

Identification of a novel kisspeptin with high gonadotrophin stimulatory activity in the dog.

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Abstract

Kisspeptin (kiss1) and its receptor (kiss1r) are essential for normal reproductive function in many species, but the role of kiss1/kiss1r signalling in the dog has not yet been elucidated. The aims of this study were to identify the canine *kiss1* and *kiss1r* genes and to determine gonadotrophin and oestradiol stimulatory activity of KP-10, the shortest biologically active form of KISS1. Canine *kiss1* and *kiss1r* genes were localised by

comparing the reference dog genome with relevant human cDNA sequences, using BLASTn software. The amino acid sequence of canine KP-10 (YNWNVFGLRY) differs at two positions from human KP-10 (YNWNSFGLRF). A single bolus of canine KP-10 was administered intravenously to anoestrous Beagle bitches in dosages of 0, 0.1, 0.2, 0.3, 0.5, 1, 5, 10, and 30 µg/kg. Blood samples were collected before and after canine KP-10 administration for measurement of plasma LH (all doses), FSH and oestradiol (1 to 30 µg/kg). From 0.2 µg/kg onward, canine KP-10 resulted in a rapid and robust rise in plasma LH concentration (maximum at 10 min). KP-10 also resulted in a rapid and robust rise in plasma FSH concentration (maximum at 10–20 min). Plasma oestradiol concentration increased significantly after dosages of 1, 5, and 10 µg/kg and reached a maximum at 60-90 min. In conclusion, canine KP-10 is a potent kisspeptin which elicits robust gonadotrophin and oestradiol responses in anoestrous bitches, suggesting that canine kiss1/kiss1r are cogent targets for modulating reproduction in dogs.

Keywords

Luteinizing Hormone, Follicle Stimulating Hormone, kiss1, kiss1r, GPR54, canine, bitch, reproduction

1. Introduction

Kisspeptins are a family of structurally related neuropeptides, encoded by the *kiss1* gene. The initial product of the human *KISS1* gene is a 145 amino acid peptide (KP145) that is cleaved into shorter peptides having a common C-terminal RF-amide, such as KP54, KP14, KP13, and KP-10. The kisspeptins are the ligands for the G-protein-coupled receptor KISS1R (also known as GPR54). All kisspeptins show the same affinity of binding to the receptor, indicating that the C-terminal end is responsible for binding and activation [1-4].

Kisspeptins are potent stimulators of GnRH-producing neurons and together with their receptor, *kiss1r*, they are required for normal functioning of the hypothalamic-pituitary-gonadal (HPG) axis. Loss-of-function mutations in the *KISS1R* gene and in the *KISS1* gene, demonstrated in humans and mice, lead to isolated hypogonadotropic hypogonadism (IHH), caused by deficient GnRH secretion and consequently deficient pituitary secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This results in impairment of both pubertal maturation and reproductive function [3,5-10]. The relevance of kisspeptin signalling is further underlined by the identification of activating mutations of the *KISS1R* and *KISS1* genes in children with precocious puberty, due to premature activation of GnRH secretion [11,12]. Central (intracerebroventricular) or peripheral (intravenous, subcutaneous, or intraperitoneal) administration of different types of kisspeptin stimulates gonadotrophin secretion in several mammalian species, including humans [13-18]. Furthermore, intracerebroventricular administration of kisspeptin antagonists prevents the preovulatory LH surge in rats and blocks the post-castration rise in circulating LH in mice, rats, and sheep [19,20]. Additionally, kisspeptins are involved in the feedback regulation of the HPG axis by gonadal steroids [4,17,21-23]. Based on these observations, kisspeptin operates proximally (upstream) to GnRH and the *kiss1/kiss1r* system is thus regarded as the gatekeeper of

reproductive function. Kisspeptin is thus an interesting target for therapeutic interventions in reproduction, such as induction of ovulation and puberty. Furthermore, via the inhibition of kisspeptin signalling, non-surgical oestrus prevention may be achieved.

The reproductive cycle of the domestic bitch (*Canis lupus familiaris*) is complex and differs from that of most other mammalian species. A follicular phase with spontaneous ovulations is followed by a luteal phase lasting about 2 months, almost irrespective of whether the bitch is pregnant, and a non-seasonal anoestrus of 2-10 months [24].

The role of kiss1/kiss1r system in the HPG axis in dogs has not been determined. Since the kiss1/kiss1r system is not operative in some species (e.g., chicken and zebra finch) it is important to establish its functionality in dogs. The presence of a functional kiss1/kiss1r system in the dog may lead to new therapeutic opportunities, such as non-surgical contraception, oestrus prevention, and ovulation induction. The aim of the present study was therefore to identify the existence of the canine *kiss1* and *kiss1r* genes and to determine whether the system is operative in the dog. We report here the identification and primary sequence of the canine *kiss1* and *kiss1r* genes and the effects of different dosages of intravenously administered canine KP-10 on the gonadotropic hormones and oestradiol in anoestrous bitches.

2. Materials and methods

2.1 Identification of canine kiss1 and kiss1r genes

The canine *kiss1* and *kiss1r* genes were localised by comparing the reference dog genome build 2.2 with the relevant human cDNA sequences using BLASTn software [25]. The Genbank accession of human cDNA was NM_002256 for *KISS1* and NM_032551 for

KISSIR. In addition, we analysed the DNA sequence of bacterial artificial chromosome (BAC) clones selected from the chromosomal region of *KISSIR* using the UCSC genome browser (<http://genome.ucsc.edu>). The BAC clones CH82-333G1, CH82-325G21, and CH82-156G16 were obtained from CHORI (<http://bacpac.chori.org/library.php?id=253>) and used to complete the coding DNA sequences of the canine *kissIr* gene. BAC DNA was isolated using the HiSpeed® Plasmid Maxi Kit (Qiagen). A standard PCR using primers 5'-GACCTCAAGCCTCCACTGTC-3' and 5'-CGAGTTGCTGTAGGACATGC-3' was performed with BAC DNA as a template. These primers bridge a DNA sequence missing from the reference genome build 2.2 and were designed using high quality trace files of the dog genome project (<http://www.ncbi.nlm.nih.gov/Traces/trace.cgi>). These and additional primers 5'-CATCTGGGGAGTGGGCTCAA-3' and 5'-GAGGAGGGAGGAGGCAAGGT-3' were used in the subsequent tercycle reactions with BigDye v3.1 according to the protocol of the manufacturer (Applied Biosystems). The reaction products were purified by ethanol precipitation in the presence of EDTA and analysed on a 3130xl Genetic Analyzer (Applied Biosystems).

The splice sites of *kissI* were identified by first blocking out repetitive DNA sequences from the chromosome region of the gene using RepeatMasker (<http://www.repeatmasker.org>), followed by prediction of the splice sites using NNSPLICE 0.9, available at http://www.fruitfly.org/seq_tools/splice.html [26].

2.2 *Animals, experimental design, and collection of blood samples*

Six healthy Beagle bitches, with a median age of 70 months (range 21-111 months), and a median weight of 12.7 kg (range 11-18.2 kg) were used for the first part of the study (dosages of 1-30 µg/kg canine KP-10: high dosage group). Afterwards another group of six

healthy Beagle bitches, with a median age of 36 months (range 16-95 months), and a median weight of 13.0 kg (range 12-15.8 kg) were used for the second part of this study (dosages of 0-1 µg/kg canine KP-10: low dosage group). All were born and raised in the Department of Clinical Sciences of Companion Animals and were accustomed to the laboratory environment and procedures such as the collection of blood. They were housed in pairs in indoor–outdoor pens, fed a standard commercial dog food once daily, and provided with water *ad libitum*.

All dogs were examined thrice weekly for swelling of the vulva and serosanguineous vaginal discharge, signifying the onset of pro-oestrus. Plasma progesterone concentration was measured thrice weekly from the start of pro-oestrus until it exceeded 13-16 nmol/l, at which time ovulation is assumed to occur [27-29]. Anoestrus was defined as the period from 100 days after ovulation to the onset of pro-oestrus, indicated by vulvar swelling and serosanguineous discharge.

Canine KP-10 was administered as a single bolus via the cephalic vein at weekly intervals during anoestrus. Blood samples were collected from the jugular vein by repeated venipuncture directly into heparinised tubes at 40 and 0 min before and at 10, 20, 30, 40, 60, 90, and 120 min after canine KP-10 administration. In the high dosage group, LH and FSH concentrations were measured in all plasma samples and the oestradiol concentration in samples collected before and at 20, 60, 90, and 120 min after canine KP-10. To determine whether the plasma gonadotrophin and oestradiol response was influenced by the time in anoestrus and whether there was a cumulative effect of repeated administration of different doses of canine KP-10, three bitches received canine KP-10 starting at 30 µg/kg (23.1 nmol/kg) and then decreasing at weekly intervals to 10, 5, 1, and 0 µg/kg (7.7, 3.8, 0.77 and 0 nmol/kg respectively), and three bitches received canine KP-10 in the reverse order starting with 0 µg/kg and then increasing to 1, 5, 10, and 30 µg/kg.

Next, lower dosages of canine KP-10 were used to determine the lowest canine KP-10 dose causing a significant LH response. Dosages of 0, 0.2, 0.3, 0.5 and 1 µg/kg (0, 0.15, 0.23, 0.38 and 0.77 nmol/kg, respectively) were administered as a single bolus via the cephalic vein during anoestrus and blood samples for determination of plasma LH concentrations were collected from the jugular vein by repeated venipuncture directly into heparinised tubes at 40 and 0 min before and at 10, 20, 30, 40, 60, 90, and 120 min after canine KP-10 administration.

2.3 *Peptides*

Canine KP-10 (YNWNVFGLRYNH₂) was produced by Eurogentec (Maastricht, The Netherlands) with a purity of 99.5%, dissolved in saline, and divided into individual doses in glass vials which were stored at -20°C and then thawed at room temperature on the day of use.

2.4 *Hormone measurements*

Plasma progesterone concentration was measured thrice weekly during the follicular phase to determine the ovulation period, using a ¹²⁵I radioimmunoassay (RIA) previously validated for ovulation timing [30]. The intra-assay and interassay coefficients of variation (CVs) were 6% and 10.8%, respectively, and the limit of quantitation was 0.13 nmol/L.

Plasma LH concentration was measured with a heterologous RIA as described previously [31,32]. The intra-assay and interassay CVs for values above 0.5 µg/L were 2.3% and 10.5%, respectively, and the limit of quantitation was 0.3 µg/L.

Plasma FSH was measured by immunoradiometric assay (IRMA) (AHROO4, Biocode SA, Liège, Belgium), according to the manufacturer's instructions and as described previously by Beijerink et al. (2007) [33]. The intra-assay and interassay CVs were 3.0% and 6.0%, respectively. The lower limit of quantitation was 0.5 µg/L.

Plasma oestradiol-17β was measured by RIA (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA) according to the manufacturer's instructions with modifications as described previously [34] and validated for the dog [35]. The intra-assay and interassay CVs were 14% and 11.8%, respectively. The lower limit of quantitation was 7 pmol/L.

2.5 *Data analysis*

Basal plasma LH, FSH, and oestradiol concentrations were calculated for each dog as the mean of the values at -40 and 0 min before canine KP-10 administration. The mean of all plasma LH concentrations measured before administration of canine KP-10 was also calculated. All LH values before KP-10 administration that exceeded this overall mean plus three SD were ascribed to pulsatile secretion and were treated as outliers and excluded from statistical analysis.

The area under the curve (AUC) for LH, FSH, and oestradiol was calculated from 0 to 120 min after administration of canine KP-10. Differences in AUCs between different dosages of canine KP-10 were analysed by a two-way ANOVA followed by post hoc Student Newman Keuls test.

Plasma LH, FSH, and oestradiol responses to different doses of canine KP-10 were analysed by nonparametric tests because not all data were normally distributed. A Friedmann test followed by a post hoc Dunnett's test was used to determine differences in plasma LH, FSH, and oestradiol concentration before and after canine KP-10 administration.

The influence of the order of administered doses of canine KP-10 was analysed by a Mann-Whitney U test. $P < 0.05$ was considered significant.

2.6 *Ethics of experimentation*

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Utrecht University.

3 **Results**

3.1 *In silico* identification of the canine *kiss1* and *kiss1r* genes

The chromosomal location of the canine *kiss1* gene was identified by a similarity search of the dog genome with the human cDNA sequence. Two closely situated fragments on chromosome 38 of the dog are highly similar to parts of human cDNA. The region containing these fragments is flanked by orthologs of *REN* and *GOLTIA*, which also flank human *KISS1*, confirming the identity of the canine gene. The alignment of human cDNA with the region on CFA38 was incomplete because of a lack of similarity between parts of the DNA sequences. The two highly similar fragments are respective parts of the two coding exons of the gene. The first part contains the putative start codon of the gene, the second part the putative KP-10 coding sequence. The putatively complete *kiss1* coding sequence was identified by prediction of the intron between the two coding exons and by localization of the first stop codon in frame with the KP-10 coding sequence. The splice donor site was predicted at nucleotide position 103 from the translation start codon and the predicted length of the intron was 1,738 nucleotides.

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Canine  1  MNSLVSQWQLMLLLCATSFRETLIKVAPMETPGPAGQRLGAQALPAPWERSPP-----
          ||||| | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Human   1  MNSLVSQWQLLLFLCATHFGEPLIKVASVGNSRPTGQQLESLLAPGEQSLPCTERKPAAT

Canine  53  -----RAPQRHLM PARRGA-----DLPAYNWNV FGLRYG
          ||      ||  ||      || | | | | | | | | | |
Human   62  ARLSRRGTSLSPPESSGSPQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRFG

Canine  82  RRRAATPGLRGGTPSPRLRVPVWGWLGLRS
          | | | | |
Human  123  KREAA-PGNHGRSAGRG

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Fig. 1. The alignment of canine and human preprokisspeptin. Identical residues are marked by vertical lines and the sequences of canine and human KP-10 are shown in the gray area.

The predicted mRNA of preprokisspeptin encodes a peptide of 111 amino acids (Fig. 1). The amino acid sequence of canine KP-10 differs from that of human KP-10 at positions 5 and 10. The amino acid sequence is YNWNVFGLRY in canine KP-10 and YNWN SFGLRF in human KP-10.

A portion of the canine *kiss1r* cDNA sequence had already been predicted from the reference genome build 2.2 (Genbank accession XM_850105.1). Because of a gap in the reference genome sequence that overlapped with exon 4 and intron 4, it was not possible to predict the correct mRNA sequence. By DNA sequence analysis of BAC clones from the region, exon 4 could be completed and it fully aligned with the corresponding part of the human cDNA sequence of *KISS1R*. The DNA sequence traces obtained with one of the primers showed a sudden loss of signal, indicating the presence of DNA elements that hamper the processivity of the DNA polymerase of the BigDye terminator kit (data not shown). Hence we were not able to complete the DNA sequence of intron 4. The unconserved 3'-part of the gene was based on the GNOMON prediction of Genbank. The predicted mRNA for canine *KISS1R* encodes a protein of 397 amino acid residues (Fig. 2). The identity of amino acid sequences between the canine and the human *KISS1R* is 75% and it increases to 90% when the unconserved C-terminal domain is ignored.

3.2 *Stimulation tests with canine KP-10 in the high dosage group*

The control saline administered at T=0 did not alter plasma LH, FSH, and oestradiol concentrations, whereas all dosages of canine KP-10 in the high dosage group resulted in significant increases in all three hormones measured (Fig. 3 and Table 1). There was no difference in the LH, FSH, and oestradiol responses between the dogs in which the weekly dosage of canine KP-10 increased and those in which it decreased.

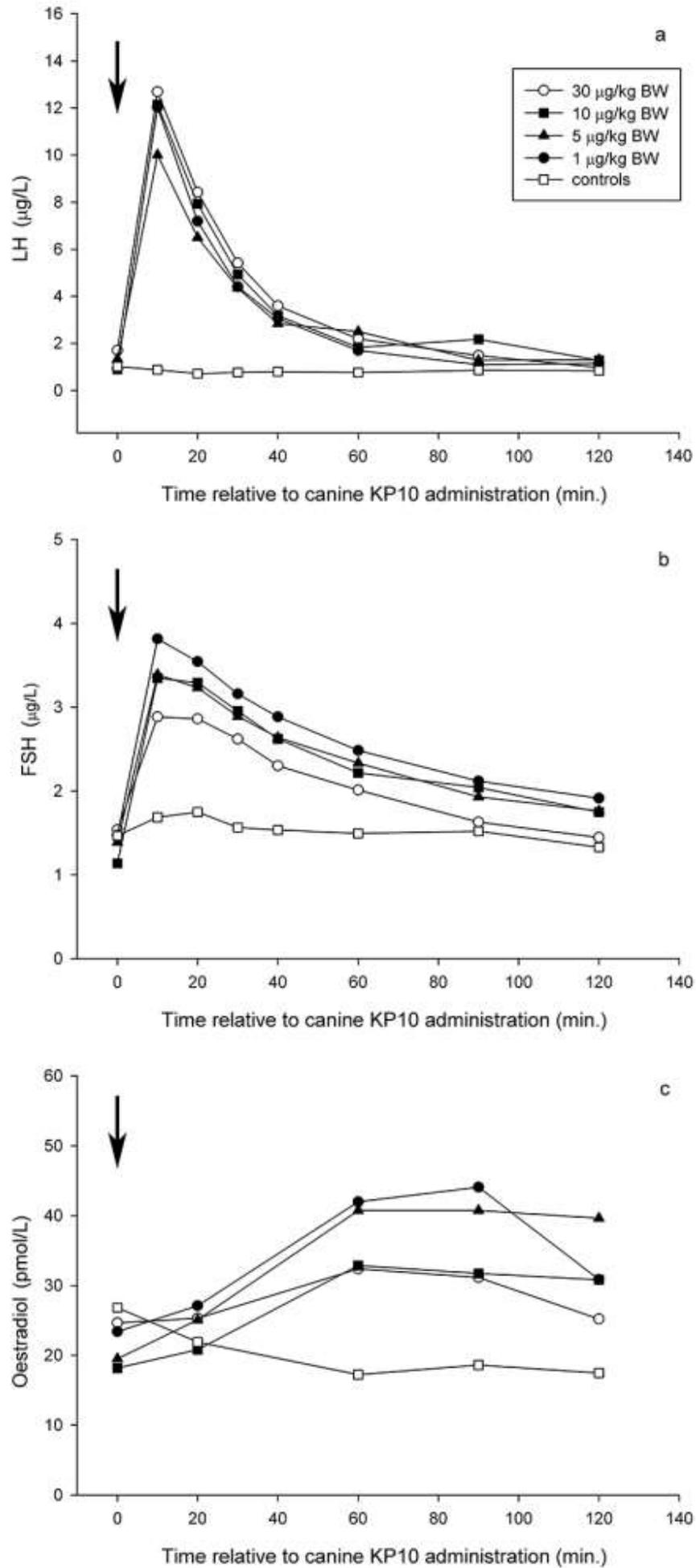


Fig. 3. Median plasma concentrations of LH (3a), FSH (3b), and oestradiol (3c) after intravenous administration (arrow) of different doses of canine KP-10 to six anoestrous bitches in the high dosage group

Table 1. Plasma LH, FSH and oestradiol concentrations before and after intravenous administration of different doses of canine KP-10. T=time in min after intravenous administration of canine KP-10.

| Hormone | Dose | Median Basal | Range | Median T=10 | Range | Median T=20 | Range | Median T=30 | Range |
|---------------------|------|--------------------|--------------|--------------------|--------------|--------------------|--------------|---------------------|--------------|
| LH (µg/L) | 0 | 1.0 | 3.3-1.9 | 1.0 | 0.5-1.1 | 0.72 | 0.4-1.0 | 0.70 | 0.4-1.0 |
| LH (µg/L) | 1 | 1.0 | 0.6-1.2 | 11.0 | 7.4-18.9 | 7.0 | 3.9-11.4 | 4.1 | 2.9-6.9 |
| LH (µg/L) | 5 | 0.83 | 0.7-1.0 | 9.2 | 5.8-16.9 | 6.1 | 3.5-10.9 | 3.8 | 2.7-6.6 |
| LH (µg/L) | 10 | 0.89 | 0.8-1.1 | 11.4 | 5.6-21.8 | 7.8 | 3.7-14.7 | 4.3 | 2.7-9.4 |
| LH (µg/L) | 30 | 1.4 | 0.9-2.2 | 11.9 | 5.7-26.2 | 8.0 | 3.7-14.3 | 5.2 | 2.6-10.9 |
| FSH (µg/L) | 0 | 1.6 | 1.1-2.0 | 1.3 | 1.0-1.7 | 1.3 | 1.1-1.7 | 1.3 | 0.9-1.6 |
| FSH (µg/L) | 1 | 1.3 | 1.1-2.1 | 3.9 | 2.3-4.9 | 3.4 | 2.3-4.6 | 3.0 | 2.0-4.2 |
| FSH (µg/L) | 5 | 1.4 | 1.0-1.9 | 3.3 | 1.9-4.5 | 3.3 | 1.7-4.2 | 3.0 | 1.7-3.6 |
| FSH (µg/L) | 10 | 1.1 | 0.8-1.7 | 3.3 | 1.6-5.3 | 3.5 | 1.7-4.9 | 3.0 | 1.6-4.6 |
| FSH (µg/L) | 30 | 1.3 | 0.7-2.2 | 3.3 | 1.7-5.7 | 3.2 | 1.6-5.5 | 2.8 | 1.4-5.1 |
| Oestradiol (pmol/L) | 0 | 25.1 | 13.0-38.4 | n/a | n/a | 18.4 | 7.0-37.3 | n/a | n/a |
| Oestradiol (pmol/L) | 1 | 21.7 | 14.1-36.5 | n/a | n/a | 27.1 | 15.2-38.6 | n/a | n/a |
| Oestradiol (pmol/L) | 5 | 13.1 | 7.0-57.1 | n/a | n/a | 22.6 | 7.0-49.9 | n/a | n/a |
| Oestradiol (pmol/L) | 10 | 16.3 | 11.8-32.5 | n/a | n/a | 18.1 | 15.9-36.8 | n/a | n/a |
| Oestradiol (pmol/L) | 30 | 21.3 | 8.0-54.4 | n/a | n/a | 21.0 | 7.3-53.8 | n/a | n/a |
| | | Median T=40 | Range | Median T=60 | Range | Median T=90 | Range | Median T=120 | Range |
| LH (µg/L) | 0 | 0.84 | 0.5-0.9 | 0.82 | 0.5-0.9 | 0.94 | 0.6-1.1 | 0.81 | 0.6-1.1 |
| LH (µg/L) | 1 | 2.9 | 2.1-4.4 | 1.5 | 1.3-2.4 | 1.0 | 0.9-1.6 | 1.1 | 0.8-1.9 |
| LH (µg/L) | 5 | 2.6 | 1.9-4.0 | 1.8 | 1.4-6.2 | 1.2 | 0.9-2.1 | 0.90 | 0.7-2.6 |
| LH (µg/L) | 10 | 3.1 | 1.9-5.1 | 1.8 | 1.1-2.4 | 1.1 | 0.9-7.6 | 1.3 | 0.8-1.9 |
| LH (µg/L) | 30 | 3.2 | 2.2-6.6 | 2.2 | 1.5-3.3 | 1.2 | 0.9-3.1 | 1.1 | 0.7-5.7 |
| FSH (µg/L) | 0 | 1.3 | 1.1-1.6 | 1.3 | 1.1-1.6 | 1.2 | 1.0-1.6 | 1.1 | 1.1-1.5 |
| FSH (µg/L) | 1 | 2.7 | 2.0-3.7 | 2.3 | 1.7-3.4 | 2.0 | 1.7-2.9 | 1.9 | 1.4-2.6 |
| FSH (µg/L) | 5 | 2.8 | 1.5-3.2 | 2.3 | 1.4-3.4 | 1.9 | 1.2-2.8 | 1.7 | 1.3-2.4 |
| FSH (µg/L) | 10 | 2.8 | 1.5-3.9 | 2.3 | 1.4-3.4 | 2.0 | 1.2-3.0 | 1.8 | 1.2-2.2 |
| FSH (µg/L) | 30 | 2.3 | 1.3-4.6 | 2.0 | 1.3-3.8 | 1.8 | 1.1-3.0 | 1.5 | 0.9-2.6 |
| Oestradiol (pmol/L) | 0 | n/a | n/a | 11.9 | 9.0-31.1 | 12.9 | 10.8-33.9 | 10.5 | 7.0-36.9 |
| Oestradiol (pmol/L) | 1 | n/a | n/a | 38.4 | 34.6-55.1 | 42.5 | 32.4-59.2 | 29.2 | 12.8-56.2 |
| Oestradiol (pmol/L) | 5 | n/a | n/a | 37.9 | 29.7-63.1 | 35.6 | 26.8-67.4 | 27.2 | 21.6-49.2 |
| Oestradiol (pmol/L) | 10 | n/a | n/a | 31.2 | 21.5-55.8 | 29.1 | 18.7-49.7 | 30.5 | 18.8-43.7 |
| Oestradiol (pmol/L) | 30 | n/a | n/a | 31.2 | 17.1-51.5 | 30.6 | 20.7-50.0 | 26.2 | 10.6-44.3 |

Peak plasma LH concentrations occurred at 10 min after canine KP-10 administration at all doses. The mean maximal LH responses were 13-fold over basal plasma LH concentrations for 1 µg/kg, 12-fold for 5 µg/kg, 14-fold for 10 µg/kg, and 10-fold for 30 µg/kg. Plasma LH concentrations had returned to basal levels at 40 min after every dose of canine KP-10.

The maximal FSH response was usually observed at 10 min after canine KP-10 administration but in dogs 1-3 it was at 20 min with every dosage. The mean maximal FSH responses were 3-fold over basal plasma FSH concentrations for all dosages. Plasma FSH concentrations had returned to basal levels at 60 min after 1 µg/kg canine KP-10 and at 40 min after 5, 10, and 30 µg/kg.

Maximal plasma oestradiol concentrations were reached at 60 min after administration of 5 and 10 µg/kg canine KP-10 and at 90 min after 1 µg/kg. After the administration of 30 µg/kg canine KP-10 there was no significant increase in plasma oestradiol concentration; 1 dog showed no evident oestradiol response, but in the other 5 dogs the response was similar to that with other dosages. The mean maximal oestradiol responses were 2-fold over basal concentrations for dosage of 1 and 10 µg/kg and 3-fold for 5 µg/kg. Plasma oestradiol concentrations returned to basal levels at 120 min after 1 µg/kg canine KP-10, but still differed from basal levels after 5 and 10 µg/kg canine KP-10 administration.

The AUCs of plasma LH and FSH concentrations were significantly higher than controls after canine KP-10 administration at all doses, but the differences in AUCs between dosages were not significant. The AUCs of plasma oestradiol concentrations after canine KP-10 administration were significantly higher than controls at 1, 5, and 10 µg/kg (Fig. 4). There were no significant differences in AUCs of oestradiol between the dosages.

3.3. *Stimulation tests with canine KP-10 in the low dosage group*

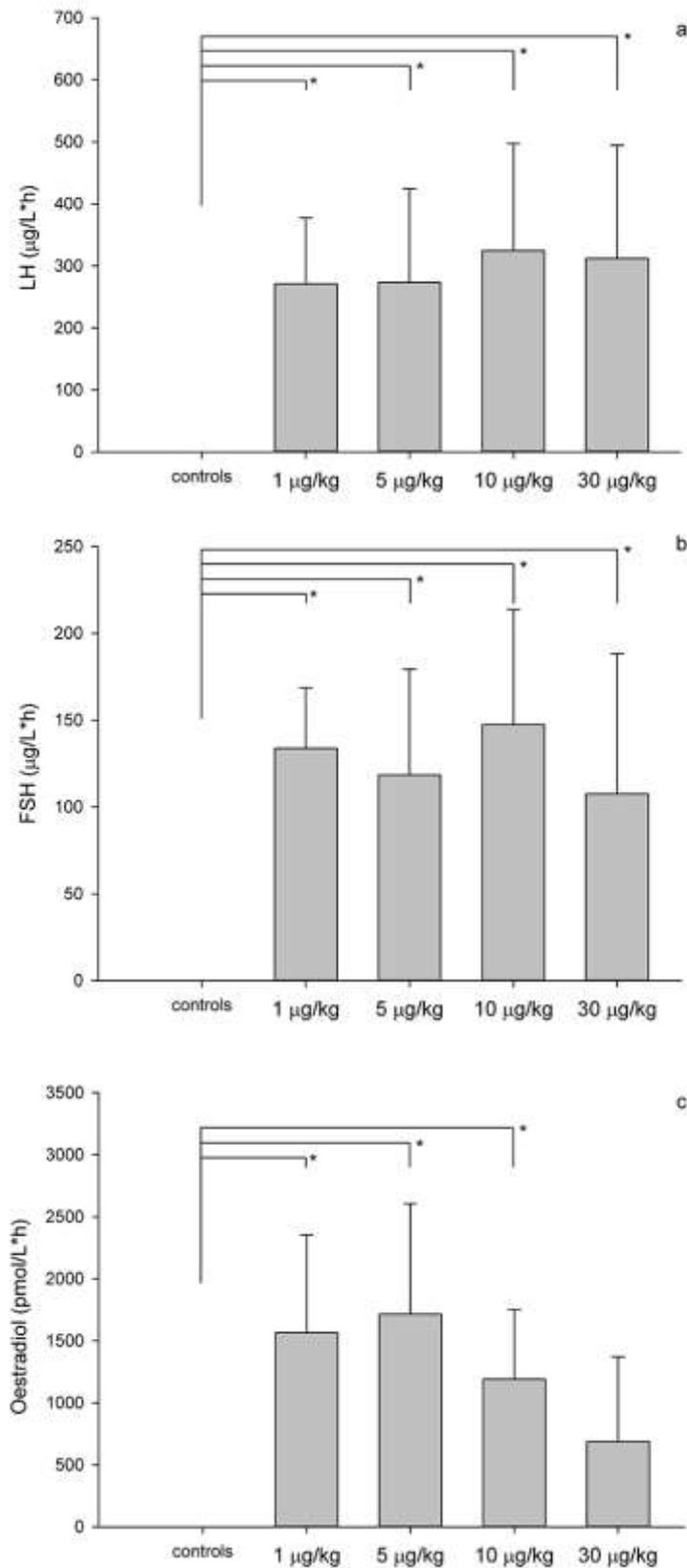


Fig. 4. Mean AUC of plasma concentrations of LH (4a), FSH (4b), and oestradiol (4c) after intravenous administration of different doses of canine KP-10 to six anoestrous bitches in the high dosage group. * indicates a significant difference from controls.

Table 2. Plasma LH concentrations before and after intravenous administration of different doses of canine KP-10 in the low dosage group. T=time in min after intravenous administration of canine KP-10.

| Dose | Median Basal | Range | Median T=10 | Range | Median T=20 | Range | Median T=30 | Range |
|-------------|---------------------|--------------|--------------------|--------------|--------------------|--------------|---------------------|--------------|
| 0 | 2.6 | 1.5-3.4 | 2.6 | 1.8-3.3 | 2.6 | 1.8-3.4 | 2.3 | 1.9-3.4 |
| 0.1 | 2.1 | 1.5-3.0 | 3.3 | 1.8-7.7 | 2.9 | 1.4-5.8 | 2.6 | 1.6-4.6 |
| 0.2 | 2.4 | 1.6-3.2 | 9.6 | 2.1-13.8 | 7.3 | 1.8-11.9 | 5.6 | 1.5-8.5 |
| 0.3 | 2.9 | 1.7-4.8 | 12.0 | 8.8-15.0 | 8.2 | 5.7-13.0 | 5.4 | 3.7-7.4 |
| 0.5 | 2.2 | 1.9-3.3 | 13.1 | 8.3-20.8 | 8.4 | 5.8-12.3 | 5.8 | 4.1-8.6 |
| 1.0 | 2.6 | 1.6-3.1 | 15.0 | 12.2-20.4 | 8.8 | 7.9-12.9 | 5.5 | 5.4-7.4 |
| Dose | Median T=40 | Range | Median T=60 | Range | Median T=90 | Range | Median T=120 | Range |
| 0 | 2.2 | 1.7-7.0 | 2.3 | 1.7-7.8 | 2.3 | 1.5-3.7 | 2.2 | 1.5-3.5 |
| 0.1 | 2.6 | 1.9-4.0 | 2.3 | 1.5-10.2 | 2.1 | 1.5-4.7 | 2.4 | 1.7-5.8 |
| 0.2 | 4.7 | 1.7-7.0 | 3.3 | 1.5-5.2 | 3.2 | 1.9-6.6 | 2.9 | 1.5-3.1 |
| 0.3 | 3.6 | 2.8-6.4 | 3.1 | 1.8-5.4 | 2.3 | 1.7-3.5 | 2.2 | 1.6-4.1 |
| 0.5 | 4.5 | 3.5-6.5 | 3.4 | 2.2-10.1 | 3.1 | 1.6-4.4 | 2.6 | 1.8-3.4 |
| 1.0 | 4.5 | 4.1-5.5 | 2.8 | 2.1-3.4 | 2.5 | 1.8-3.2 | 2.2 | 1.7-3.0 |

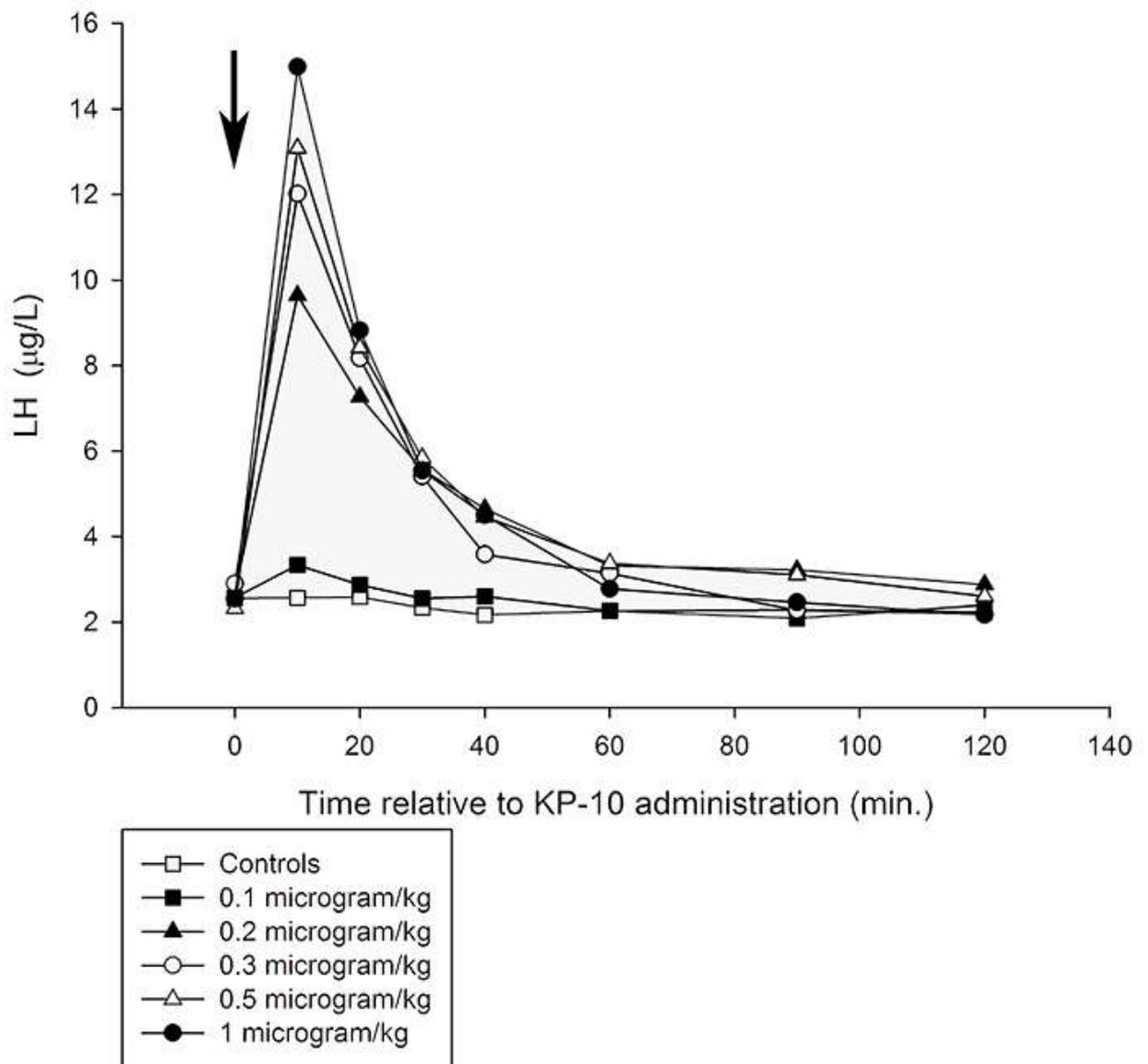


Fig. 5. Median plasma LH concentrations after intravenous administration (arrow) of different doses of canine KP-10 to six anoestrous bitches from the low dosage group.

Both the control saline and 0.1 µg/kg canine KP-10 did not result in a significant increase of plasma LH concentrations. In contrast, plasma LH concentrations significantly increased after the administration of 0.2, 0.3, 0.5 and 1 µg/kg canine KP-10 (Fig. 5 and Table 2). Plasma LH concentrations reached a maximum at T=10 at all of these dosages and returned to basal levels at 30 min after 0.2, and 0.3 µg/kg canine KP-10 and at 40 min after 0.5 and 1 µg/kg. Mean maximum LH responses were 3-, 4-, 5- and 6-fold over basal concentrations after 0.2, 0.3, 0.5 and 1 µg/kg canine KP-10, respectively.

The AUC of plasma LH concentration of the controls did not differ significantly from the AUC of plasma LH concentration after 0.1 µg/kg canine KP-10 (Fig. 6). AUCs of plasma LH concentration with 0.2, 0.3, 0.5 and 1 µg/kg were significantly higher than that of the control group. There was no difference in AUCs between 0.2, 0.3, 0.5 and 1 µg/kg

4 Discussion

4.1 Canine kiss1 and kiss1r genes

This study shows that coding sequences of both *kiss1* and *kiss1r* are conserved in the canine genome. In humans the *KISS1* gene encodes a preproprotein of 138 amino acids. The predicted canine preprokisspeptin consists of 111 amino acids. The size difference is due to divergence of the central part of the protein in the evolution of canids. This central part is not well conserved in mammals and its size in some species other than the dog also differs considerably from that in humans. The putative N-terminal signal sequence has identical residues at 15 of the 19 positions of the KISS1 preproprotein in the human and the dog. This level of similarity is higher than between the human and the mouse, which have 11 of the 19 N-terminal residues in common. The dog preproprotein has a potential dibasic cleavage site at

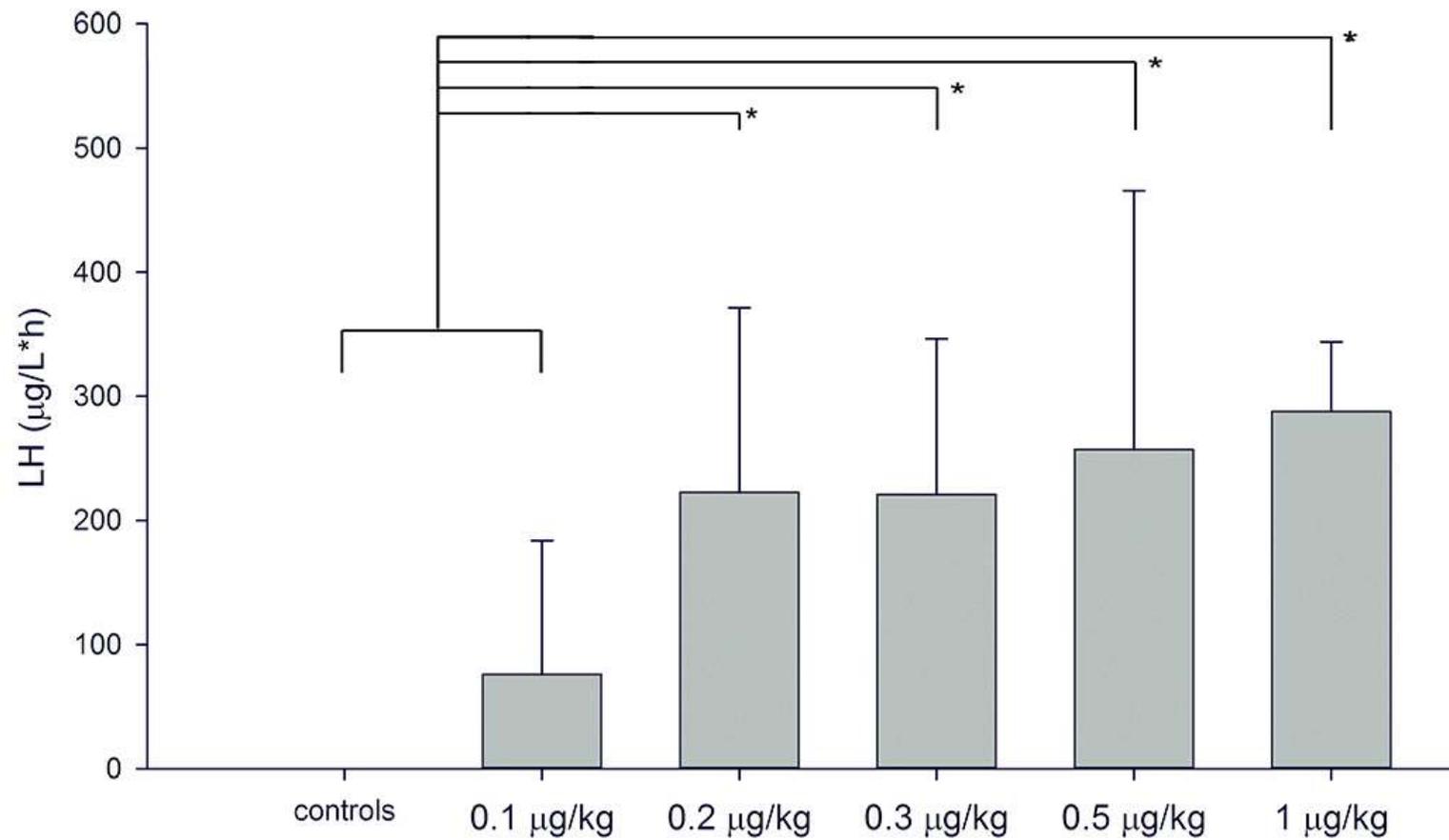


Fig. 6. Mean AUC of plasma LH concentrations after intravenous administration of different doses of canine KP-10 to six anoustrous bitches in the low dosage group. * indicates a significant difference from controls.

position 65 and a RYGR cleavage/amidation site at position 80. Use of these sites would yield a kisspeptin of only 16 residues, compared with KP-54 in humans. The amidated C-terminal stretch of 10 amino acid residues of the bioactive kisspeptin peptides is responsible for binding and activation of the receptor [36]. It is interesting to note that human and canine KP-10 differ in two amino acids. There is more similarity with other mammalian species such as rats, mice, sheep, and cattle. All of these species have kisspeptins with an RY sequence at the C-terminus, while humans have an RF sequence. The conservative substitution of F and Y terminal residues between species is common for peptides that are cleaved and amidated at this position [1-4] It indicates that the hydroxyl moiety is not required for receptor binding and activation. This is supported by structure/activity studies which show that any aromatic residue at this site is tolerable for binding and activation of the receptor [36]. The second structural difference is, however, intriguing. The substitution of Ser with Val is unexpected, because structure/activity studies indicate that a hydroxyl side chain is required, such that the conservative substitution of Thr is tolerated. This may suggest that the dog receptor differs in its requirements for the amino acid in this position and interacts with a methyl side chain rather than a hydroxyl as occurs in Val. Cloning and mutational in vitro studies comparing the canine receptor with the human receptor will clarify this issue. Such studies will also indicate whether the canine KP-10 might be the starting point for the development of a new series of agonists.

The similarity between the human kisspeptin receptor and the predicted canine kisspeptin receptor is striking. Overall there is about 75% similarity, with the largest differences at the (unconserved) C-terminus. The central 333 residues of the human and canine proteins have a similarity of 91%. This is also the case for other mammals, such as mice, rats, and cattle [37]. The canine kisspeptin receptor retains the classical hallmarks of rhodopsin family G-protein-coupled receptors such as the conserved prolines in

transmembrane (TM) domains TM1, TM3-TM7, asparagine in TM1, and aspartate in TM2 interacting with asparagine in TM7. It also has the DRY/W motif on the intracellular end of TM3 and the NPXXY motif in TM7 which are involved in receptor activation. The similarities in both kisspeptin and its receptor suggest that the role of the kisspeptin system in the dog is similar to that in other species.

4.2 *Canine KP-10 stimulation of LH, FSH, and oestradiol*

Canine KP-10 robustly and rapidly stimulated LH secretion at all dosages except 0.1 µg/kg. The plasma LH concentration reached a peak at 10 min, which is much earlier than in humans, calves, goats, and rats (about 30 min) [16,38-41]. Since the half-life of LH is similar among mammalian species, this suggests that canine KP-10 has a more rapid receptor on-rate [31]. The maximum plasma LH concentration at 10 min after canine KP-10 administration in the high dosage group represented a mean maximum of > 10-fold increase above the basal plasma LH concentration, substantially more than that reported in other mammals and more rapidly reached, 10 min versus 20 min. The mean maximum rise in plasma LH concentration after intravenous KP-10 administration was about 3-fold in men and women, 6-fold in goats and sheep, and 7-fold in prepubertal calves [16,38-40,42]. The greater response of LH in anoestrous bitches is unlikely to be due to physiological/sex steroid status, as the above studies represent a spectrum of anoestrous, castrate, postmenopausal, steroid treated, and amenorrhoeic conditions. It therefore appears likely that either the canine GnRH neuron is particularly responsive to kisspeptin or the canine gonadotroph is more sensitive to GnRH. It is possible that canine KP-10 is more efficacious in its coupling to the canine kiss1r. Resolution of this question will require in vitro studies of the responses of cloned canine

kiss1r. We are currently unable to address this question as we have yet to clone the canine receptor.

The maximum plasma LH concentration was in both the low- and the high dosage group rapidly reached 10 min after canine KP-10 administration. There was, however, a difference between the fold change from basal to maximum LH response after administration of 1 µg/kg in both groups: 13-fold in the high dosage group and 6-fold in the low dosage group. The difference in fold change may be related to the fact that the basal plasma LH concentrations in the high dosage group were twice as high as in the low dosage group. Because the maximum LH responses were similar in both groups, this resulted in a difference in fold changes. Furthermore, also the fold changes in LH after administration of GnRH differ considerably in dogs. The rise in plasma LH concentration compared to basal values after intravenous administration of the GnRH analogue gonadorelin to anoestrous bitches has been reported to be between 16-fold and 91-fold [43,45].

The stimulating effect of kisspeptin on plasma LH concentration is ascribed to the kisspeptin-induced release of hypothalamic GnRH, since GnRH antagonists ablate this stimulation [4,17,21-23]. However, in comparison with GnRH antagonists, kisspeptin antagonists only partially inhibit LH, suggesting that GnRH secretion is only partially dependent on kisspeptin input [44]. It is therefore not surprising that GnRH induces even higher plasma LH concentrations in the bitch than does canine KP-10 [33,35,43,45]. Similar results were observed by Hashizume et al. in GnRH and KP-10 stimulation tests in female goats [16]. The plasma LH response to GnRH was about three times higher and the FSH response 1.5 times higher than the response to the same dose of KP-10. In contrast, in pre- and postpubertal male goats there was no difference in the plasma profiles of LH and testosterone after administration of GnRH or KP-10 [46].

FSH concentration also rose rapidly after canine KP-10 administration. The 3-fold increase over basal FSH was similar to that after intravenous GnRH administration in anoestrous bitches [43,45]. Plasma FSH concentrations remained above basal levels for a longer period than did plasma LH concentrations. This is not surprising, as the half-life of FSH is longer than that of LH [47].

Canine KP-10 administration resulted in a 2- to 3-fold increase in plasma oestradiol, similar to the effect of GnRH stimulation in anoestrous bitches [44,45]. We observed maximum oestradiol concentrations at 60-90 min after canine KP-10 administration, similar to reported responses to GnRH in anoestrous bitches [32,35,43,45]. This is not surprising, as kisspeptin indirectly stimulate gonadal steroids via hypothalamic GnRH release [1,2,4].

In anoestrus bitches we found FSH and oestradiol responses to canine KP-10 to be similar to the responses to GnRH, but the response of LH was much higher after GnRH. LH and FSH are both secreted by pituitary gonadotrophs, which are stimulated by GnRH. However, differential regulation of LH and FSH secretion has been demonstrated in this species, indicating the presence of additional regulatory mechanisms. For example, plasma LH concentrations before and after the preovulatory LH surge were similar, while FSH concentrations were lower before than after the concomitant FSH surge [31,48]. Furthermore, FSH secretion is controlled by oestradiol and inhibin, while LH is not influenced by inhibin [49].

In this study we performed canine KP-10 stimulation tests in anoestrous bitches, beginning in early anoestrus (about 100 days after ovulation). The response to exogenous KP-10 may be influenced by the phase of the oestrous cycle, pubertal stage, and sex steroid environment [14,39,42,46]. The sensitivity of the canine pituitary to GnRH increases during the progression of anoestrus [35] and it is probable that the response to KP-10 may differ during the different phases of the cycle, since gonadal hormones exert positive or negative

feedback on GnRH production and secretion. In this respect it is interesting that GnRH neurons do not express sex steroid receptors [4,17,23,50]. With the discovery of the kiss1/kiss1r system and the finding that kisspeptin-producing neurons do express androgen and oestradiol receptors, and that GnRH neurons express kiss1r, it is likely that kisspeptin neurons form, also in the dog, the missing link in the communication between sex steroids and the GnRH neurons [4,17,21-23,50]. It has been demonstrated that negative and positive feedback of the HPG axis is mediated by a down- or up-regulation, respectively, of kiss1 mRNA in the hypothalamus. Furthermore, kiss1 mRNA expression in the hypothalamus is dependent on reproductive status and cycle stage [51,52]. It is therefore not surprising that the gonadotrophin response to exogenous KP may differ throughout the different cycle stages as has been shown in women [53]. However, data about gonadotrophin response to exogenous KP in the dog in different cycle phases, is missing.

In the high dosage group, there were no significant differences in the maximal plasma gonadotrophin and oestradiol responses, or in the AUCs, between different dosages of canine KP-10 (1-30 µg/kg). This suggests that maximum receptor occupancy and hormone responses had already been achieved at the dose of 1 µg/kg. Therefore, we also studied the effect of lower dosages of canine KP-10 on the plasma LH concentration. In contrast to the dose of 0.1 µg/kg, dosages of 0.2 µg/kg canine KP-10 or higher led to a significant LH response. There were no significant differences in AUCs between the different dosages that led to a significant LH response, although there was a dose-related maximum LH response tendency. Furthermore, plasma LH concentrations returned to basal levels already at 30 min after intravenous administration of 0.2 and 0.3 µg/kg canine KP-10, whereas after 0.5 and 1 µg/kg canine KP-10 basal levels were reached at 40 min after canine KP-10 administration similar to the results in the high dosage group. This suggests that full receptor binding and activation is reached with a dose as low as 0.5 microgram/kg canine KP-10 in anoestrous bitches. The

dosage causing the maximum LH response after an intravenous KP-10 bolus varied from 0.13 µg/kg in ovariectomised cows to 34.5 µg/kg in male monkeys [54,55]. A dose-response study in humans demonstrated a maximum LH response at 1 µg/kg, and showed a clear dose-related LH response with lower doses [38]. It can be concluded that the dog is rather sensitive to exogenous KP-10.

4.3 Conclusions

In this study we have demonstrated that both *kiss1* and *kiss1r* are present in the canine genome and that intravenously administered KP-10 rapidly and robustly stimulates the secretion of LH, FSH, and oestradiol in anoestrous bitches. These results suggest that kiss1/kiss1r signalling plays an important role in reproductive function in the dog, as in many other species. Kiss1/kiss1r, therefore offers a promising target for therapeutic intervention in dogs, such as non-surgical contraception and ovulation induction.

5 Acknowledgments

The authors thank Ms. C.H.Y. Oei for technical assistance, Ms. S.J. Mesu for technical assistance in the pharmacy, and Dr. B.E. Belshaw for editing. Prof R.P. Millar acknowledges support from the Medical Research Council, the National Research Foundation, the Technology Innovation Agency and the University of Pretoria.

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