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KISSPEPTIN SIGNALING AND ITS ROLE IN CANINE REPRODUCTION: A BASIC UNDERSTANDING

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ABSTRACT

There are several central and peripheral mechanisms that influence the GnRH secretion. For a long time it has been a mystery how these mechanisms influence the GnRH neurons. In recent years there have been exciting new discoveries in this field, especially with the discovery of kisspeptin and its receptor. Kisspeptin is a neuropeptide that seems to have an important role in the regulation of GnRH neurons in the hypothalamus in various mammalian species. It is, among other things, supposed to be involved in the onset of puberty and in the mediation of the positive feedback exhibited by estrogens on the GnRH neurons in females, which leads to the GnRH/LH surge.

Although a lot of research has already been done, to our best knowledge, kisspeptin and its receptor have not yet been researched in the dog. Because the kisspeptin signaling has been called a major gatekeeper of the gonadotropic axis, it would be interesting to discover its role in the canine reproduction. To lessen the amount of dogs that are yearly euthanized all over the world because of overpopulation, it is important to find a non-surgical method of contraception that prevents estrous cyclicity. The kisspeptin signaling pathway could be a suitable target for developing an effective, safe, long-term or permanent non-surgical contraceptive for the bitch.

The aims of the present study were to identify the canine *KISS1* and *KISS1R* genes and to characterize the amino acid sequences of canine kisspeptin and the kisspeptin receptor. The identification and characterization were done *in silico*. The results show that the *KiSS1* and the *KiSS1R* gene are present at the canine genome and a large homology is found between the canine peptides and the peptides found in other mammalian species. This gives a basis to believe that kisspeptin signaling is also important in GnRH secretion in the dog and that it may indeed be a suitable target for a non-surgical contraceptive.

INTRODUCTION

All over the world large numbers of dogs are yearly euthanized because of overpopulation. This overpopulation is the result of unregulated reproduction in populations of stray and feral dogs. It is a worldwide problem affecting developed and developing countries, which may have serious consequences for the health and welfare of both people and animals. Projects to sterilize the animals are mostly based on trap, neuter and release programs that fail to solve the problem, because the surgical methods used are time consuming and expensive and sufficient amounts of money and adequate human resources are not available. Surgery and anesthesia also bring potential health risks for the animals (Bille, Brodbelt, Eugster, Wilson). For these reasons it would be helpful to find a non-surgical contraceptive that would be safe, permanently or long term effective, inexpensive and easy to administer. Not only contraception, but also prevention of estrous cyclicity is preferred. There are a lot of progesterone-related health problems in dogs, like mammary gland tumors, endometritis, diabetes mellitus and acromegaly, which make prevention of estrous cyclicity beneficial for the long term general health (Eigenmann, Rao, Smith 2006). A suitable target for non-surgical contraception and prevention of estrous cyclicity is therefore of key importance.

In the bitch the gonadotropic axis is the central endocrinological control system of reproduction. This axis consists of three important components, namely: gonadotropin-releasing hormone (GnRH) from the hypothalamus, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland and estrogen from the ovaries. Estrogen functions as an important regulator of GnRH secretion through positive and negative feedback activity on the hypothalamus (García-Galiano). The exact mechanisms behind this control on GnRH release are unknown as there are no estrogen receptors present on GnRH neurons (Clarkson 2009).

In recent years it has been discovered that kisspeptins play an important role in reproduction in various mammalian species as regulators of GnRH release (García-Galiano, Shahab, Tena-Sempere). Kisspeptins are peptides cleaved from the *KISS1* gene product. The *KiSS1* gene was identified in 1996 as a new human malignant melanoma metastasis-suppressor gene (Lee 1996). The initial product of the *KISS1* gene is the 145 amino acid peptide KP145. The peptides that are the result of cleavage of KP145 vary in length and are named according to the number of amino acids: KP54, KP14, KP13, and KP10. They were isolated from the human placenta in 2001. Kisspeptins are RF-amide peptides. This means that like many other neuropeptides, they terminate with the amino acid sequence Arg-Phe and an amide group. Kisspeptins share the ten amino acids long C-terminus. They are the ligands for the G-protein-coupled receptor KiSS1R, also known as GPR54. The *KiSS1R* gene was characterized first in 1999 as an orphan receptor in rat brain. It was found that this G-protein-coupled receptor has a significant similarity in the transmembrane regions with rat galanin receptors (Lee 1999). It wasn't until 2001, when the *KiSS1R* gene was also found on the human genome, that it was discovered that the products of the *KiSS1* gene are in fact the ligands of this receptor. Binding studies were performed with synthetic KP54, KP14, KP13 and KP10 and it was found that all four kisspeptins were equipotent in their agonistic

activity, indicating that the ten amino acids at the C-terminal end are responsible for binding to and activation of the receptor (Kotani, Muir, Othaki).

Kisspeptins are involved in a multitude of physiological and pathological processes. The 54 amino acid long kisspeptin-54 (KP54) was the first kisspeptin to be discovered and it was named metastin, because of its involvement in suppressing metastasis of human melanomas and breast carcinomas (Lee 1996, Othaki). Mutations in the *KISS1R* gene were linked to idiopathic hypogonadotropic hypogonadism (IHH) in humans (de Roux, Seminara). IHH is caused by deficient pituitary secretion of the gonadotropic hormones FSH and LH leading to impairment of pubertal maturation and reproductive function. Later it was discovered that IHH can also be caused by mutations in the *KISS1* gene (d'Anglemont de Tassigny). These were the first discoveries that linked the *KiSS1* and *KiSS1R* gene to the reproductive system.

Studies with *KiSS1R* and *KiSS1* deficient mice showed that they displayed a phenotype very similar to human patients suffering from IHH. The deficiencies caused a lack of adult sexual development and low circulating gonadotropin concentrations. Despite severe hypogonadotropism and sexual immaturity, the mice had preserved hypothalamic gonadotropin releasing hormone (GnRH) content and also the pituitary gonadotropin responsiveness to exogenous GnRH was conserved. There were no large developmental or anatomical anomalies besides those in the reproductive system, so it appears that the lack of kisspeptin signalling didn't interfere with other important functions of the body (d'Anglemont de Tassigny, Seminara).

Other studies have found that central and peripheral administration of kisspeptin to adult mammals stimulates GnRH release, leading to a rise in LH and FSH levels (Han, Kinoshita, Navarro, Shahab) and that kisspeptin neurons lay in proximity of GnRH neurons and even project to GnRH neurons (Clarkson 2006, 2011). While GnRH neurons don't express estrogen receptors, kisspeptin neurons do (Franceschini, Smith 2005), and kisspeptin neurons are capable of activating GnRH neuron firing (Han, Liu). This provides evidence that kisspeptin is indeed the missing link between estrogen feedback and the gonadotropic axis.

Since the discovery of the importance of the KiSS1/KiSS1R system to mammalian reproduction, the *KiSS1* gene has been identified and characterized in human, in the mouse, the rat (provisional), the gray short-tailed opossum (provisional), the pig (provisional) and for many other species the *KiSS1* gene is annotated in the NCBI database (goat, bovine, rhesus macaque, chimpanzee etc.). In humans the *KiSS1* gene is found on chromosome 1. It is flanked by the *REN* gene and the *GOLT1A* gene. The gene is 6,151 base pairs (bp) long and consists of three exons, of which two are coding exons. The mRNA is 731 bp long and encodes a 138 amino acids long peptide named metastasis-suppressor KiSS-1 preproprotein. This preproprotein is cleaved to form the kisspeptins KP54, KP14, KP13 and KP10. In rat and mouse the gene is also flanked by the *REN* gene and the *GOLT1A* gene. The genes also consist of two coding exons and the KiSS1 mRNA encodes a 126 aa peptide in the mouse and a 130 aa peptide in the rat. In the mouse and rat these preproproteins are cleaved to form the kisspeptins KP52 and KP10. By comparing the mouse and rat peptides with the human peptides, it has been found that there is about 50 % homology between the preproproteins,

about 60 % between the KP54 and KP52 peptides and 90 % between the human KP10 and the mouse and rat KP10, which only differs in the last amino acid at the C-terminus (Hiden).

The *KiSS1R* gene has been identified and characterized in human, in the mouse, the rat (provisional) and the pig (provisional). For many other species the *KiSS1R* gene is annotated in the NCBI database, under which also the canine *KiSS1R* gene. In humans the *KiSS1R* gene is found on chromosome 19. In its proximity are the genes *MED16* and *ARID3A*. The gene is 6,374 bp long and consists of five exons, all containing coding sequence. The mRNA is 1650 bp long and encodes a 398 aa long peptide named the KiSS1 receptor. In rat and mouse the gene is flanked by the same genes as on the human genome. It also consists of five coding exons and the KiSS1R mRNA encodes a 396 aa peptide in the mouse and a 395 aa peptide in the rat. By comparing the mouse and rat KiSS1 receptors with the human KiSS1 receptor, it has been found that there is about 80 % homology (Hiden). The homologies in both ligands and receptor indicate a similar function of the kisspeptin systems in human, mouse and rat.

Research has also been done to the expression patterns of KiSS1 and KiSS1R mRNA. First studies showed that the highest levels of expression of KiSS1R mRNA were in the pituitary gland, pancreas, spinal cord and placenta. It was found at lower levels in other tissues, including various brain regions, stomach, small intestine, thymus, spleen, lung, testis, kidney, and fetal liver The highest levels of *KiSS1* gene expression were observed in the placenta and brain (Kotani, Muir, Othaki). Later studies also showed the presence of the kisspeptin receptor in cardiovascular tissue of human, mouse and rat, KiSS1R mRNA expression in heart and aorta tissue of mice and KiSS1R and KiSS1 mRNA expression in the human female genital tract (Cejudo Roman, Maguire, Sawyer).

To the best of our knowledge, no research on this subject has been done in dogs. Therefore, it remains unclear if kisspeptin also fills the gap between estrogen and GnRH in the reproductive axis of the bitch and if it would be a suitable target for non-surgical contraception through prevention of estrous cyclicity. So far the only information known about the dog is the annotation of the canine *KiSS1R* gene in the NCBI database. Because the *KiSS1R* gene was not sequenced completely in the dog genome project, the predicted mRNA sequence is incorrect and contains a gap compared to the human mRNA sequence. The aim of our study was to gain basic understanding of the role of kisspeptin in reproduction of the female dog by identifying and characterizing the canine *KiSS1* and *KiSS1R* genes and their products to make a comparison between the dog and other mammalian species. We identified the canine *KISS1* and *KISS1R* genes and characterized the amino acid sequences of canine kisspeptin and the kisspeptin receptor *in silico* and found large homology with other mammalian species. This indicates a similar function of the kisspeptin systems in the dog.

MATERIAL AND METHODS

IN SILICO IDENTIFICATION OF THE CANINE KISS1 AND KISS1R GENES

KiSS1

The canine kisspeptin gene was identified using the dog reference genome build 2.1 of the NCBI database. A DNA sequence comparison between the canine genome and the cDNA sequence of human preprokisspeptin (accession NM_002256) was performed using BLASTn software. The intron between the coding exons of the canine gene was defined by first blocking out repetitive DNA sequences using RepeatMasker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) followed by prediction of the splice sites using NNSPLICE 0.9) (http://www.fruitfly.org/seq_tools/splice.html).

KiSS1R

The canine kisspeptin receptor cDNA had been annotated in build 2.1 (accession XM_850105). However, due to a gap in the reference DNA sequence of the dog genome this prediction was not correct. The gap was filled through DNA sequence analysis of BAC clones that were selected from the chromosomal region using the UCSC genome browser (http://genome.ucsc.edu). These BAC clones were obtained from CHORI (http://bacpac.chori.org/library.php?id=253).

SEQUENCING THE GAP IN CANINE KISS1R

Canine gDNA and BAC clones CH82-333G1, CH82-325G21 and CH82-156G16 were used to sequence the gap in the canine kisspeptin receptor gene. The clones were cultured at 37° C for 24 hours on a solid LB agar medium containing 12.5 µl/ml chloramphenicol. Subsequently they were inoculated in a liquid LB medium containing 12.5 µl/ml chloramphenicol and incubated overnight at 37° C with shaking. Plasmid DNA was isolated using the HiSpeed® Plasmid Maxi Kit (Qiagen).

A PCR was performed at 55° C using Pfx DNA polymerase and the following primers: 5'-GACCTCAAGCCTCCACTGTC-3' and 5'-CGAGTTGCTGTAGGACATGC-3'. These primers bridge the gap and were designed using high quality trace files of the dog reference genome build 2.1. The same primers and additional primers 5'-CATCTGGGGAGTGGGCTCAA-3' and 5'-GAGGAGGGAGGAGGCAAGGT-3' were used in the subsequent sequencing 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 analyzed on a 3130xl genetic Analyzer (Applied Biosystems).

CDNA SYNTHESIS, PCR AND SEQUENCING

The following tissues were used for cDNA synthesis: placenta, pancreas, ovary, hypothalamus and liver. The placenta came from a Dobermann breeder. It was stored at -18 degrees Celsius right after birth. After delivery to the university, it was stored at -70 degrees Celsius. The other tissues were collected from healthy Beagle bitches from the university's own colony. They were euthanized because of old age or their involvement in other

research. Tissues were collected within 10 minutes following euthanization and kept in liquid nitrogen. Later they were stored at -70 degrees Celsius.

RNA was extracted using the RNeasy Mini kit of Qiagen following the protocol 'Purification of total RNA from Animal Tissues' and appendix D of the RNeasy Mini Handbook. 30 mg of frozen tissue was used as starting material. The tissue was first disrupted and homogenized in buffer RLT with a rotar-stator homogenizer. The lysate was centrifuged at full speed for three minutes at 22° C. 600 µl of supernatant was pipetted into a new tube and one volume of 70 % ethanol was added. 600 µl of the sample was transferred to an RNeasy spin column placed on a 2 ml collection tube and centrifuged for 30 seconds at full speed. Flow through was discarded. 350 µl of buffer RW1 was added to the RNeasy spin column and centrifuged for 30 seconds at full speed. Again flow through was discarded. 80 µl of DNse I incubation mix was added directly to the RNeasy spin column membrane. After 15 minutes at room temperature 350 µl buffer RW1 was added to the RNeasy spin column, the column was centrifuged for 30 seconds at full speed and the flow through was discarded. 500 µl buffer RBE was added to the RNeasy spin column twice. The first time it was centrifuged for 30 seconds, and the second time for 2 minutes at full speed to wash the spin column membrane. The RNeasy spin column was then placed on a new 1.5 ml collection tube and 30-50 µl RNase-free water was added directly to the spin column membrane. It was centrifuged for 1 minute at full speed to elute the RNA. The eluate was added to the column membrane again and centrifuged for 1 minute to raise the RNA yield.

The concentration of isolated RNA was measured using Nanodrop. First 1 µl of RNase-free water was added to the Nanodrop to provide a zero point. After this 1 µl of sample was added for RNA measurement.

Two methods were used for cDNA synthesis. The first was the iScript cDNA synthesis kit based on random primers. Approximately 500 ng of RNA (1-5 µl) was used of all five tissues. Nuclease-free water was added to reach a volume of 15 µl, after which 4 µl of 5x iScript reaction mix and 1 µl of iScript reverse transcriptase were added. The complete reaction mix was centrifuged for 5 seconds and incubated for 5 minutes at 25° C, 30 minutes at 42° C and 5 minutes at 85° C. For the second method SuperScript II Reverse Transcriptase was used. Instead of random primers, this method involved the use of two gene specific reverse primers, one for KiSS1 and one for KiSS1R. From all the primers available, the outer two were selected, so all primers were usable for PCR. Per reaction, only one primer was used. Approximately 500 ng of RNA (1-5 µl) was used of placenta, pancreas and hypothalamus. To reach a volume of 10 µl, mQ was added, followed by 1 µl of 2 µM reverse primer for KiSS1 or KiSS1R and 1 µl of dNTP mix. The mixture was heated to 65° C for 5 minutes, followed by a quick chill on ice. After brief centrifugation, 4 µl of 5x first strand buffer, 2 µl of 0.1 M DTT and 1 µl of mQ were added and the mixture was heated to 42° C for 2 minutes. 1 µl of SuperScript II RT was added and the mixture was again heated to 42° C for 50 minutes. The reaction was inactivated by heating to 70° C for 15 minutes.

Primers were designed with primer.exe. The T_m of forward en reverse primer were within 2° C of each other. All possible primer combinations were used (Table 1).

#	Name	Sequence	Gene
51	Kiss1R_cDNA_F1	GCT-GCG-ACG-GAT-CAC-AGA-AG	*KISS1R*
52	Kiss1R_cDNA_R1	GCA-GTG-TCC-AGC-AGC-AAG-C	*KISS1R*
53	Kiss1_cDNA_F2	TCT-CAG-CCT-CAG-GAC-ACT-GC	*KISS1*
54	Kiss1_cDNA_R2	GGT-CTC-GTC-TGT-GCT-GCT-TG	*KISS1*
73	KISS1_F3	GGA-CAC-TGC-CAG-CAC-CT	*KISS1*
74	KISS1_R3	CCA-AAA-GAG-GGT-AGA-ATG-AGA-GC	*KISS1*
75	KISS1R_F4	TGC-CCA-GCC-CTG-CGG-AGT	*KISS1R*
76	KISS1R_R4	GGA-TTG-TGT-GGC-ACA-GAA-AGG	*KISS1R*
77	kiss1_cDNA_FW3	CAG-CAC-CTG-CAT-CAT-CTC-AC	*KISS1*
78	kiss1_cDNA_RV3	CAT-CGC-AAC-ACA-TCG-CTA-GT	*KISS1*
79	kiss1_cDNA_FWrv	CGC-CTA-CAA-CTG-GAA-CGT-CT	*KISS1*
80	kiss1R_cDNA_RV4	TTCA-AGG-CAG-CAT-GGA-CGA-A	*KISS1R*

Table 1 The forward en reverse primers used in the attempts to sequence KiSS1 and KiSS1R mRNA. All the primer combinations for KiSS1 and KiSS1R were made.

A mix was prepared for the PCR, containing 10x PCR buffer, $MgCl_2$, dNTPs, forward and reverse primer, Platinum Taq, mQ and cDNA (Table 2). The 10x PCR Buffer and $MgCl_2$ (50 mM) were vortexed before use. dNTP (10 nM) was mixed before use and Platinum Taq was centrifuged before use and kept on ice the entire time.

The cDNA of placenta, pancreas, ovary, hypothalamus and liver was used and with every reaction a blank was added containing no cDNA. The PCR protocol was: 5 minutes at 95° C, 35 cycli with 30 seconds at 95° C, 30 seconds at 60° C and 30 seconds at 72° C, followed by a 10 minute step at 72° C. The amount of cycli was later changed to 40 and the temperature of the second step during the cycli was ranged from 55 to 65° C.

Loading dye was added to the PCR products and two ladders (100 bp and 1 kb) and the mixtures were put on an 1.5 % agarose gel made with TBE buffer for separation by gel electrophoresis. 5 µl of ethidium bromide was added per 100 ml of agarose gel. The power supply was set at 100 V for approximately 45 minutes. Results were assessed with GelDoc. If multiple bands appeared per PCR product, they were cut from the gel and put in 20 µl of mQ for two days. If only one band appeared of the appropriate size, the total PCR product was used for the sequencing steps. Because the total PCR product still contains primers and lose nucleotides, a sap/exo step was done. Per 7.5 µl of PCR product, 3 µl of sap/exo mix

(containing 2.5 µl of shrimp alkaline phosphatase and 0.5 µl of 2 units/µl exonuclease I) was added. The mix was incubated at 37° C for 60 minutes and 75° C for 20 minutes.

Components	1x (µl)	Concentration
10x PCR Buffer	2.5	1x
MgCl2 (50 mM)	1.25	2.5 mM
dNTP's (10 mM)	0.5	200 µM
Primer F (10 µM)	1.25	0,5 µM
Primer R (10 µM)	1.25	0,5 µM
Platinum Taq (5 mQ)	0.2	1 unit
mQ	16.05	***
Totaal	23	
gDNA/cDNA	2	
Totaal	25	

Table 2 The PCR mix. A new mix was made for every primer combination and type of cDNA.

The sap/exo products and the products from the bands cut from the gel were then put through the tercycle. The mix that was prepared, contained Terminator reaction mix, PCR product, primer, 5x sequence buffer and mQ (Table 3).

The mix was put through 25 heating cycles of 96° C for 30 seconds, 50° C for 15 seconds and 60° C for 30 seconds. The second step was also done at 55° C.

The tercycle products were purified in two different ways. First sephadex purification was used and later ethanol precipitation was chosen. Products were analyzed by the ABI Prism 3130xl. Found sequences were visualized with SeqMan and blast against the canine genome using the NCBI database.

Components	PCR-product from Sap/Exo	PCR-product from gel
Terminator reaction mix	1 µl	1 µl
PCR-product	2 µl	6 µl
Primer 3,2 µM	1 µl	1 µl
5x Sequence buffer	2 µl	2 µl
mQ	4 µl	X
Totaal	10 µl	10 µl

Table 3 The mix that was prepared for the tercycle reaction. The amount of PCR product differed between the PCR products from the Sap/Exo reaction and the PCR products from the gel.

RESULTS

IN SILICO IDENTIFICATION OF THE CANINE *KISS1* AND *KISS1R* GENES

KiSS1

Significant similarities with the cDNA sequence of human preprokisspeptin (accession NM_002256) were found on chromosome 38 of the canine genome. The probability that the found location indeed contained *KISS1* was raised by comparing the genes flanking the human *KISS1* gene and the genes flanking the matching parts on the canine genome. Both locations are surrounded by the same genes, namely *REN* and *GOLT1A*.

The alignment was not complete for *KISS1* because of low similarity between the middle parts of the genes of the two species. Only two smaller fragments of the *KISS1* gene are found by the similarity search (Fig. 1). These parts are derived from the two coding exons of the gene. The first part contains the putative start codon of the gene, while the second part contains the putative KP10 coding sequence. The full *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 KP10 coding sequence. Splice sites were predicted at 103 base pairs (donor site) and 1840 base pairs (acceptor site) from the start codon.

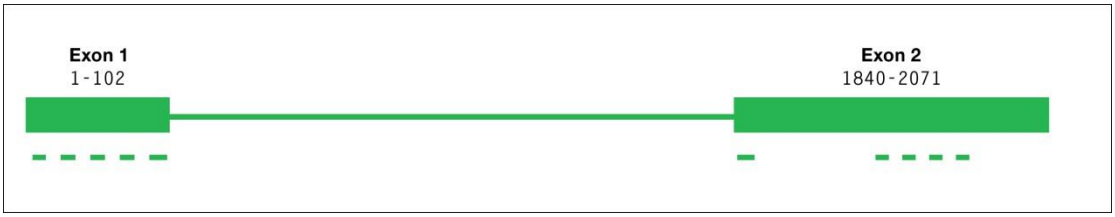

Fig. 1 The *KiSS1* gene with two coding exons and one intron. The striped line below shows the parts that were aligned using the cDNA sequence of human preprokisspeptin (accession NM_002256).

The predicted mRNA of preprokisspeptin encodes a 111 amino acid long peptide, which shows approximately 40 % identities with human preprokisspeptin. The amino acid sequence of canine kisspeptin 10 differs only two amino acids from human kisspeptin 10. Canine kisspeptin 10 has an RY instead of an RF at its C-terminus, which is the same in mouse and rat kisspeptin 10, and the fifth amino acid is a valine instead of a serine (Fig. 2).

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Canine   1    MNSLVSWQLMLLLCATSFRETLEKVAPMETPGPAGQRLGAQALPAPWERSPP---------  53
              MNSLVSWQL+L LCAT F E LEKVA +    P GQ+L +  L AP E+S P
Human    1    MNSLVSWQLLLFLCATHFGEPLEKVASVGNSRPTGQQLESLGLLAPGEQSLPCTERKPAAT  61

Canine   54   -------------------------RAPQRHLMPARRGA-------DLPAYNWNVFGLRYG  81
                                       AP    +PA +GA        DLP YNWN FGLR+G
Human    62   ARLSRRGTSLSPPPESSGSPQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRFG  122

Canine   82   RRRAATPGLRGGTPSPRLRVPVGWGLGLRS 111
              +R AA PG  G
Human    123  KREAA-PGNHGRSAGRG  138
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Fig. 2 The alignment between canine and human preprokisspeptin. In green the similarities between the two amino acid sequences and in grey the sequences of canine and human KP10.

KiSS1R

A portion of the canine kisspeptin receptor sequence had already been predicted (accession XM_850105.1). Because a part of exon 4 of the gene was missing in the reference genome sequence, it was not possible to predict the correct mRNA sequence.

SEQUENCING THE GAP IN CANINE *KISS1R*

The predicted size of the gap in canine *KiSS1R* in the NCBI database was 410 bp (Fig. 3). A segment of 368 bp of the gap was sequenced using BAC clones CH82-333G1, -325G21 and -156G16. This segment contained the predicted splice site of exon 4 at 73 bp from the start of the gap. The other part of the gap is located on intron 4. Attempts to sequence the final part of the gap, a smaller segment of approximately 42 bp, were not successful. Neither was the use of canine gDNA during the first attempts to sequence the gap.

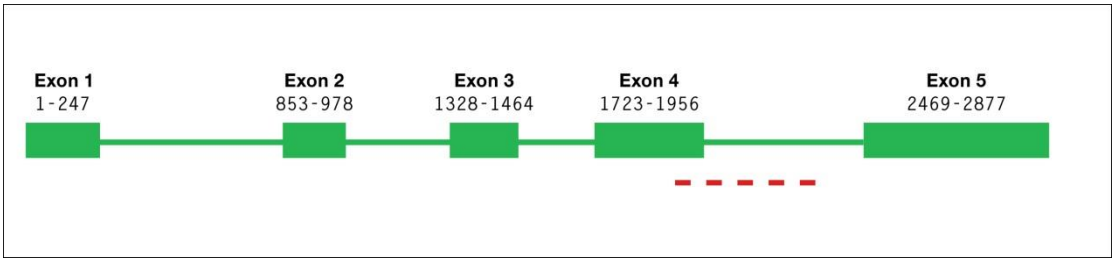

Fig. 3 The *KiSS1R* gene with its five exons and four introns. The red striped line shows the part where the gap was situated in the dog reference genome build 2.1 of the NCBI database.

After sequencing the large segment of the gap, the complete exons could be identified by alignment of the canine gene and the cDNA sequence of the human kisspeptin receptor. Both genes consist of five exons. The predicted mRNA of the canine kisspeptin receptor encodes a protein of 382 amino acid residues. The amino acid sequences of the canine and the human kisspeptin receptor show 75% identities. In the transmembrane regions there is >95 % homology (Fig. 4).

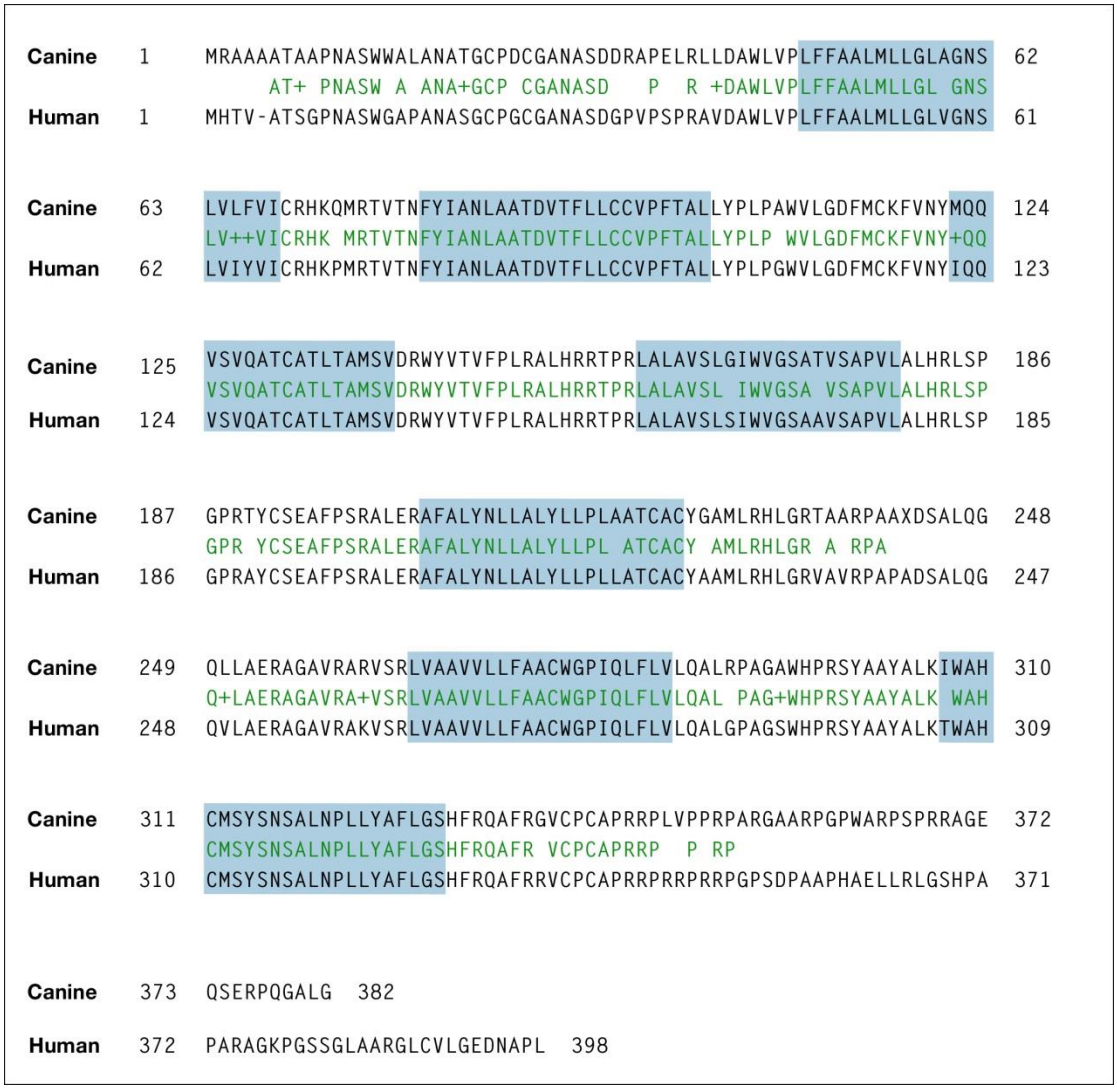

Fig. 4 The alignment between the canine and human kisspeptin receptor. In green the similarities between the two amino acid sequences and in grey the seven transmembrane regions of the human kisspeptin receptor (NP_115940.2).

CDNA SYNTHESIS, PCR AND SEQUENCING

None of the attempts to sequence KiSS1 and KiSS1R mRNA succeeded. The positive control with primers for RPS19 mRNA gave good results, which showed that cDNA synthesis and PCR procedures worked well (Fig. 5). All possible primer combinations were used on all five

tissues using temperatures ranging from 55 to 65° C. The tissues were chosen for their known connection to KiSS/KiSS1R signaling. For each tissue, except the liver, studies confirm the presence of KiSS1 and/or KiSS1R mRNA in different mammalian species, the placenta and hypothalamus being the tissues with the highest expression levels of KiSS1 and KiSS1R mRNA.

Fig. 5 The positive controle with primers for RPS19 gave good results in all five tissues (lane 1-5), whereas the PCR performed with primers for KiSS1 (lane 8-12) and KiSS1R (lane 15-19) didn't show any bands after gelelectrophoresis. Lane 7 and 14 show a 100 bp size standard.

DISCUSSION

This study shows that both *KiSS1* and *KiSS1R* are present on the canine genome. In humans the *KiSS1* gene encodes a preproprotein of 138 amino acids. The predicted canine preprokisspeptin consists of only 111 amino acids. Although it appears that there is a part missing in the canine preproprotein (Fig. 2), blast searches for this specific area of the human sequence in the canine genomic database of the NCBI were not successful, leading to the conclusion that during evolution this part was lost in canines. A large similarity of 80 % is found between human and canine kisspeptin 10 (Fig. 2), the part that is believed to be important in binding to and activating the kisspeptin receptor. The homology with other mammalian species is even larger. The canine kisspeptin 10 differs only one amino acid with the kisspeptin 10 of rat, mouse, sheep and cattle. These kisspeptins all have an RY at their C-terminus, opposed to the RF at the human C-terminus.

The homology between the human kisspeptin receptor and the predicted canine kisspeptin receptor is striking. Overall there is about 75 % of homology, with the biggest differences at the C- terminus. This is also true for other mammalian species, like the mouse, rat and cattle (Hiden). These homologies in both kisspeptin and its receptor indicate a similar function of the kisspeptin system in human and dog.

The gap in the *KiSS1R* gene was not completely sequenced. The trace files directly surrounding the gap were not of sufficient quality to be used to design primers. Primers were designed at >300 bp from the gap at trace files of higher quality. The forward and reverse primer were used successfully, resulting in sequences of enough quality to design new primers closer to the gap. Although the new forward primer was successful, the new reverse primer was not. The trace files show a sudden loss of signal (Fig. 6), which could be the result of a certain sequence at that part of the gene causing a conformation that blocks the polymerase.

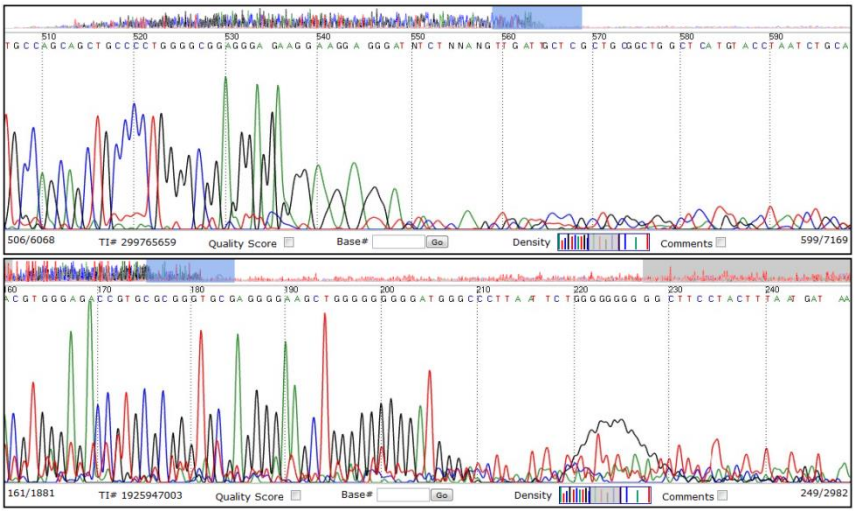

Fig. 6 Two trace files from the NCBI database that lay in close proximity to the gap in the canine *KiSS1R* gene. A clear loss of signal is seen at 550 bp in the upper trace file and between 200 and 210 bp in the lower trace file.

It is unfortunate that the attempts to sequence KiSS1 and KiSS1R mRNA weren't successful. One of the reasons for this could be that the mRNA levels weren't sufficient. Although the placenta is mentioned often as the tissue with the highest expression levels, it appears that this is especially true during the time of trophoblast invasion, which is during the first trimester in pregnancy of women and at 12.5 days in rat. In rat, the levels of KiSS1 and KiSS1R mRNA expression gradually decline after day 12.5 during placental maturation and they are not detectable anymore at day 18.5, which is approximately 4 days before birth (Hiden). The placenta used in this study was collected at birth and this could very well be the reason for the insufficient mRNA levels. Canine pregnancy can be divided into three stages: preimplantation from day 0 to day 20, embryonic and placental development from day 20 to day 45, and fetal and placental maturation from day 45 to day 65 (Sahlfeld). For further studies that aim to sequence KiSS1 and KiSS1R mRNA it would be useful to collect placentas between day 20 and day 45 of pregnancy.

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