The entire brain structure, except the cerebellum, was examined, and kisspeptin-ir cell bodies were located almost exclusively in the ARC, with several positive cells extending from the boundary of the nucleus. Kisspeptin-ir cells were relatively few and scattered in the rostral part (Fig. 2A, B). Their number gradually increased in the middle part (Fig. 2C, D), and reached a maximum around a level that was slightly caudal to the midpoint of the ARC (Fig. 2E). A densely packed kisspeptin-ir cluster was observed throughout the caudal part of the ARC (Fig. 2E–H, shadows). The size of the cluster and the number of surrounding positive cells eventually decreased towards the caudal edge of the nucleus. Although a quantitative analysis of kisspeptin/NKB coexpression was only performed in the middle part (Supplementary Fig. 3), it appeared that nearly all of the kisspeptin-ir cell somata in the ARC, and those extending from the boundary of the nucleus, contained NKB-ir signals.

Figure 3 shows the distribution of kisspeptin-ir fibers. Their distributions were restricted to within the septo-preoptic-hypothalamic continuum, and no immunoreactivity was found in other areas, such as the OB, hippocampus, amygdaloid complex, or brain stem. A high density of positive fibers was observed throughout the ARC, which were most abundant in its caudal part (Fig. 3F–H). The internal layer of the ME also exhibited rich projections of positive fibers (Figs. 1D, 3E–G), and some of them formed dense plexuses towards the pars tuberalis at the level of the tuberoinfundibular sulcus (Figs. 1D, 3F, G). A considerable number of positive fibers also extended from the internal layer to the ventral margin of the external layer of the ME. The medial preoptic nucleus (MPN) contained a moderate density of kisspeptin-ir fibers, whereas they were found in relatively small amounts in other hypothalamic areas, such as the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST), median preoptic nucleus (MnPO), paraventricular nucleus, ventromedial nucleus (VMN), and dorsomedial nucleus (DMN). The supraoptic nucleus and premammillary nucleus (PM) were devoid of immunoreactivity. Double-labeling immunohistochemistry revealed that the majority of kisspeptin-positive fibers also contained NKB-ir products (Supplementary Fig. 4, arrows: online only). There were also a few

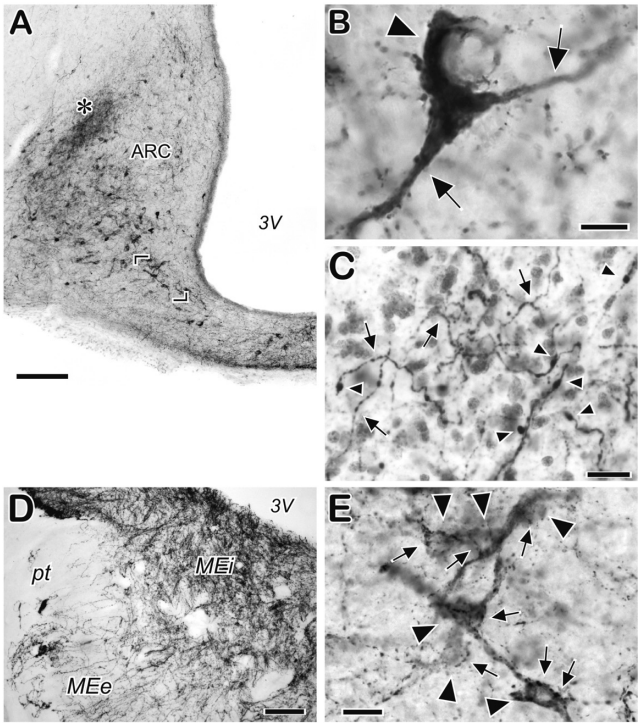

Fig. 1. Photomicrographs showing the general morphology of kisspeptin-immunoreactive (ir) neurons in the goat brain. (A) Representative profile of kisspeptin-ir signals in the caudal part of the arcuate nucleus (ARC). An asterisk indicates a densely packed immunopositive structure. (B) Kisspeptin-ir cell soma (arrowhead) and neural processes (arrows) at high magnification. (C) Kisspeptin-ir fibers (arrows) in the medial preoptic nucleus in a section subjected to brief Nissl counter-staining after immunohistochemistry. Arrowheads indicate *en passant* or terminal buttons. (D) Kisspeptin-ir fibers in the internal (*MEi*) and external (*MEe*) layers of the median eminence. (E) The area indicated by a pair of brackets in (A) at high magnification. Note that kisspeptin-ir cell somata (arrowheads) are surrounded by a number of kisspeptin-positive thin fibers (arrows). *pt*, par tuberalis; 3*V*, third ventricle. Scale bars: (A), 200 μm; (B), 10 μm; (C) and (E), 20 μm; (D), 100 μm.

fibers with only kisspeptin immunoreactivity (Supplementary Fig. 4, closed arrowheads) in the rostral structures of the hypothalamus such as the MPOA and MPN. However, the ratio of kisspeptin single-labeled fibers to kisspeptin/NKB double-labeled fibers appeared to be low in all of the nuclei, although this was not quantitatively analyzed.

Interaction between the kisspeptin and SP neural systems

Pre-incubation of the anti-SP antibody with a vehicle had no effect on SP immunoreactivity (Supplementary Fig. 2D). Pre-incubation of the antibody with 10 nmol SP resulted in a complete elimination of SP-positive signals in all areas, including the ARC (Supplementary Fig. 2E), whereas NKB (10 nmol) did not affect SP immunoreactivity (Supplementary Fig. 2F). According to the manufacturer's instructions, this antibody does not cross-react with NKA or kassinin.

To examine whether kisspeptin- and SP-ir signals colocalize in the same neuron, we first conducted fluorescence double-labeling immunohistochemistry. Both signals existed in the rostral (not shown), middle (Supplementary Fig. 5A, B: online only), and caudal (Supplementary Fig. 5D, E) parts of the ARC. However, no cell bodies concomitantly expressed two signals, except in two instances (Supplementary Fig. 5G–I) out of approximately 2,000 cases of clearly distinguishable kisspeptin-ir cell somata and numerous indistinguishable ones forming the kisspeptin-ir cluster in 27 sections of three goats. In addition, we found no evidence for the colocalization of kisspeptin and SP immunoreactivities in neural fibers, in any areas examined.

In DAB-nickel double-labeled sections, SP-ir fibers were observed in several structures of the goat brain, including the hypothalamus. Throughout the rostro (Fig. 4A)-caudal (Fig. 4B) extent of the ARC, in particular, a substantial number of SP-positive fibers were projected in areas where kisspeptin-positive cell somata were distributed, and some were very close to kisspeptin-ir cell bodies. At a high magnification, SP-ir fibers appeared to be in contact with kisspeptin-ir cells (Fig. 4C, D). The quantitative analysis revealed that the majority of kisspeptin-positive cell somata (87.1 ± 3.4%, n = 3) exhibited this morphological feature. To investigate the interaction between the kisspeptin and SP systems in detail, confocal microscopy was performed on fluorescence double-labeled sections. A stacked image of 15 serial confocal planes revealed that several kisspeptin-positive cell somata were surrounded by a number of SP-ir fibers (Fig. 4E). Analysis of a single, 1-μm-thick plane of the same section confirmed that the SP-ir fibers directly apposed on the surface of the kisspeptin-ir cell body at several points (Fig. 4F). This was the case for other kisspeptin-ir cells in Fig. 4E, as well as for most of those, if not all, in other sections. In the hypothalamus, a few SP-positive cells were observed in the VMN (Fig. 4G) and PM (Supplementary Fig. 5E). The ARC also contained SP-ir cells (Fig. 4H), but only a few (10 cells in 18 sections from three goats).

Discussion

A polyclonal antiserum (gC2) against the C-terminal part of goat kisspeptin was produced. The C-terminal structure of the kisspeptin molecule appears to share a common, or very similar, antigenic motif with other hypothalamic peptides, which raises the possibility of generating antibodies that are not specific to kisspeptin [3, 35]. Indeed, an anti-human Kp-10 antiserum [36, 37] has been shown to cross-react with molecules other than kisspeptin [3, 8]. Therefore, we carefully assessed the specificity of the gC2 antiserum. Several lines of evidence confirmed that gC2 specifically recognizes the kisspeptin molecule. Firstly, immunohistochemistry and *in situ* hybridization revealed that cell bodies containing gC2- and *Kiss1*-positive signals perfectly overlapped each other in the ARC (Supplementary Fig. 1). Secondly, gC2 immunoreactivity was completely abolished by pre-treatment with rat- or human-type Kp-10, whereas other peptides, including RFRP, had no effect on immunohistochemistry (Supplementary Fig. 2). The results indicate that gC2 reacts with kisspeptins regardless of its C-terminal residue (Y or F). Thirdly, gC2-ir signals colocalized with NKB and Dyn (Supplementary Fig. 3), which is a unique characteristic of ARC kisspeptin neurons and has been found in various species [6, 8–11]. Moreover, the colocalization rates obtained by gC2 were almost identical to those observed using the anti-mouse kisspeptin antibody (Takeda, #254)

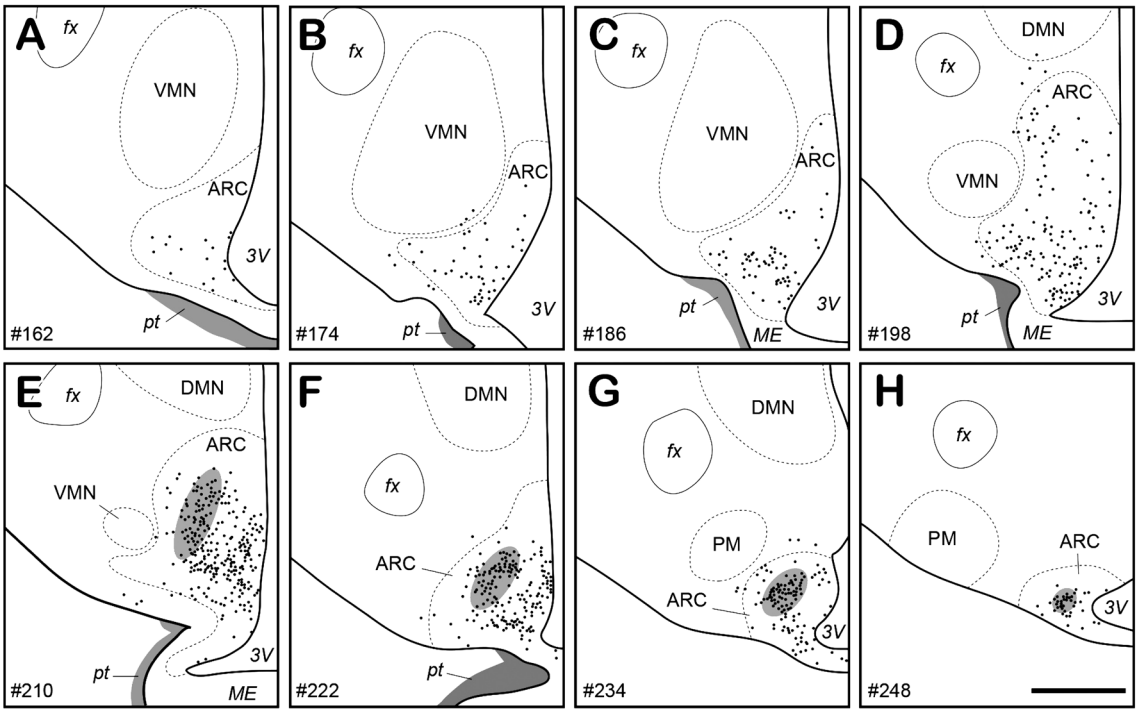

Fig. 2. Drawings of kisspeptin-ir cell soma in the goat brain. Black dots represent kisspeptin-ir cell somata. Shadows in (E)–(H) indicate densely packed immunopositive structures consisting of distinguishable (dots) and indistinguishable positive cell somata (see Fig. 1A, asterisk). Each panel is separated by 600 μm. The section number (assigned serially, in a rostral to caudal direction) is shown on the bottom-left corner of each panel. ARC, arcuate nucleus; DMN, dorsomedial nucleus of the hypothalamus; PM, premammillary nucleus; VMN, ventromedial nucleus of the hypothalamus; *fx*, fornix; *ME*, median eminence; *pt*, par tuberalis; *3V*, third ventricle. Scale bar for (A)–(H), 1 mm.

[9]. Finally, distributions of gC2-positive cell bodies (Fig. 2) and the projection patterns of gC2-ir fibers (Fig. 3) were essentially comparable with those containing kisspeptin- or NKB-ir cells and fibers in other species [3, 4, 21, 35, 38, 39] and to some extent in goats [40, 41], albeit with slight species differences. Because amino acid sequences of the 12 C-terminal residues of kisspeptin are identical in goats, cattle, sheep, pigs, rats, and hamsters, the gC2 serum can be employed in studies that use those animals.

The general morphology of the kisspeptin cell bodies detected by gC2 (Fig. 1B) appeared to be similar to that described in mice and rats [35], sheep [38], and monkeys [42] using different antibodies. We found that kisspeptin-ir cell somata were distributed throughout the ARC and were particularly abundant in its middle to caudal parts (Fig. 2), which is consistent with previous reports in ewes [38, 43], monkeys [42], and orchidectomized (ORX) [31] and OVX [40] goats. However, we have failed to identify a rostral population of kisspeptin neurons in ORX [31] or OVX (this study) goats, whereas an *in situ* hybridization study successfully demonstrated the presence of *Kiss1* mRNA in the POA of the same species [44]. It has been shown that colchicine treatment is necessary to immunohistochemically detect Kiss1 peptides in the AVPV of rats [35, 39], but not mice [35]. This suggests that although AVPV/POA kisspeptin neurons exist across mammalian species, Kiss1 peptides in their cell bodies are rapidly released and/or subjected to turnover [35]; consequently, they are rarely detected by immunohistochemistry without colchicine treatment in animals such as rats and goats.

Several immunohistochemical studies have indicated the presence of a scattered population of kisspeptin cells in the VMN of ewes [38] and the DMN of mice and rats [35], guinea pigs [45], ewes [38], and mares [46]. We also observed a very few positive cells in the VMN (Fig. 2B) and DMN (Fig. 2D), but they were only found in areas adjacent to the ARC. Moreover, their morphological appearance was identical to that of positive cells in the ARC, and they maintained the KNDy composition. Therefore, all of the kisspeptin-ir cells in the mediobasal hypothlamus may belong to a single population of KNDy neurons in the ARC, with some of them extending laterally from the border of the nucleus to the VMN and dorsally to the DMN in goats. It is not clear whether this was also the case in other studies [35, 38, 45, 46].

Kisspeptin-ir fibers projected into the septo-preoptic-hypothalamic continuum (Fig. 3), and we found no kisspeptin-positive signals outside the continuum. We found two types of kisspeptin-ir fiber: one had both kisspeptin and NKB immunoreactivity, and the other only kisspeptin immunoreactivity (Supplementary Fig. 4). Considering that almost all of the kisspeptin-ir neurons in the ARC had NKB immunoreactivity (Supplementary Fig. 3) and that POA kisspeptin neurons do not contain NKB [5, 8], the former type of kisspeptin-ir fiber might originate from the ARC population, whereas the latter might originate from the POA population, although their cell bodies were not detected in the colchicine-untreated OVX goat. Because

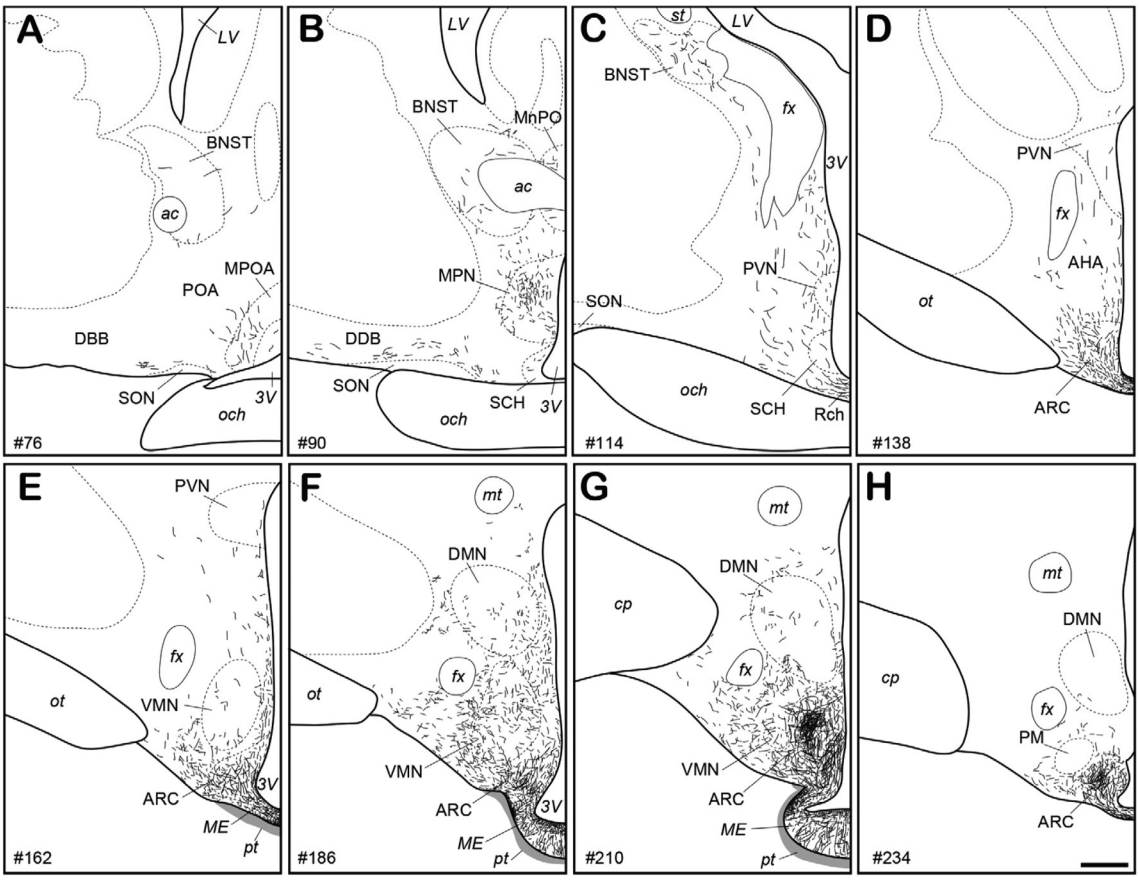

Fig. 3. Drawings of kisspeptin-immunoreactive (ir) fiber projection in the goat brain. Black lines represent kisspeptin-ir fibers. Each panel is separated by 1,200 μm. The section number (assigned serially, in a rostral to caudal direction) is shown on the bottom-left corner of each panel. AHA, anterior hypothalamic area; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; DBB, diagonal band of Broca; DMN, dorsomedial nucleus of the hypothalamus; MnPO, median preoptic nucleus; MPN, medial preoptic nucleus; MPOA, medial preoptic area; PM, premammillary nucleus; POA; preoptic area; PVN, paraventricular nucleus of the hypothalamus; Rch, retrochiasmatic area; SCH, suprachiasmatic nucleus; SON, supra optic nucleus; VMN, ventromedial nucleus of the hypothalamus; ac, anterior commissure; *cp*; cerebral peduncle; *fx*, fornix; *LV*, lateral ventricle; *ME*, median eminence; *mt*, mammillothalamic tract; och, optic chiasm; ot, optic tract; *pt*, par tuberalis; *st*, stria terminalis; *3V*, third ventricle. Scale bar for (A)–(H), 1 mm.

the ratio of kisspeptin single-labeled fibers to double-labeled ones was low in all of the nuclei, it is plausible that Fig. 3 displays KNDy neuronal projections. The map described for the goat is essentially similar to that produced in previous studies that partly described the distributions of kisspeptin, NKB, or KNDy neuronal fibers in rats [47], sheep [5, 8], goats [9, 48], horses [46], monkeys [42], and humans [49].

The ARC and ME contained dense accumulations of kisspeptin-ir fibers (Fig. 3D–H). In the ARC, positive fibers surrounded kisspeptin-ir cell bodies themselves (Fig. 1E), which is a unique feature of KNDy neurons and has been demonstrated in a variety of mammalian species [8, 9, 11, 50]. It has been suggested that such a KNDy construct represents reciprocal connections among KNDy neurons that synchronize neural activity with GnRH pulse generation [6, 14–16]. Moreover, because KNDy neuronal fibers have been shown to directly appose on GnRH axon terminals in the ME [42, 48, 51], it is possible that GnRH-pulse-generating activity is conveyed via dense plexuses of kisspeptin fibers to the ME to stimulate GnRH release [6, 14–16]. A few kisspeptin-ir fibers were also observed in other nuclei within the septo-preoptic-hypothalamic continuum, such as the MPOA, MPN, BNST, and MnPO (Fig. 3A–C), in which they often exhibited *en passant* and terminal buttons (Fig. 1C). A wide range of KNDy neuronal projections suggests that the KNDy neural system is involved in a variety of brain functions, such as thermoregulation [17]. However, the precise physiological significance of each anatomical structure is largely unknown.

To investigate the anatomical relationship between the SP and KNDy neural systems, we first addressed the issue of whether KNDy neurons coexpress SP in the OVX goat by double-labeling fluorescence immunohistochemistry. We observed no somata that concomitantly contained SP- and kisspeptin-positive signals, except in two instances out of approximately 2,000 clearly distinguishable kisspeptin-ir cells and numerous indistinguishable ones forming the kisspeptin-ir cluster (Supplementary Fig. 5). Therefore, it appears

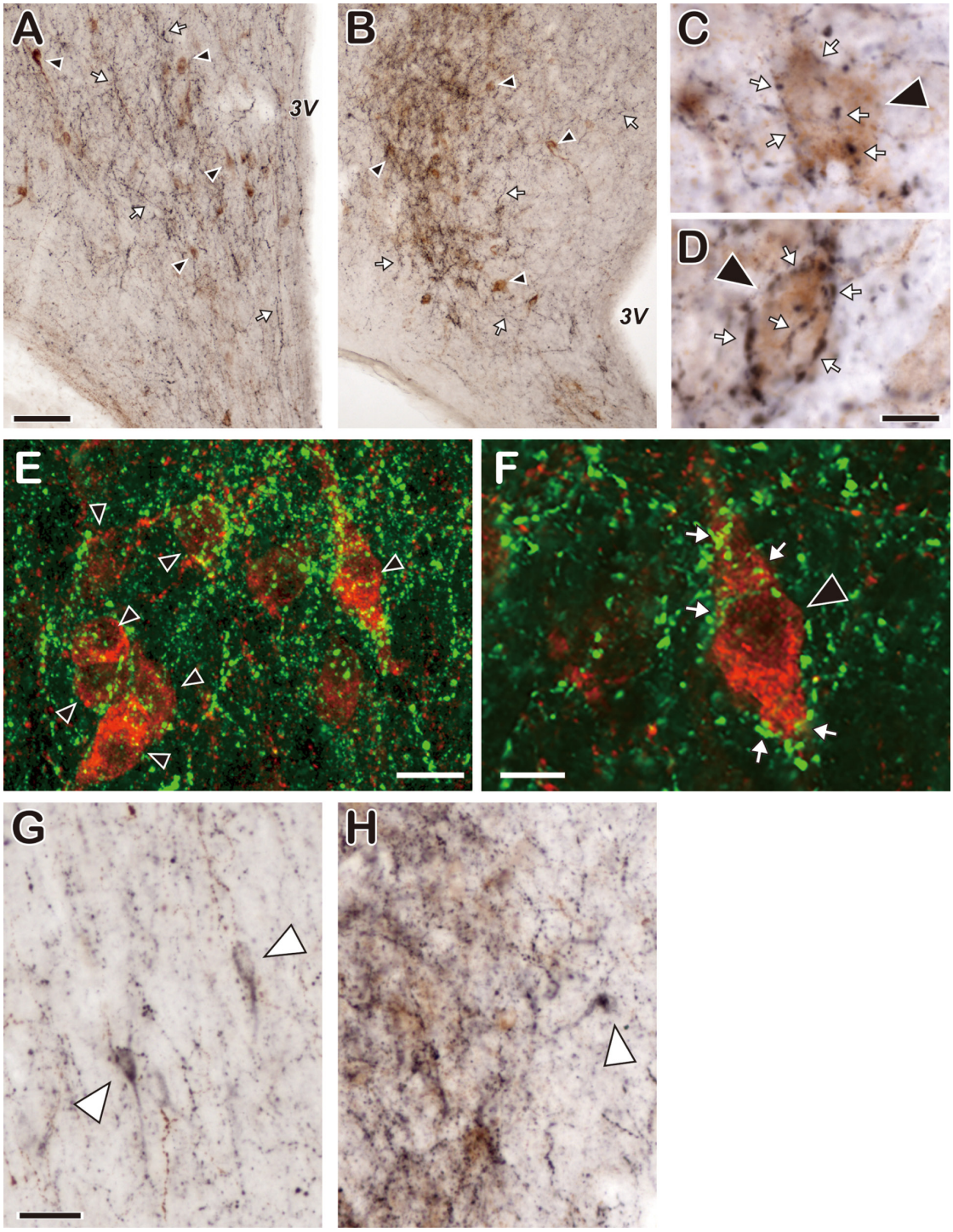

Fig. 4. Photomicrographs showing kisspeptin/substance P (SP) double-labeling immunohistochemistry. (A)–(D), (G), and (H) Light microscope images of diaminobenzidine (DAB)-nickel-stained sections. Kisspeptin and SP immunoreactivities are indicated, respectively, as brown and purple-black reaction products. (E) and (F) Confocal microscope images of fluorescence-stained sections. Kisspeptin and SP immunoreactivities are indicated, respectively, as red and green fluorescence signals. (A) Rostral part of the arcuate nucleus (ARC). (B) Caudal part of the ARC. (C) and (D) Representative images of kisspeptin-immunoreactive (ir) cells surrounded by SP-ir fibers. (E) Stacked image of 15 serial confocal planes. (F) Single 1-μm-thick confocal plane of (E). (G) SP-ir cell somata in the ventromedial nucleus. (H) SP-ir cell soma in the caudal part of the ARC. Closed and open arrowheads represent kisspeptin-ir and SP-ir cell somata, respectively. Arrows indicate SP-ir fibers. Scale bars: (A) and (B), 100 μm; (C), (D), and (F), 10 μm; (E), 20 μm; (G) and (H), 30 μm.

that KNDy neurons do not coexpress SP in OVX goats, which agrees with results in mice [26], monkeys [25], and ewes [27], but not in humans [24], suggesting species differences in the KNDy construct, as postulated previously [24, 25, 27].

Although ARC kisspeptin-ir neurons did not have SP-positive signals in their cell bodies, they were surrounded by a substantial number of fibers with SP immunoreactivity (Fig. 4A, B). Surprisingly, the quantitative analysis revealed that close appositions of SP-ir fibers (Fig. 4C, D) were seen on nearly all (87%) of the kisspeptin-ir cell somata in the rostral-middle parts of the ARC. Confocal microscopy confirmed that SP-ir fibers were in direct contact with kisspeptin-ir cell somata at several points (Fig. 4F). In the caudal part of the nucleus, although dense kisspeptin-ir signals made quantification difficult, it appeared that kisspeptin-ir cells forming the cluster were also surrounded by numerous SP-ir fibers (Supplementary Fig. 5F). Therefore, it is probable that SP-ir fiber apposition is a common anatomical feature of the majority of KNDy neurons. Several neural systems input into KNDy neurons. For example, 17, 13–30, and 32–44% of KNDy neurons are contacted by GnRH-ir [52], NPY-ir [53], and proopiomelanocortin-ir [53] fibers, respectively, in ewes. However, the extent of SP-ir fiber apposition was much higher than those peptides, and equivalent to that of the KNDy fiber-somata apposition [8, 9]. These results provide anatomical evidence that the SP system is heavily involved in the KNDy construct. The source of the SP-ir fibers is unclear. It is unlikely that they arise from KNDy neurons, because they do not contain SP. Populations of SP-ir cells were observed, albeit in relatively low numbers, in several hypothalamic nuclei, such as the VMN (Fig. 4G), PM (Supplementary Fig. 5E), and ARC (Fig. 4H). They may be possible candidates for the SP system associating with KNDy neurons. Because estrogen enhances SP immunoreactivity in the hypothalamus [54] and the number of SP-positive cells is substantially increased by colchicine treatment [55], future studies on estrogen- and colchicine-treated animals may provide further information in this regard.

The physiological function of the SP/KNDy anatomical substrate is completely unknown. NK1R (SP receptor) immunoreactivity has been observed in only 6% of ARC kisspeptin cells, and neither the frequency nor amplitude of LH pulses are affected by an NK1R agonist in anestrus ewes [27]. Similarly, although both NK1R and NK3R (NKB receptor) agonists stimulate GnRH pulse generator activity and the accompanying pulsatile LH secretion, the efficacy of the former is much lower than that of the latter in OVX goats [28]. These results suggest that the contribution of SP/NK1R signaling to GnRH pulse generation may be small or merely supplemental, at least in mature sheep and goats. Alternatively, SP/NK1R signaling in KNDy neurons may play a role in the onset of puberty, because in mice, the maximum expression of *Tac1* and *Tacr1* (that encode SP receptor) is observed at prepubertal periods, and repeatedly treating prepubertal females with an NK1R agonist increases serum LH concentrations and advances the onset of puberty [56].

In conclusion, a polyclonal antiserum (gC2) against the C-terminal residue of goat kisspeptin was successfully produced. Using the colocalization of kisspeptin and NKB immunoreactivity as a marker for KNDy neurons, we mapped the distributions of their cell bodies and fibers in the OVX goat brain. This is the first report describing a detailed map of the KNDy neural system in the ruminant species. Furthermore, the present study demonstrated anatomical evidence for a robust association between the KNDy and SP neural systems in the OVX goat.

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References

1. **Okamura H, Yamamura T, Wakabayashi Y.** Kisspeptin as a master player in the central control of reproduction in mammals: an overview of kisspeptin research in domestic animals. *Anim Sci J* 2013; **84**: 369–381. [Medline] [CrossRef]
2. **Uenoyama Y, Pheng V, Tsukamura H, Maeda K-I.** The roles of kisspeptin revisited: inside and outside the hypothalamus. *J Reprod Dev* 2016; **62**: 537–545. [Medline] [CrossRef]
3. **Mikkelsen JD, Simonneaux V.** The neuroanatomy of the kisspeptin system in the mammalian brain. *Peptides* 2009; **30**: 26–33. [Medline] [CrossRef]
4. **Lehman MN, Merkley CM, Coolen LM, Goodman RL.** Anatomy of the kisspeptin neural network in mammals. *Brain Res* 2010; **1364**: 90–102. [Medline] [CrossRef]
5. **Lehman MN, Coolen LM, Goodman RL.** Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* 2010; **151**: 3479–3489. [Medline] [CrossRef]
6. **Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA.** Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* 2009; **29**: 11859–11866. [Medline] [CrossRef]
7. **True C, Kirigiti M, Ciofi P, Grove KL, Smith MS.** Characterisation of arcuate nucleus kisspeptin/neurokinin B neuronal projections and regulation during lactation in the rat. *J Neuroendocrinol* 2011; **23**: 52–64. [Medline] [CrossRef]
8. **Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CVR, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ.** Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* 2007; **148**: 5752–5760. [Medline] [CrossRef]
9. **Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H.** Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J Neurosci* 2010; **30**: 3124–3132. [Medline] [CrossRef]
10. **Hassaneen A, Naniwa Y, Suetomi Y, Matsuyama S, Kimura K, Ieda N, Inoue N, Uenoyama Y, Tsukamura H, Maeda K-I, Matsuda F, Ohkura S.** Immunohistochemical characterization of the arcuate kisspeptin/neurokinin B/dynorphin (KNDy) and preoptic kisspeptin neuronal populations in the hypothalamus during the estrous cycle in heifers. *J Reprod Dev* 2016; **62**: 471–477. [Medline] [CrossRef]
11. **Ramaswamy S, Seminara SB, Ali B, Ciofi P, Amin NA, Plant TM.** Neurokinin B stimulates GnRH release in the male monkey (*Macaca mulatta*) and is colocalized with kisspeptin in the arcuate nucleus. *Endocrinology* 2010; **151**: 4494–4503. [Medline] [CrossRef]
12. **Hrabovszky E.** Neuroanatomy of the human hypothalamic kisspeptin system. *Neuroendocrinology* 2014; **99**: 33–48. [Medline] [CrossRef]
13. **Karsch FJ.** The hypothalamus and anterior pituitary gland. *In*: Austin CR, Short RV (eds.), Reproduction in mammals, Hormonal control of reproduction 2nd ed, Cambridge: Cambridge University Press; 1984: 1–20.
14. **Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA.** Neurokinin B and the hypothalamic regulation of reproduction. *Brain Res* 2010; **1364**: 116–128. [Medline] [CrossRef]
15. **Okamura H, Tsukamura H, Ohkura S, Uenoyama Y, Wakabayashi Y, Maeda K-I.** Kisspeptin and GnRH pulse generation. *In*: Kauffman AS, Smith JT (eds.), Kisspeptin signaling in reproductive biology. Advances in experimental medicine and biology 784, Springer: Springer; 2013: 297–323.
16. **Goodman RL, Coolen LM, Lehman MN.** A role for neurokinin B in pulsatile GnRH secretion in the ewe. *Neuroendocrinology* 2014; **99**: 18–32. [Medline] [CrossRef]
17. **Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, McMullen NT, Rance NE.** Role for kisspeptin/neurokinin B/dynorphin (KNDy) neurons in cutaneous vasodilatation and the estrogen modulation of body temperature. *Proc Natl Acad Sci USA* 2012; **109**: 19846–19851. [Medline] [CrossRef]
18. **Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, Lai J, Ciofi P, McMullen NT,**

Rance NE. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology* 2012; **153**: 2800–2812. [Medline] [CrossRef]

19. **Higo S, Iijima N, Ozawa H.** Characterisation of *Kiss1r* (*Gpr54*)-expressing neurones in the arcuate nucleus of the female rat hypothalamus. *J Neuroendocrinol* 2017; **29**. [CrossRef]. [Medline]
20. **Clarkson J, dAnglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE.** Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol* 2009; **21**: 673–682. [Medline] [CrossRef]
21. **Desroziers E, Mikkelsen J, Simonneaux V, Keller M, Tillet Y, Caraty A, Franceschini I.** Mapping of kisspeptin fibres in the brain of the pro-oestrous rat. *J Neuroendocrinol* 2010; **22**: 1101–1112. [Medline] [CrossRef]
22. **Almeida TA, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martín JD, Candenas ML.** Tachykinins and tachykinin receptors: structure and activity relationships. *Curr Med Chem* 2004; **11**: 2045–2081. [Medline] [CrossRef]
23. **Lasaga M, Debeljuk L.** Tachykinins and the hypothalamo-pituitary-gonadal axis: An update. *Peptides* 2011; **32**: 1972–1978. [Medline] [CrossRef]
24. **Hrabovszky E, Borsay BÁ, Rácz K, Herczeg L, Ciofi P, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z.** Substance P immunoreactivity exhibits frequent colocalization with kisspeptin and neurokinin B in the human infundibular region. *PLoS ONE* 2013; **8**: e72369. [Medline] [CrossRef]
25. **Kalil B, Ramaswamy S, Plant TM.** The distribution of substance P and kisspeptin in the mediobasal hypothalamus of the male rhesus monkey and a comparison of intravenous administration of these peptides to release GnRH as reflected by LH secretion. *Neuroendocrinology* 2016; **103**: 711–723. [Medline] [CrossRef]
26. **Navarro VM, Bosch MA, León S, Simavli S, True C, Pinilla L, Carroll RS, Seminara SB, Tena-Sempere M, Rønnekleiv OK, Kaiser UB.** The integrated hypothalamic tachykinin-kisspeptin system as a central coordinator for reproduction. *Endocrinology* 2015; **156**: 627–637. [Medline] [CrossRef]
27. **Fergani C, Mazzella L, Coolen LM, McCosh RB, Hardy SL, Newcomb N, Grachev P, Lehman MN, Goodman RL.** Do substance P and neurokinin A play important roles in the control of LH secretion in ewes? *Endocrinology* 2016; **157**: 4829–4841. [Medline] [CrossRef]
28. **Yamamura T, Wakabayashi Y, Ohkura S, Navarro VM, Okamura H.** Effects of intravenous administration of neurokinin receptor subtype-selective agonists on gonadotropin-releasing hormone pulse generator activity and luteinizing hormone secretion in goats. *J Reprod Dev* 2015; **61**: 20–29. [Medline] [CrossRef]
29. **Smith JT.** Kisspeptin signalling in the brain: steroid regulation in the rodent and ewe. *Brain Res Brain Res Rev* 2008; **57**: 288–298. [Medline] [CrossRef]
30. **Kano Y, Sawasaki T, Oyama T.** Biological characteristics of miniature Shiba goats (authors transl). *Jikken Dobutsu* 1977; **26**: 239–246 (In Japanese). [Medline]
31. **Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda K-I, Okamura H.** Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. *J Neuroendocrinol* 2009; **21**: 813–821. [Medline] [CrossRef]
32. **Watson RE Jr, Wiegand SJ, Clough RW, Hoffman GE.** Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* 1986; **7**: 155–159. [Medline] [CrossRef]
33. **Hunyady B, Krempels K, Harta G, Mezey E.** Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. *J Histochem Cytochem* 1996; **44**: 1353–1362. [Medline] [CrossRef]
34. **Zuccolilli GO, Hayashi S, Mori Y.** Hypothalamic structures of the goat on stereotaxic coordinates. *J Vet Med Sci* 1995; **57**: 459–467. [Medline] [CrossRef]
35. **Franceschini I, Yeo SH, Beltramo M, Desroziers E, Okamura H, Herbison AE, Caraty A.** Immunohistochemical evidence for the presence of various kisspeptin isoforms in the mammalian brain. *J Neuroendocrinol* 2013; **25**: 839–851. [Medline] [CrossRef]
36. **Brailoiu GC, Dun SL, Ohsawa M, Yin D, Yang J, Chang JK, Brailoiu E, Dun NJ.** KiSS-1 expression and metastin-like immunoreactivity in the rat brain. *J Comp Neurol* 2005; **481**: 314–329. [Medline] [CrossRef]
37. **Pompolo S, Pereira A, Estrada KM, Clarke IJ.** Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. *Endocrinology* 2006; **147**: 804–810. [Medline] [CrossRef]
38. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A.** Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* 2006; **401**: 225–230. [Medline] [CrossRef]
39. **Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K.** Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev* 2007; **53**: 367–378. [Medline] [CrossRef]
40. **Wakabayashi Y, Yamamura T, Sakamoto K, Mori Y, Okamura H.** Electrophysiological and morphological evidence for synchronized GnRH pulse generator activity among Kisspeptin/neurokinin B/dynorphin A (KNDy) neurons in goats. *J Reprod Dev* 2013; **59**: 40–48. [Medline]
41. **Okamura H, Mori Y.** Multiple-unit activity recording of the gonadotropin-releasing hormone pulse generator. *In*: Armstrong WE, Tasker JG (eds.), Neurophysiology of neuroendocrine neurons. Chichester: John Wiley & Sons, 2014: 323–346.
42. **Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM.** Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (*Macaca mulatta*) as revealed by double immunofluorescence and confocal microscopy. *Endocrinology* 2008; **149**: 4387–4395. [Medline] [CrossRef]
43. **Merkley CM, Porter KL, Coolen LM, Hileman SM, Billings HJ, Drews S, Goodman RL, Lehman MN.** KNDy (kisspeptin/neurokinin B/dynorphin) neurons are activated during both pulsatile and surge secretion of LH in the ewe. *Endocrinology* 2012; **153**: 5406–5414. [Medline] [CrossRef]
44. **Matsuda F, Nakatsukasa K, Suetomi Y, Naniwa Y, Ito D, Inoue N, Wakabayashi Y, Okamura H, Maeda K-I, Uenoyama Y, Tsukamura H, Ohkura S.** The luteinising hormone surge-generating system is functional in male goats as in females: involvement of kisspeptin neurones in the medial preoptic area. *J Neuroendocrinol* 2015; **27**: 57–65. [Medline] [CrossRef]
45. **Bosch MA, Xue C, Rønnekleiv OK.** Kisspeptin expression in guinea pig hypothalamus: effects of 17β-estradiol. *J Comp Neurol* 2012; **520**: 2143–2162. [Medline] [CrossRef]
46. **Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C.** Kisspeptin immunoreactive neurons in the equine hypothalamus Interactions with GnRH neuronal system. *J Chem Neuroanat* 2008; **36**: 131–137. [Medline] [CrossRef]
47. **Krajewski SJ, Burke MC, Anderson MJ, McMullen NT, Rance NE.** Forebrain projections of arcuate neurokinin B neurons demonstrated by anterograde tract-tracing and monosodium glutamate lesions in the rat. *Neuroscience* 2010; **166**: 680–697. [Medline] [CrossRef]
48. **Matsuyama S, Ohkura S, Mogi K, Wakabayashi Y, Mori Y, Tsukamura H, Maeda K, Ichikawa M, Okamura H.** Morphological evidence for direct interaction between kisspeptin and gonadotropin-releasing hormone neurons at the median eminence of the male goat: an immunoelectron microscopic study. *Neuroendocrinology* 2011; **94**: 323–332. [Medline] [CrossRef]
49. **Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Kallo I.** The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. *Eur J Neurosci* 2010; **31**: 1984–1998. [Medline] [CrossRef]
50. **Burke MC, Letts PA, Krajewski SJ, Rance NE.** Coexpression of dynorphin and neurokinin B immunoreactivity in the rat hypothalamus: Morphologic evidence of interrelated function within the arcuate nucleus. *J Comp Neurol* 2006; **498**: 712–726. [Medline] [CrossRef]
51. **Uenoyama Y, Inoue N, Pheng V, Homma T, Takase K, Yamada S, Ajiki K, Ichikawa M, Okamura H, Maeda K-I, Tsukamura H.** Ultrastructural evidence of kisspeptin-gonadotrophin-releasing hormone (GnRH) interaction in the median eminence of female rats: implication of axo-axonal regulation of GnRH release. *J Neuroendocrinol* 2011; **23**: 863–870. [Medline] [CrossRef]
52. **Goodman RL, Hileman SM, Nestor CC, Porter KL, Connors JM, Hardy SL, Millar RP, Cernea M, Coolen LM, Lehman MN.** Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology* 2013; **154**: 4259–4269. [Medline] [CrossRef]
53. **Backholer K, Smith JT, Rao A, Pereira A, Iqbal J, Ogawa S, Li Q, Clarke IJ.** Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells. *Endocrinology* 2010; **151**: 2233–2243. [Medline] [CrossRef]
54. **Okamura H, Yokosuka M, Hayashi S.** Induction of substance P-immunoreactivity by estrogen in neurons containing estrogen receptors in the anteroventral periventricular nucleus of female but not male rats. *J Neuroendocrinol* 1994; **6**: 609–615. [Medline] [CrossRef]
55. **Simerly RB, Gorski RA, Swanson LW.** Neurotransmitter specificity of cells and fibers in the medial preoptic nucleus: an immunohistochemical study in the rat. *J Comp Neurol* 1986; **246**: 343–363. [Medline] [CrossRef]
56. **Simavli S, Thompson IR, Maguire CA, Gill JC, Carroll RS, Wolfe A, Kaiser UB, Navarro VM.** Substance p regulates puberty onset and fertility in the female mouse. *Endocrinology* 2015; **156**: 2313–2322. [Medline] [CrossRef]