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The effects of kisspeptin agonist canine KP-10 and kisspeptin antagonist p271 on plasma LH concentrations during different stages of the estrous cycle and anestrus in the bitch

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ABSTRACT

Kisspeptin (KP) plays a key role in the regulation of the hypothalamic-pituitary-gonadal axis via the release of GnRH. As normal KP signaling is essential for reproductive function, it could be an interesting new target for therapeutic interventions, e.g., nonsurgical contraception in dogs. The aims of the present study were to investigate the effect of KP-10 administration on plasma LH concentration in different stages of the reproductive cycle and to investigate the suitability of p271 as KP antagonist in the bitch. Two groups of six adult Beagle bitches were used. In one group, plasma LH concentration was determined before (40 and 0 minutes) and 10, 20, 40, and 60 minutes after the intravenous administration of 0.5-μg/kg body weight (BW) canine KP-10. In the other group, the bitches received a continuous intravenous infusion with p271 (50 μg/kg BW/h) for 3 hours, and 0.5-μg/kg BW canine KP-10 was administered intravenously 2 hours after the start of the p271 infusion. Their plasma LH concentration was determined before (−40 and 0 minutes) and 30, 60, 90, 120, 130, 140, 160, and 180 minutes after the start of the p271 infusion. In both groups, the experiments were performed during the follicular phase, the first and second half of the luteal phase, and during anestrus. Canine KP-10 induced an increase of plasma LH concentration during all estrous cycle stages and anestrus. There was no difference in LH response between the two groups. The lowest LH response was seen during the follicular phase and the highest response during anestrus. The area under the curve (AUC) for LH and LH increment in the follicular phase were lower than those in anestrus. The AUC LH and LH increment in the first half of the luteal phase were lower than those in the second half of the luteal phase and anestrus. The AUC LH and LH increment in the second half of the luteal phase were not different from those in anestrus. Continuous administration of the antagonist p271 did not alter basal plasma LH concentration and could not prevent or lower the LH response to KP-10 in any of the cycle stages and anestrus. It can be concluded that the LH response to KP-10 is dependent on estrous cycle stage and that peripheral administrated p271 cannot be used as KP antagonist in the dog.

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This provides new insight in reproductive endocrinology of the bitch, which is important when KP signaling is considered for therapeutic interventions, such as for estrus induction or nonsurgical contraception in the bitch.

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1. Introduction

The discovery in 2003 that kisspeptin (KP) signaling appears to be the gateway to normal reproductive function in both males and females was a milestone in the unraveling of the endocrinological regulation of reproduction [1,2]. Humans and mice with a dysfunctional KP system because of a deactivating mutation of the KP gene (*KISS1*) or the KP receptor gene (*KISS1R*) fail to go into puberty and display hypogonadotropic hypogonadism [1,3]. The relevance of KP signaling is further underlined by the identification of activating mutations of the *KISS1R* and *KISS1* genes in children with precocious puberty [4,5].

The human *KISS1* gene encodes a 145-amino acid peptide that is cleaved into four shorter peptides that have a common C-terminal RF-amide: KP-54, KP-14, KP-13, and KP-10. These four KPs are the ligands for the KISS1R, a G-protein–coupled receptor (also known as GPR54), and have the same binding affinity to the receptor, indicating that the C-terminal end is responsible for binding and activation [6–8].

Kisspeptins play a key role in the hypothalamic-pituitary-gonadal (HPG) axis and are highly expressed in the hypothalamus. Here, KP neurons directly stimulate GnRH neurons to secrete GnRH, which stimulates LH and FSH secretion by the gonadotropes in the pituitary gland [7,9,10]. It has been demonstrated that central (intracerebroventricular [ICV]) or peripheral intravenous administration of KP (e.g., KP-10) results in an increase of LH and FSH secretion in many species (human, pig, cow, goat, dog, rat, and mouse) [11–17]. Negative and positive feedback on the HPG axis by gonadal steroids is mediated by a downregulation or upregulation, respectively, of kiss1 messenger RNA (mRNA) in the hypothalamus [7]. In several species, it has been demonstrated that hypothalamic kiss1 mRNA expression is dependent on reproductive status [18,19]. Also, the LH response to exogenous KP has been shown to be dependent on estrous cycle stage and anestrus in women, ewes, and female rats [14,20,21].

An even better understanding of KP signaling followed when Roseweir et al. [22] developed a KP antagonist. Peripheral or ICV administration of the antagonist (p271 for peripheral use and p234 for ICV use) prevented the preovulatory LH surge in female rats and blocked the postcastration rise in circulating LH in male mice, rats, and sheep [22–24].

It can be concluded that KP signaling plays a key role in mammalian reproduction; however, relatively little is known about KP signaling in dogs. The reproductive cycle of the domestic bitch (*Canis lupus familiaris*) is complex and differs in many aspects 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 or not the bitch is pregnant, and a nonseasonal anestrus of 2 to 10 months [25]. Plasma progesterone concentrations are solely dependent on CL function. During the first half of the luteal phase, progesterone production is independent of pituitary support, and therefore, this part of the cycle may be called the pituitary-independent phase [26]. During the second half of the luteal phase, pituitary support is necessary to maintain luteal function, with prolactin as the main luteotropic factor, and therefore, this part of the cycle may be called the pituitary-dependent phase [26–33].

The bitch appears to be quite sensitive to exogenous KP-10, on the basis of a robust rise in plasma LH, FSH, and estradiol concentrations in anestrous bitches after peripheral administration of KP-10 [12]. These findings suggest that KP signaling also plays an important role in reproductive endocrinology in the dog. This knowledge opens new windows of opportunities for therapeutic interventions, such as estrus induction or estrus prevention. The latter is of great importance, not only for pet dogs but also for stray dogs. In many countries, pet overpopulation and stray dogs cause major problems, leading in the United States of America, e.g., to euthanasia of over 4 million animals annually [34]. In third world countries, stray dog populations are an important reservoir for zoonoses such as rabies. To reduce the incidence of rabies in humans caused by dog bites, the number of stray dogs needs to be reduced, e.g., by contraceptive methods [35]. However, currently the only effective, reliable, and permanent method for canine contraception is gonadectomy. This procedure is time-consuming, costly, needs specially trained people, and presents surgical and anesthetic risks for the animal [36]. There is a need for alternative methods that are faster, easier to perform, and less expensive. Because KP plays a key role in regulation of the HPG axis, a nonsurgical contraceptive method on the basis of influencing KP signaling using KP might be ideal, making application of a KP antagonist in dogs very interesting. It is therefore tempting to study whether p271 also has KP antagonistic properties in the dog.

The first aim of the present study was to investigate whether the effect of KP-10 administration on plasma LH concentration is dependent on estrous cycle stage or anestrus in the bitch, which is important for future research on KP or its receptor as possible targets for nonsurgical contraceptive methods. The second aim was to investigate the suitability of p271 as KP antagonist in the bitch by examining basal and KP-10–stimulated plasma LH concentrations during infusion of p271 in different stages of the estrous cycle and anestrus.

2. Materials and methods

2.1. Animals and experimental design

To study the effects of KP and p271 on LH secretion during different stages of the estrous cycle and anestrus, two experiments (Experiment 1 and Experiment 2) were

conducted. Twelve healthy Beagle bitches were used, six for each experiment. The median age of the six bitches at the start of Experiment 1 was 86 months (range 39–118 months) and that of Experiment 2 was 41 months (range 28–112 months). The median body weight of the dogs in Experiment 1 was 14.0 kg (range 12.6–16.0 kg) and in Experiment 2 was 15.5 kg (range 14.3–18.0 kg). 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 runs, 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 proestrus. Plasma progesterone concentration was measured thrice weekly from the start of proestrus until it exceeded 13 to 16 nmol/L, at which time ovulation is assumed to occur [27–29]. Anestrus was defined as the period from 100 days after ovulation to the onset of the next proestrus.

2.1.1. Experiment 1

Kisspeptin stimulation tests were performed in six dogs during different stages of the estrous cycle and anestrus: once in the follicular phase (median 5 days before ovulation, range 4–8 days), once in the first half of the luteal phase (median 18 days after ovulation, range 13–20 days), once in the second half of the luteal phase (median 44 days after ovulation, range 42–55 days), and once in anestrus (median 106 days after ovulation, range 101–108 days). Canine KP-10, 0.5-μg/kg body weight (BW), was administered in the cephalic vein as a single bolus, and blood samples for measurement of the plasma LH concentration were collected from the jugular vein by repeated venipuncture at 40 minutes and immediately before and at 10, 20, 40, and 60 minutes after the administration of KP-10.

2.1.2. Experiment 2

Kisspeptin stimulation tests during intravenous infusion of the KP antagonist p271 were performed in six dogs during the same cycle phases as described in Experiment 1: the follicular phase (median 5 days before ovulation, range 1–8 days), the first half of the luteal phase (median 17 days after ovulation, range 11–20 days), the second half of the luteal phase (median 42 days after ovulation, range 33–50 days), and anestrus (median 109 days after ovulation, range 103–112 days).

Peptide 271, 50-μg/kg BW/h, was continuously administered via a catheter in the cephalic vein for 3 hours. Two hours after the start of the p271 infusion, a single intravenous bolus of 0.5-μg/kg BW canine KP-10 was administered intravenously. Blood samples were collected from the jugular vein by repeated venipuncture at 40 minutes and immediately before and every 30 minutes for two hours after the start of p271 infusion. After the administration of canine KP-10, blood samples for measurement of the plasma LH concentration were collected at 10, 20, 40, and 60 minutes (i.e., 130, 140, 160, and 180 minutes after the start of the p271 infusion). Figure 1 provides a schematic overview of the KP stimulation tests in both experiments.

2.2. Peptides

Canine KP-10 (YNWNVFGLRYNH2), produced by the American Peptide Company with a purity of 99.5%, was dissolved in 0.05% dimethylsulfoxide and divided into individual doses in glass vials that were stored at −20 °C and then thawed at room temperature on the day of use.

Kisspeptin antagonist p271 (RRMKWKKY[D-A]NWNGFG[D-W]RF-NH2), produced by the American Peptide Company with a purity of 96.9%, was dissolved in saline on the day of the experiment and used immediately.

2.3. Hormone measurements

Plasma progesterone concentration was measured thrice weekly during the follicular phase to determine the ovulation period, using a ^{125}I RIA previously validated for ovulation timing [37]. The intra-assay and interassay coefficients of variation 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 validated for the dog, as described previously [38,39]. The intra-assay and interassay coefficients of variation for values above 0.5 μg/L were 2.3% and 10.5%, respectively, and the limit of quantitation was 0.3 μg/L.

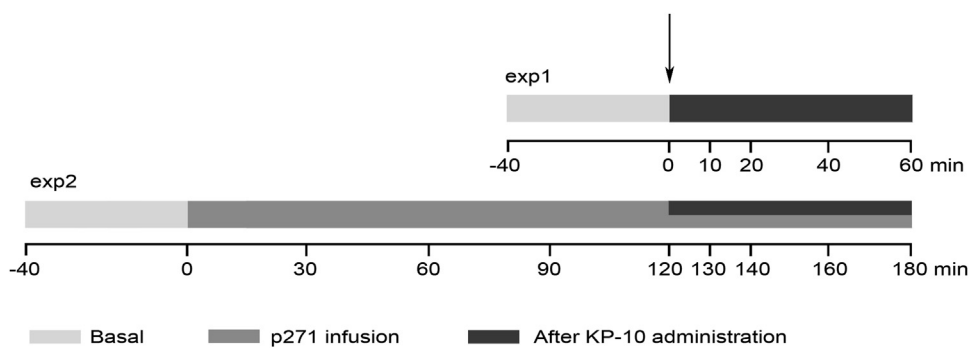

Fig. 1. Experimental design. Time points (min) of blood sample collection relative to peptide administration. The arrow indicates the administration of 0.5-μg/kg BW canine KP-10.

2.4. Data analysis

The mean of all basal plasma LH concentrations was calculated. All basal LH values that exceeded this overall mean plus 3 standard deviation were ascribed to pulsatile secretion and were treated as outliers and excluded from statistical analysis. In both groups, the basal plasma LH concentration used for further analysis was calculated for each dog as the mean of the values at −40 and 0 minutes before KP-10 or p271 administration.

A log transformation of plasma LH concentrations was used because not all data were normally distributed. To determine the effect of KP-10 on LH secretion in Experiment 2, the mean of the two samples at 90 and 120 minutes after the start of p271 infusion was calculated and used as basal LH concentration to determine the effect of KP-10 administration. For both groups, AUC LH above zero was calculated for the period from KP-10 administration until 60 minutes after KP-10 administration. In addition, the increment was calculated by subtracting the basal LH concentration from the maximum LH concentration.

Statistical analysis was performed using SPSS for Windows, version 22 (SPSS Inc., Chicago, USA).

To analyze whether there were differences in age or body weight between the groups, an independent *t* test was performed. A linear mixed model was used to analyze differences in plasma LH concentrations before and after KP-10 and to analyze differences of cycle stages and group effects (Experiment 1 and Experiment 2) of AUC LH and LH increment, with Bonferroni correction. A P-value less than 0.05 was considered to be significant.

2.5. Ethics of experimentation

This study was approved by the Ethics Committee of Utrecht University.

3. Results

There were no significant differences in age and body weight between the two groups of bitches in both experimental groups. For both experimental groups, the median plasma LH concentrations before and after KP-10 administration are shown in Table 1. Administration of KP-10 resulted in a significant increase of plasma LH concentration in all cycle stages and anestrus in both groups.

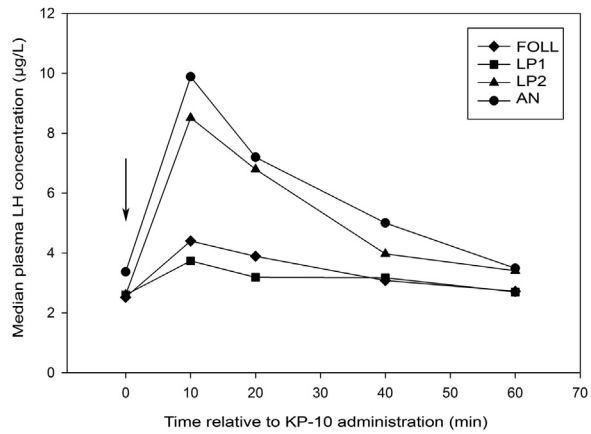

Fig. 2. Median plasma LH concentration (in µg/L) before and after intravenous administration of 0.5-µg/kg BW canine KP-10 (arrow) during the follicular phase (FOLL), first half of the luteal phase (LP1), second half of the luteal phase (LP2), and anestrus (AN) (n = 12).

Compared to basal values, the antagonist p271 did not decrease the plasma LH concentration in any of the studied estrous cycle stages or anestrus. Further, p271 infusion failed to lower or prevent the LH response to KP-10. The two groups showed no significant differences in LH increment and AUC LH. Consequently, the groups were fused (n = 12) for further analysis of the effects of KP-10 on plasma LH concentrations.

Peak LH concentrations were reached at 10 minutes after KP-10 administration and declined thereafter (Fig. 2). The LH response to KP-10 was most pronounced during anestrus (median increment 8.2 µg/L, range 2.8–24.7 µg/L) and least pronounced during the first half of the luteal phase (median increment 1.4 µg/L, range −0.3 to 3.7). The increment of LH during the follicular phase (median 2.2 µg/L, range 1.2–8.2 µg/L) was lower than during the second half of the luteal phase (median 5.6 µg/L, range 1.1–14.4 µg/L, $P = 0.033$) and anestrus ($P < 0.001$). The increment of LH during the first half of the luteal phase was also lower than those during the second half of the luteal phase and anestrus (both $P < 0.001$) but was not different from that in the follicular phase ($P = 0.077$). The increment of LH during the second half of the luteal phase was not different from that in anestrus ($P = 0.399$).

Table 1
Median (and range) plasma LH concentrations (in µg/L) before (basal) and 10, 20, 40, and 60 min after intravenous administration of 0.5 µg/kg BW canine KP-10 in both experimental groups (Experiment 1 and Experiment 2) during the follicular phase (FOLL), first half of the luteal phase (LP1), second half of the luteal phase (LP2), and anestrus (AN).

Cycle stage	Median (and range) plasma LH concentrations (µg/L)									
	Basal	Range	t = 10	Range	t = 20	Range	t = 40	Range	t = 60	Range
FOLL (exp1)	2.2	1.5–2.6	4.1	3.6–7.0	3.3	2.8–5.1	2.7	1.9–3.3	2.5	1.7–3.1
FOLL (exp2)	3.1	1.6–9.6	5.2	3.2–14.6	4.2	2.4–13.5	4.1	2.5–11.5	3.3	2.3–9.7
LP1 (exp1)	2.4	1.2–3.3	3.8	2.4–7.0	3.1	2.2–4.8	2.8	2.2–4.0	2.5	1.8–15.2
LP1 (exp2)	2.9	1.8–5.5	3.7	1.5–9.1	3.2	1.2–7.6	3.9	1.1–6.4	3.3	1.2–5.6
LP2 (exp1)	2.1	1.5–3.7	8.5	5.3–15.9	6.8	4.5–10.2	3.3	2.3–8.9	2.7	1.7–5.2
LP2 (exp2)	3.4	1.9–6.1	9.0	5.1–13.7	6.5	4.1–8.7	4.7	3.5–6.4	4.3	3.4–5.1
AN (exp1)	2.1	1.5–3.7	8.5	5.1–26.8	5.2	4.0–11.9	3.3	2.5–5.2	2.5	1.3–3.5
AN (exp2)	5.7	3.2–6.2	16.2	9.3–24.1	10.0	5.7–12.6	6.4	4.8–8.8	4.8	3.5–7.3

Abbreviations: exp1, Experiment 1; exp2, Experiment 2.

The AUC LH in the follicular phase (median 208 μg/L × min, range 149–723 μg/L × min) was lower (P = 0.006) than that in anestrus (median 359 μg/L × min, range 186–709 μg/L × min). The AUC LH in the follicular phase was not significantly different from that in the first (median 209 μg/L × min, range 75–415 μg/L × min, P = 1.000) and second half of the luteal phase (median 355 μg/L × min, range 193–436 μg/L × min, P = 0.123). The AUC LH in the first half of the luteal phase was lower than that in the second half of the luteal phase (P = 0.018) and anestrus (P = 0.001). The AUC LH during the second half of the luteal phase was not significantly different (P = 1.000) from that in anestrus (Fig. 3).

4. Discussion

This study shows that KP-10 administration results in an LH response in all estrous cycle phases and anestrus but with differences in the magnitude of LH responses between the cycle stages. The LH response to KP-10 stimulation in anestrus was significantly higher than that in the follicular phase. This is in agreement with observations in other species like ewes, where the LH response to KP was significantly higher during seasonal anestrus compared to other cycle stages [20,40]. An explanation may be the expression of kiss1r on GnRH neurons in the hypothalamus, which has been reported to be significantly higher during seasonal anestrus than that in other cycle stages in ewes [40]. In line with this observation, the GnRH response to KP-10, determined in pituitary portal blood, was also higher during anestrus compared to other cycle stages in sheep [40]. In sheep, the LH pulsatility in anestrus is lower than that in other cycle stages, which may lead to accumulation of LH in the gonadotropes and, subsequently, allow more LH release after stimulation with KP-10 [20]. However, plasma LH profiles of bitches during different stages of the estrous cycle and anestrus did not show a lower LH pulse frequency in anestrus [38].

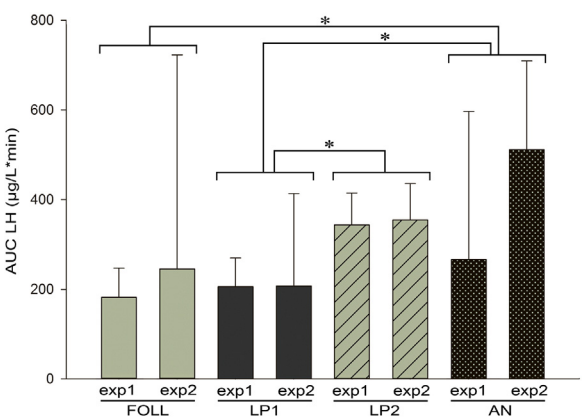

Fig. 3. Median AUC of plasma LH concentration (in μg/L × min) after intravenous administration of 0.5-μg/kg BW canine KP-10 to six beagle dogs in both experimental groups (Experiment 1 and Experiment 2) during the follicular phase (FOLL), first half of the luteal phase (LP1), second half of the luteal phase (LP2), and anestrus (AN). Significant differences are indicated with an asterisk (*; P < 0.05). AUC, area under the curve.

The lower LH response to KP-10 during the follicular phase, compared to the second half of the luteal phase and anestrus, may also be explained by ovarian feedback. The follicular phase is characterized by elevated plasma concentrations of estradiol [41]. The negative and positive feedback of steroids on the HPG axis is mediated by a downregulation or upregulation, respectively, of kiss1 mRNA in the KP neurons in the hypothalamus [7,18,42]. In females, estradiol can exert both negative and positive feedback on the hypothalamus (the latter to induce the preovulatory LH surge) by regulating kiss1 gene expression in different nuclei of the hypothalamus. As demonstrated in rodents, estradiol results in downregulation of kiss1 in the arcuate nucleus, but in the anteroventral periventricular nucleus, kiss1 is upregulated under estradiol influence. In line with these observations, in female rats, ewes, and women the LH response to KP administration was highest during the late follicular phase, compared to the early follicular phase and the luteal phase [14,20,21]. These findings suggest that KP plays an important role in inducing the preovulatory LH surge [43]. In the present study, however, KP-10 was administered not during the preovulatory LH surge or around ovulation but during the early follicular phase (median 5 days before ovulation). In this part of the follicular phase, estradiol may still exert a negative feedback effect on KP secretion, resulting in less GnRH secretion and subsequently less LH secretion. It is therefore not surprising that the LH response to KP-10 administration during the follicular phase was lower than in the other estrous cycle stages or anestrus. It may be that the time-window of increased sensitivity of GnRH (and also KP-10) during the late follicular phase, as shown in rats, ewes, and women, is short. More studies about the sensitivity to KP-10 around the time of the preovulatory LH surge and ovulation in the dog are needed.

The LH response to KP-10 administration in the first half of the luteal phase was much lower than that in the second half of the luteal phase. The plasma progesterone concentration rises quickly after ovulation and remains elevated in the bitch for around 2 months, almost irrespective of whether the bitch is pregnant or not. About 4 weeks after ovulation, the plasma progesterone concentration starts to decline gradually [29]. A major share of the KP neurons in the hypothalamus expresses the progesterone receptor, and progesterone exerts a negative feedback effect at the hypothalamic level on kiss1 mRNA expression [44,45]. The higher circulating progesterone levels during the first half of the luteal phase of the dog may have resulted in more negative feedback at the hypothalamic level than those during the second half of this phase. As a consequence, there may be a decreased expression of the kiss1r on GnRH neurons during the first half of the luteal phase, which explains the lower LH response to exogenous KP-10 in our study. However, data about progesterone influence on kiss1r expression in the hypothalamus are lacking [40]. In line with the hypothesis that progesterone exerts a negative feedback effect on kiss1 mRNA expression is the observation that compared to the first half of the luteal phase, LH pulse amplitude and LH pulse frequency are increased during the second half of the luteal phase in bitches [46].

In addition, the LH content in the pituitary gland is low after ovulation and gradually increases during the progression of the luteal phase [47]. This, together with simultaneously decreasing plasma progesterone concentrations, may explain the higher LH response to exogenous KP-10 during the second half of the luteal phase.

Intravenous administration of the KP antagonist p271 did not lower basal LH concentration and could not prevent or decrease the LH response to exogenous KP-10. This is quite surprising, as p271 showed inhibiting properties in other mammals. For example, central (ICV) infusion of p234 to female monkeys did not decrease basal GnRH, but there was some suppression of GnRH pulsatility and mean GnRH concentration. In mice, p234 did not alter GnRH neuron firing, but the antagonist blunted the stimulating effect of KP-10. Furthermore, in male rats, central administration of p234 did not alter basal LH levels, but it blunted the LH response to KP-10 that was coadministrated with p234 [22,23]. In prepubertal female rats, ICV administration of p234 was also associated with physical changes, such as delayed vaginal opening [23,48]. It can be concluded that this KP antagonist does not affect basal GnRH or gonadotropin levels but seems to affect the pulsatile secretion of GnRH, at least in mice and rats.

An important goal of our studies was to investigate whether the KP antagonist p234 could be applied as nonsurgical contraceptive method. Of course, this excludes ICV administration, and therefore, p271 had to be used: p271 is p234 tagged with a penetratin, which allows penetration of the peptide through the blood-brain barrier after peripheral administration.

The major part of the kiss1r is well conserved among species, but at the C terminus, there are differences between species [49]. There is about 75% identity between the human and canine kiss1r, e.g., and this increases to 90% when the unconserved part of the C terminus is ignored [12]. Because of interspecies differences in amino acid sequences of kiss1r, it is possible that p234 and p271 are not antagonists in all species. Whether a KP antagonist that works in one species will have a similar effect in another is even more difficult to predict given that the binding sites on the kiss1r are yet unknown.

In conclusion, KP-10 induced a significant rise of plasma LH concentration in both the follicular phase, the first and second half of the luteal phase, and anestrus, with the highest response during anestrus. This indicates a changing hypothalamic sensitivity to KP-10 during different estrous cycle stages and anestrus. When KP and/or its receptor are used as a target for nonsurgical contraceptive methods and estrus prevention, this knowledge is of great importance, as treatment timing may affect the outcome. Further, because the KP antagonist p271 showed no effect on either basal or stimulated plasma LH concentrations, p271, administered intravenously, cannot be used as KP antagonist in dogs.

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