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—Original Article—

Identification of feline *Kiss1* and distribution of immunoreactive kisspeptin in the hypothalamus of the domestic cat

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Abstract. In recent years, the *Kiss1* gene has been reported in a number of vertebrate species, and a substantial dataset has been acquired to demonstrate the critical role of kisspeptins in the reproductive system; yet limited information is available for carnivores. In the present study, we identified and characterized feline *Kiss1* by isolating and cloning its full-length cDNA in the domestic cat hypothalamus and caracal testis, using the method of rapid amplification of cDNA ends. Additionally, we isolated and cloned the 3′ end of *Kiss1* cDNA, containing kisspeptin-10 (Kp10), from the ovaries of a clouded leopard and Siberian tiger. Nucleotide sequencing revealed that domestic cat *Kiss1* cDNA is of 711 base pairs and caracal *Kiss1* cDNA is of 792 base pairs, both having an open reading frame of 450 base pairs, encoding a precursor protein *Kiss1* of 149 amino acids. The core sequence of the feline kisspeptin Kp10 was found to be identical in all species analyzed here and is highly conserved in other vertebrate species. Using an anti-Kp10 antibody, we found the immunoreactive kisspeptin to be localized in the periventricular and infundibular nuclei of the cat hypothalamus. The results show that kisspeptin is highly conserved among different feline families, and its immunoreactive distribution in the hypothalamus may indicate its physiological function in the domestic cat.

Key words: Caracal, Clouded leopard, Hypothalamus, Kisspeptin, Kp10, Tiger

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For over a decade now, a growing pool of studies has demonstrated the essential role of kisspeptin in a variety of reproductive processes in mammals. With its central action on GnRH neurons in the hypothalamus, kisspeptin is a potent regulator of puberty onset, fertility, and ovulation processes [1, 2]. Kisspeptin and its receptor Kiss1r (alternatively designated GPR54) have been reported in rodents [3–6], primates [7–9], ungulates [10–12], the musk shrew [13], marsupials [14], fish [15–17], amphibians [18, 19], and one domestic carnivore, the dog [20]. Surprisingly, until just recently, there has been no report on the kisspeptin/Kiss1r signaling system in one of the most common carnivores —the domestic cat [21] .

The domestic cat is an interesting model for studying the ovulation process. Initially described as induced ovulators [22, 23], queens were later reported to also ovulate spontaneously in the absence of cervical stimulation [24, 25]. Indeed, the family *Felidae* includes species with a whole spectrum of reproductive patterns, from almost exclusively induced ovulation (e.g., Siberian tiger [*Panthera tigris altaica*]) to occasional [26] or regular (e.g., clouded leopard [*Neofelis nebulosa*]), spontaneous ovulation; from seasonal to non-seasonal breeders; and from monoestrous to polyestrous ovarian cyclicity [27, 28]. Research into the kisspeptin system of the domestic cat would add important pieces to the puzzle of feline reproduction, while identification of feline *Kiss1* opens up possibilities of developing ovulation induction protocols for wild felids, based on kisspeptin analogs. Such protocols have been successfully developed in humans, sheep, and rodents [29, 30] and show potential to reduce the risk of ovarian hyperstimulation, compared to other triggers [31].

The *Kiss1* gene is translated to prepro-kisspeptin (in human, kisspeptin-145, consisting of 145 amino acids [aa]); the peptide is then proteolytically cleaved, and the C terminal-RFG is amidated by carboxypeptidase to yield kisspeptin-54, designated metastin [32]. Shorter active peptides are also produced, presumably by degradation of the N terminus, and include kisspeptin-14, kisspeptin-13, and kisspeptin-10 (Kp10). All derivatives (kisspeptins) share the same C-terminal 10 aa amidated sequence and belong to the larger family of RF-amide peptides. The 10 aa sequence of Kp10 is essential and sufficient for binding to Kiss1r and activating the kisspeptin/Kiss1r signaling pathways.

Localization of kisspeptin neurons in the hypothalamus is similar in most mammalian species studied: two major populations have been reported, with localization in the preoptic area (POA) or the anteroventral periventricular nucleus (AVPV) in rodents, and in

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Table 1. Sequences of primers used for sequence and expression analyses of feline *Kiss1*, and PCR product sizes

Primer	Primer sequence 5′–3′	Product size (bp)	Use *
fKiss1	Fw: TGG CAC CCA TGG AGA ATC	250	a
	Rv: AGG TCC TTC TCC CGC TGA A		
fKiss1 3′ end	Fw: GAA AAG GTG GCA CCC ATG GAG AAT C	552	b
	RACE kit Rv: GCT GTC AAC GAT ACG CTA CGT AAC G		
Nested fKiss1 3′ end	Fw: GTG CTG GTT CAG CGG GAG AAG GA	308	b
	RACE kit Rv: CGC TAC GTA ACG GCA TGA CAG TG		
fKiss1 5′ end	RACE kit Fw: CGA CTG GAG CAC GAG GAC ACT GA	505	b
	Rv: GGA CAG GTC CTT CTC CCG CTG AA		
Nested fKiss1 5′ end	RACE kit Fw: GGA CAC TGA CAT GGA CTG AAG GAG TA	258	b
	Rv: GAG GAT TCT CCA TGG GTG CCA CCT T		
Beta-actin	Fw: GGA TTT TGA GCA GGA GAT GGC	178	a
	Rv: GTT GAA GGT GGT CTC GTG GAT G		

bp, base pair; Fw, forward; Rv, reverse; RACE kit, primers used from the commercial kit. * a, used for partial sequence analysis and expression; b, used for RACE.

the arcuate nucleus (ARC) or the equivalent infundibular region in primates [5, 10, 13, 33–36]. In the domestic cat hypothalamus atlas, these two areas are designated as the anterior periventricular and infundibular nuclei, respectively [37]. The anterior periventricular nucleus in the cat is a population of small, round or oval cells, which lie along the wall of the third ventricle in the anterior region; the infundibular nucleus in the cat is a prominent, densely packed nucleus within the walls of the infundibular and mammillary recess [37]. Kisspeptin neurons, localized in AVPV, are largely associated with GnRH surge generation and are considered targets for the positive feedback action of estrogen, while kisspeptin neurons in ARC are involved in GnRH pulse generation and are targets of estrogen's negative feedback action [38]. Additional small populations of kisspeptin neurons in the hypothalamus are localized in the ventromedial hypothalamus and paraventricular nucleus and have been suggested to be involved in reproductive behavior [39, 40].

The present study aimed to characterize, for the first time, the feline *Kiss1* and identify the localization of immunoreactive kisspeptin in the hypothalamus of the domestic cat.

Materials and Methods

Animals and tissue collection

Hypothalamus tissues were collected opportunistically *post mortem* from free-ranging mature female domestic cats that died accidentally and were delivered to the pathology department of the Small Animal Teaching Hospital, Chulalongkorn University. Upon collection, three female domestic cats were in an inactive stage of the estrous cycle, identified by ovarian gross morphology (follicle diameter ≤ 1 mm, no corpus luteum), and one female domestic cat was on day 25–28 of gestation, according to the crown-lump length of a fetus [41].

Gonads of wild felids were collected opportunistically *post mortem* from Khao Kheow Open Zoo. Testes were collected from a caracal (*Caracal caracal*) male, aged 9 years; ovaries were collected from a Siberian tiger (*Panthera tigris altaica*) multiparous female, aged 10 years, and from a clouded leopard (*Neofelis nebulosa*) multiparous female, aged 9 years.

All collected tissues were delivered to the laboratory on ice within 24 h, and either fixed in 4% paraformaldehyde for immunohistochemical analysis, or plunged into liquid nitrogen for RNA isolation.

RNA isolation and cDNA synthesis

Up to 30 mg of each tissue was homogenized in homogenization tubes (tissue grinding CKMix 2 ml, Bertin Technologies, Montigny-le-Bretonneux, France) in 100 µl of RNA lysis buffer (RNeasy Mini Kit, Qiagen, Germany) at 5000 rpm for 3 × 30 sec (Minilys® personal homogenizer, Bertin Technologies). Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with DNase (RQ1 RNase-Free DNase, Promega, USA). The Nanodrop ND-2000 (Wilmington, Delaware, USA) was used to assess the concentration and purity of isolated RNA. Additional control of RNA quality and integrity was performed *via* microfluidic analysis using an Agilent 2100 Bioanalyzer (Agilent Genomics, USA); RNA integrity number (RIN) values were from 8.0 to 9.3 for the domestic cat, 8.2 for the caracal, 8.6 for the Siberian tiger, and 5.4 for the clouded leopard. For RT-PCR, 1–2.5 µg of isolated RNA was reverse transcribed into single-stranded (ss) complementary DNA (cDNA) using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcriptase was not added to the negative control to verify the absence of genomic DNA contamination.

Amplification of partial feline Kiss1

All primers used were purchased from Ward Medic Ltd (Bangkok, Thailand). For PCR, primers for feline (f) *Kiss1* were designed based on the sequence deduced from the comparison of the predicted *Felis catus Kiss1* gene sequence (XM_023247458.1) and published *Canis lupus familiaris* (KJ512885.1), *Homo sapiens* (NM_002256.3), *Bos taurus* (AB466319.1), *Sus scrofa* (AB466320.1), *Mus musculus* (AB666166.1), and *Rattus norvegicus* (NM_181692.1) *Kiss1* gene sequences listed in the NCBI database, using Primer3 open-source software version 4.0.0 [41]. Primers for feline beta-actin were designed based on the *Felis catus* gene sequence listed in the NCBI database

(AB051104.1). Primer information is listed in Table 1. Based on cat ss cDNA templates of hypothalamic origin, the partial cDNA sequence of f*Kiss1* was amplified using AmpliTaq Gold® 360 DNA Polymerase with a built-in hot start, using 360 GC Enhancer solution (Applied Biosystems, CA, USA). The same primers and polymerase kit were used to amplify f*Kiss1* partial cDNA sequences from ss cDNA templates of wild felids' gonads. For all samples, the PCR conditions were 95°C for 10 min; 40 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, and elongation at 72°C for 45 sec; and finally, elongation at 72°C for 7 min.

Rapid amplification of cDNA ends and molecular cloning of feline Kiss1

To obtain full-length 5′ and 3′ ends, the total mRNA isolated from the hypothalamic tissue of mature female cats in the inactive stage of the estrous cycle, and from the testis tissue of the mature male caracal, was used for adaptor ligation and cDNA synthesis using a GeneRacer® Kit with AMV RT (Invitrogen). The total mRNA isolated from the ovarian tissue of the mature female clouded leopard and mature female Siberian tiger was used to obtain the 3′ end of *Kiss1* cDNA.

Gene-specific primers for rapid amplification of cDNA ends (RACE) PCR were designed based on the partial cDNA sequence of f*Kiss1* obtained here (see Table 1), and were used for all feline species in this study. The cDNA templates of the 5′ and 3′ ends were used for amplification using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). The PCR conditions were 94°C for 2 min; 40 cycles of denaturation at 95°C for 45 sec, annealing at 68°C for 45 sec, and elongation at 68°C for 60 sec; and final elongation at 68°C for 10 min. For nested PCR, the conditions were 94°C for 2 min; 25 cycles of denaturation at 95°C for 45 sec, annealing at 65°C for 30 sec, and elongation at 68°C for 60 sec; and final elongation at 68°C for 10 min.

Purified PCR products (QIAquick Gel Extraction Kit, Qiagen) were ligated to the TOPO-TA vector and transfected in One Shot™ TOP10 cells (both Invitrogen). For each animal, six positive clones per 5′ end and 3′ end of f*Kiss1* and partial f*Kiss1* were sequenced by First BASE Laboratories Sdn Bhd (Selangor, Malaysia).

Sequence analysis

Sequences were analyzed using CLC Sequence Viewer 7 (Qiagen). The cDNA sequences of f*Kiss1* were analyzed using the BLAST algorithm on the NCBI web site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame (ORF) sequence of f*Kiss1* was predicted by the ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The signal peptide and the neuropeptide prohormone cleavage sites were predicted using SignalP 4.1 [42], ProP 1.0 [43], and Neuropred software [44]. Phosphorylation sites were predicted using NetPhos 3.1, with the threshold set at 0.7 (http://www.cbs.dtu.dk/services/NetPhos/). Multiple sequence alignment was performed using ClustalW [45], and the phylogenetic tree was constructed by MEGA 7.0.26 using the neighbor-joining method [46]. All online analyses adopted the default parameters, unless otherwise stated.

For the phylogenetic tree, representatives of several orders and different kisspeptin isoforms were used. GenBank accession numbers of the sequences and signal peptide information are as follows: *Sus scrofa, Artiodactyla* (*Kiss1*, NP_001128436.1); *Felis catus, Carnivora* (*Kiss1*, MG564501); *Ovis aries, Artiodactyla* (*Kiss1*, NP_001293033.1); *Capra hircus, Artiodactyla* (*Kiss1*, NP_001272639.1); *Equus caballus, Perissodactyla* (NP_001276073.1); *Homo sapiens, Primates* (*Kiss1*, NP_002247.3); *Mus musculus*, *Rodentia* (*Kiss1*, NP_839991.2); *Rattus norvegicus*, *Rodentia* (NP_859043.1); *Mesocricetus auratus, Rodentia* (NP_001268497.1); *Canis lupus familiaris, Carnivora* (predicted *Kiss1*, XP_013966762.1); *Suncus murinus, Eulipotyphla* (*Kiss1*, BAL02985.1); *Monodelphis domestica, Didelphimorphia* (*Kiss1*, DAA06347.1); *Xenopus tropicalis*, *Anura* (*Kiss1a*, ACJ50538.1; *Kiss1b*, ACJ50539.1; *Kiss2*, ACJ50540.1), and *Danio rerio*, *Cypriniformes* (*Kiss1*, NP_001106961.1; *Kiss2*, NP_001136057.1).

Immunohistochemistry

The localization of kisspeptin protein was assessed by rabbit polyclonal antibody for Kp10 (AB9754, Millipore, MA, USA), which was previously validated on domestic cat ovary and uterus tissues [21].

Hypothalamus tissue of two mature female cats in the inactive stage of the estrous cycle and one mature cat on day 25–28 of gestation, fixed in 4% paraformaldehyde, was dehydrated, embedded in paraffin following standard procedures, and sectioned at 4 μm thickness. Sectioned tissue was mounted on gelatin-coated microscope slides (LabServ, Auckland, New Zealand), deparaffinized in xylene (Riedel-de-Haën, Seelze, Germany), and rehydrated in decreasing concentrations of ethanol before being rinsed in PBS (pH 7.4). The antigenic sites were enhanced by incubation in 0.1% trypsin at 37°C for 30 min. Thereafter, endogenous peroxidase activity was blocked by incubating the sections for 30 min in 3% H_2O_2 in methanol, followed by incubation with normal goat serum (S-1000, Vector Laboratories, Burlingame, CA, USA) for 1 h at 25°C to reduce nonspecific binding of the first antibody. Sections were then incubated overnight with 1:500 diluted anti-Kp10 antibody at 4°C. After being labeled with their primary antibodies, sections were washed with PBS-Tween 0.1% (Calbiochem, Darmstadt, Germany) and incubated with biotinylated goat anti-rabbit antibody for 30 min at 25°C (BA1000, Vector Laboratories). The immunoperoxidase color reaction was developed using an avidin-biotin immunoperoxidase kit (VECTASTAIN Elite ABC Kit, Vector Laboratories) with diaminobenzidine substrate chromogen solution (ImmPACT™ DAB peroxidase substrate, Vector Laboratories). The sections were counterstained with hematoxylin (Bio-Optica, Milan, Italy), dehydrated in increasing concentrations of ethanol, and covered with mounting medium (DePeX, BDH Laboratory Supplies, Poole, UK) and coverslips. Histologic evaluation was performed under a BX51 light microscope fitted with a DP73 digital camera (both Olympus, Tokyo, Japan). The cytoarchitectonic atlas in the Horsley-Clarke coordinate system of the domestic cat hypothalamus was used to determine the section areas [37].

To verify the specificity of anti-Kp10, the primary antibody was preabsorbed with 10 μg of metastin (445888, Millipore) at 4°C overnight, which resulted in the absence of immunoreaction, except in the optic chiasm (Fig 5-C, 5-F). Negative controls for anti-Kp10 included (1) substitution of the primary antibody with PBS and (2) substitution of the primary antibody with normal rabbit IgG (NI01, Millipore); no staining was detected in either of them.

Results

Characterization of Felis catus Kiss1

Initially, a partial fragment of 277 bp was cloned from the hypothalamus cDNA using two primers, fKiss1-fw and fKiss1-rev (Table 1). Thereafter, the unknown ends of the cDNA were cloned via 5′ and 3′ RACE. Overlapping sequences of all fragments yielded a full-length cDNA of *F. catus Kiss1* of 711 bp (GenBank accession number: MG564501) containing a 5′ UTR of 134 bp, a 3′ UTR of 127 bp, and an ORF of 450 bp, encoding a precursor protein *Kiss1* of 149 aa with a predicted signal peptide of 17 aa, a kisspeptin decapeptide, and a 2 aa proteolytic cleavage site, KR (Figs. 1 and 2). Analysis of the overall aa sequence of the kisspeptin precursor in BLAST revealed the highest similarity of *F. catus Kiss1* with the following predicted sequences: 90%, Pacific walrus (XP_004392920.1); 87%, giant panda (XP_002918259.1); 86%, ferret (XP_004756445.1); and 84%, Hawaiian monk seal (XP_021542248.1). The core sequence of kisspeptin, Kp10, was highly conserved and showed high sequence homology with other mammalian and non-mammalian species: 100%, mouse (NP_839991.2); 100%, pig (NP_001128436.1); 100%, western clawed frog (NP_001156331.1); 90%, musk shrew (BAL02985.1); 90%, human (NP_002247.3); and 90%, dog (XP_013966762.1). The *F. catus Kiss1* ORF sequence obtained here experimentally, differed from the predicted *F. catus Kiss1* ORF sequence (XM_023247458.1) in one nucleotide substitution, i.e., guanine was substituted with cytosine in the experimental *F. catus Kiss1* at nucleotide position 445 of the ORF. This resulted in the substitution of alanine for proline in the last position of the predicted kisspeptin precursor.

Characterization of Caracal caracal Kiss1

Initially, a partial fragment of 277 bp was cloned from the testis cDNA using two primers, fKiss1-fw and fKiss1-rev (Table 1). Thereafter, the unknown ends of cDNA were cloned via 5′ and 3′ RACE. Overlapping sequences of all fragments yielded a full-length cDNA of *C. caracal Kiss1* of 792 bp (GenBank accession number: MH638298) containing a 5′ UTR of 205 bp, a 3′ UTR of 137 bp, and an ORF of 450 bp, encoding a precursor protein *Kiss1* of 149 aa with a predicted signal peptide of 17 aa, a kisspeptin decapeptide, and a 2 aa proteolytic cleavage site, KR (Fig. 3A). Alignment of the aa sequence of the *C. caracal* kisspeptin precursor with the *F. catus* kisspeptin precursor revealed no differences (Fig. 3B).

Comparison of 3′ ends of feline Kiss1

Partial cDNA sequences were cloned and sequenced for *Panthera tigris altaica Kiss1* (GeneBank accession number: MH638299) and *Neofelis nebulosa Kiss1* (GeneBank accession number: MH638300). The predicted partial kisspeptin precursors covered the region from 32–149 aa of *F. catus Kiss1*. Alignment of aa sequences revealed one substitution of alanine for serine in *N. nebulosa Kiss1* at position 65 aa (position based on full feline *Kiss1*). The core aa sequence Kp-10 showed no differences between the felids analyzed (Fig. 3B).

Localization of immunoreactive kisspeptin

Immunohistochemical analysis revealed the presence of kisspeptin-immunoreactive cell bodies and fibers in the hypothalamus of two mature female cats in the inactive stage of the estrous cycle and one mature cat on day 25–28 of gestation (schematic illustration, Fig. 4). Four populations of kisspeptin-immunoreactive cell bodies were identified in the coronal sections of female cats: small population of faintly stained cells in the amygdaloid complex (Amg; Fig. 5A), large populations in the anterior periventricular nucleus (Pea; Fig. 5B), in periventricular nucleus tubular component (Pet; Figs. 5D, 5E) and the largest population in the infundibular nucleus (Figs. 5D, 5E). Anterior regions of Pea displayed some immunoreactive fibers, but no immunoreactive cells (Fig. 5A). However, more posterior regions of Pea displayed dense populations of immunoreactive fibers and cells along the third ventricle, with fibers spreading into the dorsal hypothalamic area (Fig. 5B). Populations of both immunoreactive fibers and cells, were present in the infundibular nucleus, with density increasing in the posterior region (Figs. 5D, 5E). Immunoreactive fibers and cells were distributed in Pet along the third ventricle, with fibers spreading into the dorsomedial nucleus (Figs. 5D, 5E). No immunoreactive fibers were observed in the Amg or other hypothalamic areas.

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  1  GTGAGCCCTGGAGCCCAGCTCCCCTCTCTGTGCGTGTTCATCAA 44
 45 CCCAAGAAGCACCTGGAGACCCACGGAGCTGCTACCCGGCCGGAC 89
 90 AGAGCCAAGCCTCAGGGCACTGTCAGGTCCTGTCTTCTCACCAGG 134
135 ATGAACTCGCTGGTTTCTTGGCAGCTGATGCTTTTCCTCTGTGCC 179
      M  N  S  L  V  S  W  Q  L  M  L  F  L  C  A

180 ACCTCCTTCAGGGAGACATTTGAAAAGGTGGCACCCATGGAGAAT 224
      T  S  F  R  E  T  F  E  K  V  A  P  M  E  N

225 CCTCTATCTACAGGCCAGCGGCTCGGATCCCAGGCCCTCCTGGCC 269
      P  L  S  T  G  Q  R  L  G  S  Q  A  L  L  A

270 CCGTGGGAGCAGAGCCCGCGATGCGCGGAGAGAAAGCCCGCCGGG 314
      P  W  E  Q  S  P  R  C  A  E  R  K  P  A  G

315 GCCCAGCCCAACGCGCGGGGGGCCTCGCTGTGCCCTCCTCCCGAG 359
      A  Q  P  N  A  R  G  A  S  L  C  P  P  P  E

360 AGTGCCGCGGGGCCCCAGCGGCCAGGCCTGTGCGCCCCCCGCAGC 404
      S  A  A  G  P  Q  R  P  G  L  C  A  P  R  S

405 CGCCTGATCCCCGCCCCGCGGGGCGCGGTGCTGGTTCAGCGGGAG 449
      R  L  I  P  A  P  R  G  A  V  L  V  Q  R  E

450 AAGGACCTGTCCGCCTACAACTGGAACTCCTTCGGCCTGCGCTAC 494
      K  D  L  S  A  Y  N  W  N  S  F  G  L  R  Y

495 GGGAAGCGGCAGACCGCCCCTCCCGGAAGCGTCCGCGGGGGGCGC 539
      G  K  R  Q  T  A  P  P  G  S  V  R  G  G  R

540 TGCGCTTGGCTGAGCGCCAGGTGCGGGATGGGGGCGGGGCCATGA 584
      C  A  W  L  S  A  R  C  G  M  G  A  G  P  *

585 ATTTCAAACCCCAGACTAGGCGTCTGAGCTGAGGGTGGGGGGTGG 629
630 AGGGTAGGGCAGTGGGAGGGGGACTGGGGCTTCTGTCCTGAAACA 674
675 ATAAAGGAAATGCTGCCCAAAAAAAAAAAAAAAAAAA         712
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Fig. 1. The nucleotide sequence and the deduced amino acid sequence of *Felis catus Kiss1*. The stop codon is indicated by an asterisk (*), and the start codon (ATG) is bold. The predicted sequence of the signal peptide is underlined. The kisspeptin-10 amino acid sequence is boxed. The predicted serine phosphorylation sites are marked with a diamond, and the predicted threonine phosphorylation sites, with a circle. The predicted cleavage site is indicated by double underlining. The GenBank Accession Number is MG564501.

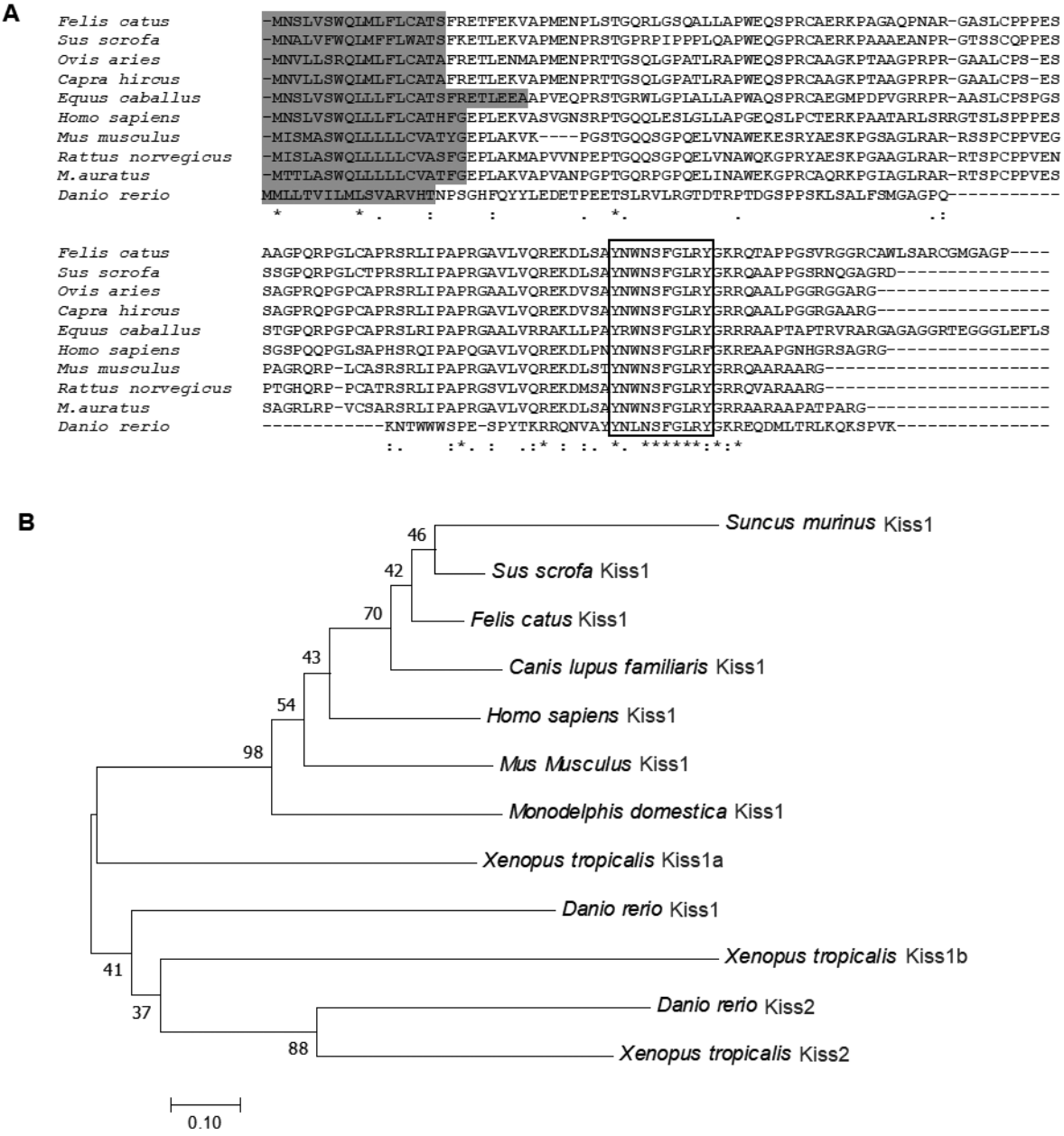

Fig. 2. Comparison of amino acid sequences (A) and phylogenetic analysis (B) of kisspeptin precursors from different species. Sequences were aligned by the ClustalW program. (A) Identical and similar amino acid residues are marked with asterisks and dots, respectively. Gaps (indicated by hyphens) are introduced in some sequences to maximize alignment. The signal peptides are shaded in grey. The box shows the kisspeptin-10 that is especially conserved throughout the species. (B) The phylogenetic tree was constructed by MEGA 7.0.26 using the neighbor-joining method with 1000 bootstrap replicates. The number shown at each branch indicates its bootstrap value (%). The evolutionary distances were computed using the Poisson correction method and are in the unit of the number of amino acid substitutions per site. For the phylogenetic tree, representatives of several orders and different kisspeptin isoforms were used; GenBank accession numbers are provided in Materials and Methods.

Discussion

To the best of our knowledge, this is the first report on the *Kiss1* sequence and hypothalamic kisspeptin localization in the family *Felidae*. In this study, we cloned and analyzed the full-length feline *Kiss1* cDNA and investigated the distribution of immunoreactive kisspeptin in the hypothalamus of the domestic cat. All immunohistochemical analyses were performed on female tissues; the existence of sexual dimorphism in feline kisspeptin hypothalamic distribution is possible, but was not investigated in this study.

The length of the feline kisspeptin precursor was found to be 149 aa, compared to 145 aa in humans [32] and 126 aa in mice [42].

Interestingly, we could predict only two cleavage sites in feline *Kiss1*: one signal peptide cleavage site between positions 17 and 18 aa, and one propeptide cleavage site at position 121–122 KR, which would result in a predicted 103 aa mature peptide. In previously reported species, generation of the mature kisspeptin requires proteolytic processing by members of the proprotein convertase family of proteases, majorly furin in humans and monkeys, with cleavage sites at the specific dibasic residues [43, 44]. Such proteolytic processing would result in, e.g., 54 aa, 52 aa, and even 29 aa mature kisspeptins in humans, mice, and musk shrews, respectively [3, 13, 43]. Feline *Kiss1* may have a cleavage site at the 66 R position, which would result in a 54 aa mature peptide; however, the prediction score was way below the threshold, with only 0.27 cleavage potential. Indeed, experimental investigation is needed to determine the true proteolytic processing mechanism of feline *Kiss1*.

The alignment analysis, which included feline *Kiss1,* revealed that the Kp10 sequence is highly conserved throughout vertebrates, as reported previously [18]. In the generated phylogenetic tree, feline *Kiss1* was included in the cluster with *Kiss1* of other mammals and showed clear segregation from *Kiss2*. The current hypothesis of kisspeptin phylogeny proposes that *Kiss1* and *Kiss2* are paralogous to each other and have arisen as a result of gene duplication at the locus level before the emergence of lampreys, probably due to the whole genome duplication of the ancestral vertebrate [45]. *Kiss2* was presumably lost after the divergence of placental and marsupial mammals from monotremes. However, both genes are reported to be present in platypuses, lampreys, clawed frogs, elephant sharks, zebrafish, medakas, and goldfish [15, 18, 46]. The single *Kiss1* has been so far reported in the families of *Hominidae, Cercopithecidae*, *Suidae*, *Bovidae*, *Equidae*, *Soricidae*, *Canidae*, *Muridae*, *Cricetidae, Caviidae*, and *Didelphidae*. The feline *Kiss1* presented here, puts the *Felidae* in the same group as these reports and further supports the proposed evolutionary history of kisspeptin.

Representatives of the different feline genera in our study (*Felis*, *Caracal*, *Panthera*, and *Neofelis*) with different ovulatory patterns (almost exclusively induced or spontaneous), all shared an identical Kp10 sequence and an identical 3′ end of *Kiss1*, except for one substitution that was observed in the clouded leopard. In addition, the absence of a predicted dibasic residue for proteolytic processing was noted. Such a highly conserved structure of *Kiss1* in *Felidae*

A

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  1                      GTGCCCTCCACTCAGTGAAACTCCT 25
 26 TGCCCTGCTTCGCTTCACCCCTCCCTCCTGGGTGCCACACACGAC 70
 71 CCCCCCCCCCCCGCCTCCAGGGCTTTCCTCTTCACACTGGGCAGC 115
116 ACCCTGCCCATGCCCTGCGCCTGACCTAGTCTCCCCTCCTTCCTT 160
161 CTATCTCAGCCTCAGGGCACTGTCAGGTCCTGTCTTCTCACCAGG 205
206 ATGAACTCGCTGGTTTCTTGGCAGCTGATGCTCTTCCTCTGTGCC 250
     M  N  S  L  V  S  W  Q  L  M  L  F  L  C  A

251 ACCTCCTTCAGAGAGACATTTGAAAAGGTGGCACCCATGGAGAAT 295
     T  S  F  R  E  T  F  E  K  V  A  P  M  E  N

296 CCTCTATCTACAGGCCAGCGGCTCGGATCCCAGGCCCTCCTGGCC 340
     P  L  S  T  G  Q  R  L  G  S  Q  A  L  L  A

341 CCGTGGGAGCAGAGCCCGCGATGCGCGGAGAGAAAGCCCGCCGGG 385
     P  W  E  Q  S  P  R  C  A  E  R  K  P  A  G

386 GCCCAGCCCAACGCGCGGGGGGCCTCGCTGTGCCCTCCTCCCGAG 430
     A  Q  P  N  A  R  G  A  S  L  C  P  P  P  E

431 AGTGCCGCGGGGCCCCAGCGGCCAGGCCTGTGCGCCCCCCGCAGC 475
     S  A  A  G  P  Q  R  P  G  L  C  A  P  R  S

476 CGCCTGATCCCCGCCCCGCGGGGCGCGGTGCTGGTTCAGCGGGAG 520
     R  L  I  P  A  P  R  G  A  V  L  V  Q  R  E

521 AAGGACCTGTCCGCCTACAACTGGAACTCCTTCGGCCTGCGCTAC 565
     K  D  L  S  A  Y  N  W  N  S  F  G  L  R  Y

566 GGGAAGCGGCAGACCGCGCCTCCCGGAAGCGTCCGCGGGGGGCGC 610
     G  K  R  Q  T  A  P  P  G  S  V  R  G  G  R

611 TGCGCTTGGCTGAGCGCCAGGTGCGGGATGGGGGCGGGGGCATGA 655
     C  A  W  L  S  A  R  C  G  M  G  A  G  A  *
656 ATTTCAAACCCCAGACTAGGCGTCTGAGCTGGGGGTGGGGGGTGG 700
701 AGGGTAGGGCAGTGGGAGGGGGACTGGGGCTTCTGTCCTGAAACA 745
746 ATAAAGGAAATGCTGCCCAAAAAAAAAAAAAAAAAAAAAAAAAA 791
792 AA                                           794
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B

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Neofelis nebulosa   ---------------------------------LSTGQRLGSQALLAPWEQSPRCAERKPAGAQPNSRGASLCPPPESA
Panthera tigris a.  ---------------------------------LSTGQRLGSQALLAPWEQSPRCAERKPAGAQPNARGASLCPPPESA
Caracal caracal     MNSLVSWQLMLFLCATSFRETFEKVAPMENPLSTGQRLGSQALLAPWEQSPRCAERKPAGAQPNARGASLCPPPESA
Felis catus         MNSLVSWQLMLFLCATSFRETFEKVAPMENPLSTGQRLGSQALLAPWEQSPRCAERKPAGAQPNARGASLCPPPESA
                                                    ********************************:************

Neofelis nebulosa   AGPQRPGLCAPRSRLIPAPRGAVLVQREKDLSAYNWNSFGLRYGKRQTAPPGSVRGGRCAWLSSRRGMGAGA
Panthera tigris a.  AGPQRPGLCAPRSRLIPAPRGAVLVQREKDLSAYNWNSFGLRYGKRQTAPPGSVRGGR--------------
Caracal caracal     AGPQRPGLCAPRSRLIPAPRGAVLVQREKDLSAYNWNSFGLRYGKRQTAPPGSVRGGRCAWLSARCGMGAGA
Felis catus         AGPQRPGLCAPRSRLIPAPRGAVLVQREKDLSAYNWNSFGLRYGKRQTAPPGSVRGGRCAWLSARCGMGAGP
                    ************************************************************
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Fig. 3. The nucleotide sequence and the deduced amino acid sequence of *Caracal caracal Kiss1* (A) and comparison of 3′ end of kisspeptin in feline species (B). (A) The stop codon is indicated by an asterisk (*), and the start codon (ATG) is bold. The predicted sequence of the signal peptide is underlined. The kisspeptin-10 amino acid sequence is boxed. The predicted serine phosphorylation sites are marked with a diamond, and the predicted threonine phosphorylation sites, with a circle. The predicted cleavage site is indicated by double underlining. The GenBank Accession Number is MH638298. (B) Sequences were aligned in ClustalW program. Identical and similar amino acid residues are marked with asterisks and dots, respectively. Gaps are indicated by hyphens. The box shows the kisspeptin-10 that is especially conserved throughout the species. GenBank accession numbers are provided in Results.

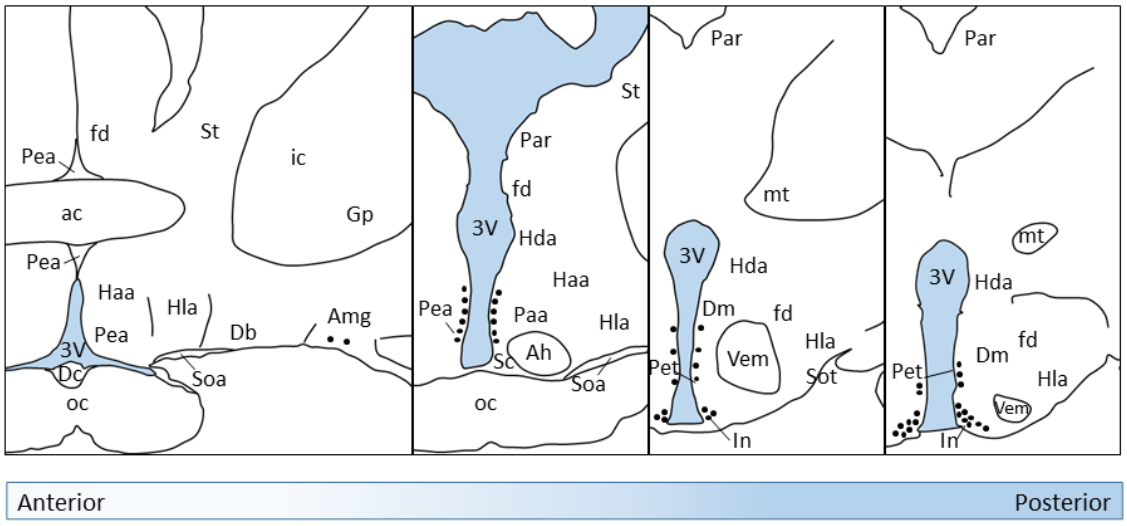

Fig. 4. Schematic illustration of the distribution of kisspeptin immunoreactive neurons in the hypothalamus of intact adult queens. Dots indicate cell bodies labeled with Kp-10 antibody. Shaded area represents the third ventricle (3V). ac, anterior commissure; Ah, anterior hypothalamic nucleus; Amg, amygdaloid complex; Db, nucleus of the diagonal band of Broca; Dc, dorsal chiasmatic nucleus; Dm, dorsomedial nucleus; fd, descending column of the fornix; Gp, globus pallidus; Haa, anterior hypothalamic area; Hda, dorsal hypothalamic area; Hla, lateral hypothalamic area; In, infundibular nucleus; ic, internal capsule; mt, mammillothalamic tract; oc, optic chiasm; Paa, paraventricular nucleus, anterior component; Par, paraventricular nucleus of the thalamus; Pea, anterior periventricular nucleus; Pet, periventricular nucleus, tuberal component; Sc, suprachiasmatic nucleus; Soa, supraoptic nucleus, anterior component; Sot, supraoptic nucleus, tuberal component; St, bed nucleus of the stria terminalis; Vem, ventromedial nucleus. The cytoarchitectonic atlas in the Horsley-Clarke coordinate system of the domestic cat hypothalamus was used to determine the section areas [37].

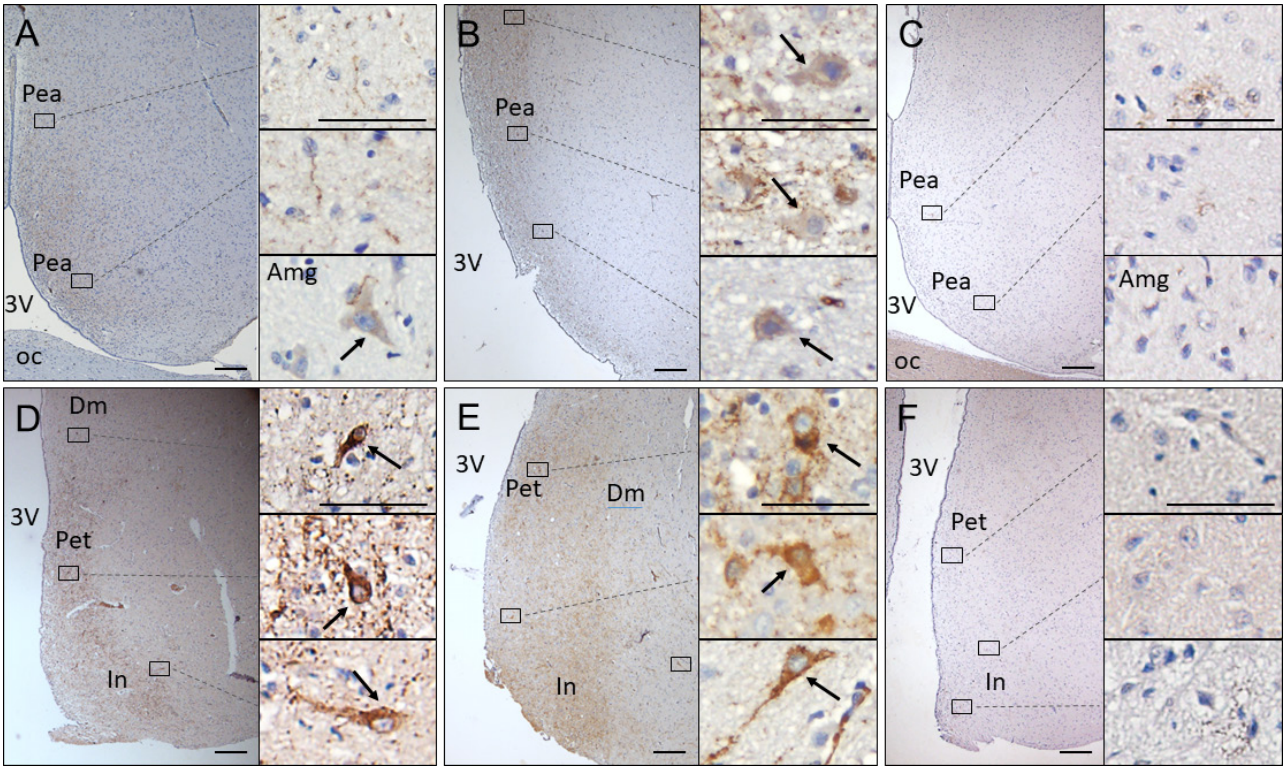

Fig. 5. Distribution of immunoreactive kisspeptin in the hypothalamus of the domestic cat. Kisspeptin immunoreactive cell bodies were identified in amygdaloid complex (Amg; A), anterior periventricular nucleus (Pea; B), periventricular nucleus, tuberal component (Pet; D, E) and infundibular nucleus (In; D, E). Immunoreactive fibers were scattered in the areas of Pea, Pet, In and dorsomedial nucleus (Dm). Black arrows indicate immunoreactive cell bodies. A preabsorption test revealed nonspecific staining in the oc and a minimal area in the Pea (C, F). The cytoarchitectonic atlas in the Horsley-Clarke coordinate system of the domestic cat hypothalamus was used to determine the section areas [37]. 3V, third ventricle. Scale bars: 200 µm, 50 µm in magnification boxes.

promises easier transfer of kisspeptin ovulation induction protocols developed in domestic cats, to wild felids [47].

We found two major distribution sites of kisspeptin-expressing neuron cell bodies and fibers in the hypothalamus of the domestic cat: the periventricular nucleus, equivalent to AVPV, and the infundibular nucleus, equivalent to ARC. This is consistent with reports on other mammals, including both spontaneous and induced ovulators: mice [48–50], rats [40, 51], hamsters [52], guinea pigs [6], sheep [12, 36], pigs [10], goats [53], monkeys [54, 55], humans [55, 56], and musk shrews [13]. The kisspeptin population in ARC is considered to be the largest group of kisspeptin cells in the mammalian hypothalamus [39]. The kisspeptin neuron population on the rostral level has been demonstrated to be involved in the estrogen-induced LH surge in many species [57]. In particular, estradiol stimulates *Kiss1* expression in the AVPV, while inhibiting it in the ARC [57]. Inhibition of *Kiss1* expression by steroids has been demonstrated specifically in the ARC of the mouse [58], Syrian hamster [59], ewe [12], and human [55]. However, in certain species, ARC is involved in both negative and positive feedback control of gonadotropins [5]. As demonstrated here, the distribution of immunoreactive kisspeptin neuron bodies and fibers in the periventricular and infundibular regions suggests the potential role of kisspeptin in gonadotropin regulation in the domestic cat, as in other studied species. Additional small population of kisspeptin–immunoreactive cell bodies was found in the amygdaloid complex in the anterior regions of the hypothalamus. In rodents, kisspeptin neuronal population was identified in medial amygdala and was shown to be upregulated by sex steroids [60]. The amygdaloid complex in the domestic cat is not the same area as medial amygdala in rodents, and the potential function of kisspeptin neurons in this region in the cat is unclear. Kisspeptin-immunoreactive fibers were also localized in dorsomedial nucleus and dorsal hypothalamic area, similar to humans [56] and rats [51].

In conclusion, based on gene structure, phylogenetic relations, and hypothalamic distribution, *Kiss1* may have an important physiological role in reproductive processes in felids, as it does in other species studied. Our study introduces the basis for further investigation into kisspeptin action in the domestic cat, providing the core sequence of *Kiss1* for development of ovulation induction protocols.

Conflict of interests: The authors declare no conflicts of interest.

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