The efficacy of kisspeptin antagonists p234 and p271 in blocking the response to KP-10 *in vivo* in the bitch and *in vitro* using flow-cytometry

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BACKGROUND: The neuropeptide kisspeptin and its receptor GPR54 play a crucial role in the reproductive function of mammals. Finding an efficient kisspeptin antagonist could be the first step towards a new method for non-surgical contraception in the dog. The aims of this study were to test kisspeptin antagonists 271 and 234 *in vitro* using flow-cytometry and to test p271 *in vivo* during different stages of the estrous cycle of the bitch. **METHODS:** ***In vitro:*** Chem-1 cells expressing the human GPR54 receptor were labeled with Fluo-3 AM, a fluorescence indicator for intracellular calcium, and examined by flow-cytometry. A concentration response study of hKP-10 and cKP-10 was designed to test if flow-cytometry is a reliable method for measuring intracellular calcium (as a measure for receptor activation) and to determine the optimal hKP-10 concentration. This data were then used to test the antagonists p234 and p271 *in vitro*. ***In vivo:*** P271 was tested during different stages of the estrous cycle of the bitch. In this study only the results in the follicular phase are discussed. Female dogs received 50 µg/kg/hour p271 by iv infusion for three hour. Two hours after the start of the infusion 0.5 µg/kg cKP-10 was iv administered. Controls received one iv injection of 0.5 µg/kg cKP-10. Blood samples before, during and after the peptide administration were collected for the determination of plasma [LH]. **RESULTS:** Both human and canine KP-10 gave a typical sigmoidal concentration-response curve *in vitro.* We found no antagonistic or agonist effect of peptide 234 and 271 *in vitro*. Furthermore, p271 was not able to reduce basal plasma LH concentrations and the LH response to cKP-10 *in vivo* in the bitch during follicular phase. **CONCLUSION:** P271 is probably not a good kisspeptin antagonist for the dog. Flow-cytometric calcium flux assays seem like a reliable method to study kisspeptin receptor activation *in vitro*.

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1. INTRODUCTION

Every year millions of dogs are euthanized in the world due to overpopulation. Preventing dogs to reproduce can solve overpopulation and may improve the welfare of dogs. Unfortunately, many so called ‘trap, neuter and release’ programs are not efficient enough in reducing the number of stray dogs. Furthermore, surgical castration and sterilization are time consuming and costly[1]. In addition there is a surgical and anesthetic risk for the animals and the intervention itself is not pain free. It is therefore desirable to find an animal-friendly alternative to surgical contraception, which hopefully can put an end to the overpopulation in various parts of the world. In finding a new method, the understanding of the reproductive system of dogs is of great importance. A promising target for a method to influence the reproductive system is the neuropeptide kisspeptin and its receptor.

Kisspeptins are structurally related peptides encoded by the *KISS1* gene acting through binding and subsequent activation of the G protein-coupled receptor GPR54[2,3]. The *KISS1*

gene encodes for a precursor polypeptide containing 145 amino acids. This peptide can be proteolytically cleaved to generate the biologically active 54 amino acid protein kisspeptin-54 (KP-54). KP-45 can be further cleaved to the lower molecular weight KP-14, KP-13 and KP-10 which show similar activities *in vitro* to KP-54[2,3]. All biologically active peptides contain a common carboxy terminal sequence, which is essential for binding with the receptor. KP-10 is the smallest peptide which results in full receptor activation[4]. The GPR54 receptor is a seven transmembrane G-protein coupled receptor that activates phospholipase C (PLC) after binding of kisspeptin. Activation of PLC leads to the conversion of phosphatidylinositol biphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3), which in turn induces the mobilization of intracellular Ca^{2+}. This results in changes in ion channel permeability, causing depolarization responses[5].

GPR54 and kisspeptins have proven to be both essential for activation of the hypothalamic-pituitary-gonadal (HPG) axis at puberty. Mutations in the *GPR54* gene in humans are associated with idiopathic hypogonadotropic hypogonadism (IHH), a rare clinical condition characterized by impuberism, low plasma gonadotropins and sex steroids concentrations and infertility[6,7]. This condition is also seen in both sexes of experimental mutant mice lacking functional GPR54[7,8] and mice with disrupted *kiss1* gene[9]. Mutant males have small testes, severe disruption of spermatogenesis and fail to develop secondary sex glands. Mutant females do not have an estrous cycle, very small uterus and ovaries with no mature antral follicle formation[10].

The regulatory mechanism of mammalian reproductive function consist of the HPG axis. In the brain, neurons in the medial preoptic area of the hypothalamus are responsible for pulsatile secretion of gonadotropin releasing hormone (GnRH). GnRH is delivered through the portal blood system to the anterior pituitary. In the anterior pituitary, GnRH stimulates gonadotropic cells to release the gonadotropic hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins act on the gonads by stimulating synthesis of the sex steroids (testosterone and estrogen). Within this axis, hormonal feedback loops exist, in which sex steroids regulate the secretion of GnRH and gonadotropin[10]. Direct action of estrogen on GnRH neurons is unlikely as the GnRH neurons do not express the estrogen receptor alpha (ERα)[11]. It now seems likely that the feedback signals from sex steroids on the GnRH neurons are acting via Kiss1 expressing neurons since virtually all of the Kiss1 neurons express ERα[12,13] and Kiss1 neurons are directly linked to GnRH neurons. Estrogen and testosterone are able to down regulate Kiss1 expression at the arcuate nucleus (ARC) of the hypothalamus in several species, including rats, mice, sheep, pig, monkey and human[5]. Contrary to the ARC, expression of Kiss1 neurons at the anteroventral periventricular nucleus (AVPV) of the hypothalamus increases due to estrogen positive feedback. This induce the preovulatory LH surge in female mouse and rat[12-15]. In addition to the transcriptional effects, sex steroids increase the GnRH neuronal sensitivity to kisspeptins[16] which cause maximal LH surge release preceding ovulation. Furthermore, systemic or intracerebroventricular (i.c.v) injection of kisspeptins into several animal species including rats, mice, sheep, primates and humans results in an increase of plasma gonadotropins and sex steroids which indicates that these peptides play a crucial role in reproductive function[5]. Actions of kisspeptins are conducted via activation of GnRH neurons since the effect of kisspeptins are eliminated by pretreatment with GnRH antagonists as demonstrated in rats, mice and monkeys[5]. The majority of GnRH neurons express the GPR54 receptor[17,18] which makes direct action of kisspeptin in activating the GnRH neurons assumable. Besides, Kiss1 expressing neurons make close contact with GnRH neurons[19,20]. Kisspeptin appears to be the missing link in the functioning of the HPG axis.

The role of kisspeptin and its receptor is unknown in the dog. However, preliminary studies (manuscript submitted) showed that kisspeptin signaling is present in the dog, as peripheral administration of both human KP-10 (hKP-10) and canine KP-10 (cKP-10) in anestrous bitches resulted in a massive increase of plasma LH, FSH and estradiol concentrations. This strongly suggests that kisspeptin and the GPR54 receptor play the same crucial role in reproductive function in the dog as it does in many other species. Finding an effective kisspeptin antagonist that could block the actions of kisspeptin would be a step toward a new method for non-surgical contraception of dogs. Roseweir *et al.*[4] developed the antagonist peptide 234 (p234) by amino acid substitution of KP-10 analogs. This peptide had the ability to block the effects of KP-10 *in vitro* and *in vivo* in monkey, sheep, rats and mice by central administration[4]. A variant peptide of p234 tagged with a penetratin extension to the NH_2 terminus was developed (p271) which potentially facilitated transfer across the blood-brain barrier[4]. The tag did not affect the binding affinity of p234 to the receptor and systemic administration of p271 successfully inhibited KP-10 stimulation of LH in male rats[21]. This antagonist seems to have a lot of potential and may be a possibility for a new method for non-surgical contraceptive in the dog. However previous *in vitro* study measuring the intra-cellular calcium using single-cell fluorescence microscopy showed no antagonistic properties of p271 (data not published) though it was difficult to draw firm conclusions because there were some problems with the experimental set-up. In this study we used a different *in vitro* testing method. The aims of this study are: 1) to test kisspeptin antagonist p271 and p234 *in vitro*, by flow-cytometric calcium flux, using celllines that express the human GPR54 and 2) to test kisspeptin antagonists p271 *in vivo* in the dog, during different stages of the estrous cycle of the bitch and anestrus. The hypothesis is that the administration of p271 suppresses the HPG axis in the bitch and that p271 and p234 both reduce the calcium flux induced by KP-10.

2. MATERIALS AND METHODS

2.1 Flow cytometric calcium flux assay

2.1.1 Cell labelling with fluo-3-acetoxylmethyl ester (Fluo-3 AM)

Chem-1 cells (Millipore BV, the Netherlands) stably expressing the human GPR54 receptor were used. Before cell labelling an external medium of Hank's Balanced Salt Solution (HBSS, Gibco, Life Technologies, The Netherlands) was supplemented with 10 mM HEPES (Gibco, Life Technologies, The Netherlands), 0.05% w/v Bovine Serum Albumin (BSA, A7030, Sigma Aldrich, The Netherlands) and 2.5 mM probenecid (P8761, Sigma Aldrich, The Netherlands, note: 500mM stock prepared freshly in 1N NaOH). Hereafter the solution is referred to as HBSS+++. Cells were counted and suspended with HBSS+++ to give a concentration of 5 x 10^6 cells/ml. 2 μM Fluo-3 AM (Molecular Probes, Life Technologies, The Netherlands, 1 mM stock prepared in dimethylsulfoxide (DMSO)) was added to the cell suspension and incubated for 1h at 37°C agitating and protected from light. After incubation, the cells were washed by adding up to 10 ml HBSS+++ followed by centrifugation (5 min, 150 xg) to remove excess of Fluo-3AM. Supernatant was discarded and the cell pellet was resuspended to 1 x 10^6 cells/ml in HBSS+++.

2.1.2 Flow cytometric calcium flux

Fluorescent labelled cells were examined on a FACSCalibur (Becton en Dickinson, The Netherlands). Changes in intra-cellular calcium were measured at a 10ms interval during 60 seconds, within a cell population using Fluo-3 AM ester as fluorescence indicator of intra-cellular calcium. Fluo-3 is coupled to an acetoxymethyl ester (AM) which masks the calcium-binding region of the molecule and makes it lipid soluble allowing it to cross the cell membrane. Following de-esterification by non-specific cytoplasmic esterases, the Fluo-3 is trapped inside the cell and the molecule can bind calcium[22]. Fluo-3 AM provides intense fluorescence upon binding calcium, with an intensity that is proportional to [Ca^{2+}], detected at a maximum emission at 526 nm which can be monitored by FL1, log setting, (green, 526 nm band pass) sensors in FACSCalibur.

2.1.3 Concentration response study KP-10

To test if flow-cytometry is a reliable method for measuring the response of KP-10 and to determine the optimal KP-10 concentration to test later with the antagonists, a concentration response study was designed. The concentrations 10^{-6} M to 10^{-13} M of hKP-10 (YNWNSFGLRF-NH2, American peptide company, USA) and the concentrations 10^{-5} M to 10^{-13} M of cKP-10 (YNWNVFGLRY-NH2, American peptide company, USA) were used in this concentration response study. Dilutions were made 10 times more concentrated in HBSS+++ to end up with the intended concentrations when adding the KP-10 to the volume cells. 225 μl labeled cells (1*10^6 cells/ml HBSS+++) were pipetted in high-optical clarity 5ml polystyrene Round-Bottom Tubes (Falcon 352052, Becton en Dickinson, The Netherlands). The cells were aspirated by the flow cytometer and fluorescence of the cells was measured for a period of 60 seconds. During the first 10-15 seconds the baseline fluorescence of the Fluo-3–Ca^{2+}-complex was measured. Thereafter the tube was shortly removed and 25 μl KP-10 was added after which aspiration and analyzing continued. For each concentration of KP-10 a new tube with cells was used. KP-10 concentrations were

tested in increasing order starting with the lowest concentration (10^{-13} M) to prevent contamination of residuals of high concentrations in the internal system of the flow-cytometer. Negative control consisted of the addition of 25µl HBSS+++ after 10-15 seconds. Experiments were repeated 3-6 times. cKP-10 dilutions were freshly prepared with every experiment. Because there was too little dry matter of hKP-10 to prepare the dilutions freshly with every experiment, the concentration of $1*10^{-4}$ M hKP-10 was stored after the first experiments in -20°C. On the next experimental days new dilution series of hKP-10 were made out of this concentration.

2.1.4 Antagonists p234 and p271

The concentrations 10^{-5} M to 10^{-13} M of both p271(RRMKWKK(D-A)NWNGFG(D-W)RF-NH2, American peptide company, USA) and p234 (ac(D-A)NWNGFG(D-W)RF-NH2, American peptide company, USA) were tested in the same manner as human and canine KP-10 concentration response study to see if these peptides could give an intrinsic calcium flux. Next it was tested whether p271 and p234 could compete with hKP-10 (10^{-8} M) for its receptor. Because 10^{-8} M hKP-10 was located on the steep slope of the concentration response curve this concentration was used in combination with the various concentrations of the antagonists. Dilutions were made 10 times more concentrated in HBSS+++ to end up with the intended concentrations after addition to the volume of cells. 25µl hKP-10 10^{-8} M was mixed with 25 µl antagonist. 200 µl labeled cells ($1*10^{6}$ cells/ml) were aspirated by the flow cytometer and after 10-15 seconds of baseline measurements the tube was shortly removed and the 50 µl of the mixture of hKP-10 and antagonist was added after which the measurement continued. The various concentrations (10^{-5} M to 10^{-13} M) of the antagonists were tested in increasing order starting with the lowest concentration (10^{-13} M). Experiments were repeated 3 times. The negative control consisted of adding a mixture of 25µl KP-10 10^{-8} M with 25 µl HBSS+++ to 200 µl cells. The negative control was repeated (3-6 times) in each experiment (with the exception of 1 experiment because of too few cells).

2.1.5 Statistical analysis

The samples were analyzed using the software FlowJo (Treestar). In the scatter plot of forward scatter (FSC) vs. side scatter (SSC) the living cell population was gated and analyzed. Changes in the fluorescence intensity of the Fluo-3–Ca^{2+}-complex were measured on the FL1 channel (log, voltage 475) and fluorescence was plotted versus time. Two time gates were set, 1 gate (named P4) before the addition of any stimulus (10 seconds, t=0 to t=10) and 1 gate (named P5) after the addition of the stimulus (30 seconds, t=30 to t=60). The difference between the mean fluorescence of gate P5 and gate P4 was presumed to be the response of the stimulus. A Kolmogorov-Smirnov test was used to check if the data was normally distributed. The data of the hKP-10 concentration response study was normally distributed. For the cKP-10 concentration response study we couldn't check for normality because of too few experiments. However we have no reason to assume non-normal distribution for the cKP-10 data. Therefore we used a one-way ANOVA followed by a post hoc Bonferroni's Multiple Comparison test to identify statistical differences between the various concentrations. For the antagonist study, the mean of the negative controls (only KP-10 10^{-8}) was calculated for each experiment. Because there were too few experiments to check if we could assume normal distribution we used non-parametric statistical tests. The differences between the antagonist concentrations and the negative control (KP-10 only) were determined using Kruskal-Wallis test. The statistical significance level was set at $p<0.05$. Statistical analysis were performed using the program GraphPad Prism 5.03. No further

statistics were needed to determine if p234 and p271 gave intrinsic calcium stimulation, based on the plots made by flowJo.

2.2 Antagonist p271 in the dog

2.2.1 Animals

Eleven healthy Beagle bitches (3-10 years of age) were used in this study which was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University (DEC.2012.III.028)**.** The dogs were born and raised at the Department of Clinical Science of Companion Animals and were habituated to laboratory environment and procedures such as the collection of blood samples. They were housed in pairs in indoor-outdoor runs and fed a standard commercial diet once a day. Water was available ad libitum.

2.2.2 Peptides

cKP-10 and p271 were synthesized by American Peptide Company (USA). cKP10 was dissolved in sterile 0,09% NaCl, stored at -20°C and thawed on the day of the experiment. P271 was stored solid at -20°C and dissolved in sterile 0,09% NaCl on the day of the experiment.

2.2.3 Experimental design

The efficacy of the antagonist p271 was investigated in four stages of the estrous cycle: the follicular phase, twice in the luteal phase (both the pituitary independent stage and the pituitary dependent stage) and once in anestrus. All dogs were examined three times per week for the presence of vulvar swelling and serosanguinous vaginal discharge, which were considered to signify the onset of proestrus[23,24]. Plasma progesterone concentration was measured thrice weekly from the start of proestrus until the day which it reached values above 13-16 nmol/L when ovulation was assumed to occur[24]. Experiments in the follicular phase were when plasma progesterone concentration was still beneath 1 ng/ml. The pituitary independent phase, the pituitary dependent phase and the anestrus were assumed to be respectively around day 12, 42 and 100 days after ovulation.

<u>*Test group*</u>

P271 was administered by iv infusion (50µg/kg/hour) for three hours to six Beagle bitches in the above described stages of the estrous cycle. Two hours after starting the p271 infusion, one iv injection of cKP-10 (0.5 µg/kg) was administered. Preliminary studies identified 0.5 µg/kg cKP-10 as the lowest dose with the optimal effect. Blood samples were collected from the jugular vein in heparinized tubes at 40 and 0 minutes before starting the infusion to determine basal values of LH and at 30, 60, 90, 120, 130, 140, 160 and 180 minutes after starting the p271 infusion.

<u>*Controls*</u>

The control study consisted of a single iv injection of cKP-10 (0.5 µg/kg) to six Beagle bitches in the different stages of the estrous cycle (1 dog was both used in the test group as well as in the control group). Blood samples were collected from the jugular vein in heparinized tubes at 40, 30, 20, 10 and 0 minutes before cKP-10 administration and at 10, 20, 30, 40, 50 and 60 minutes after cKP-10 administration. Plasma samples were stored at -20°C until assayed. Figure 1 shows a schematic representation of the experimental design.

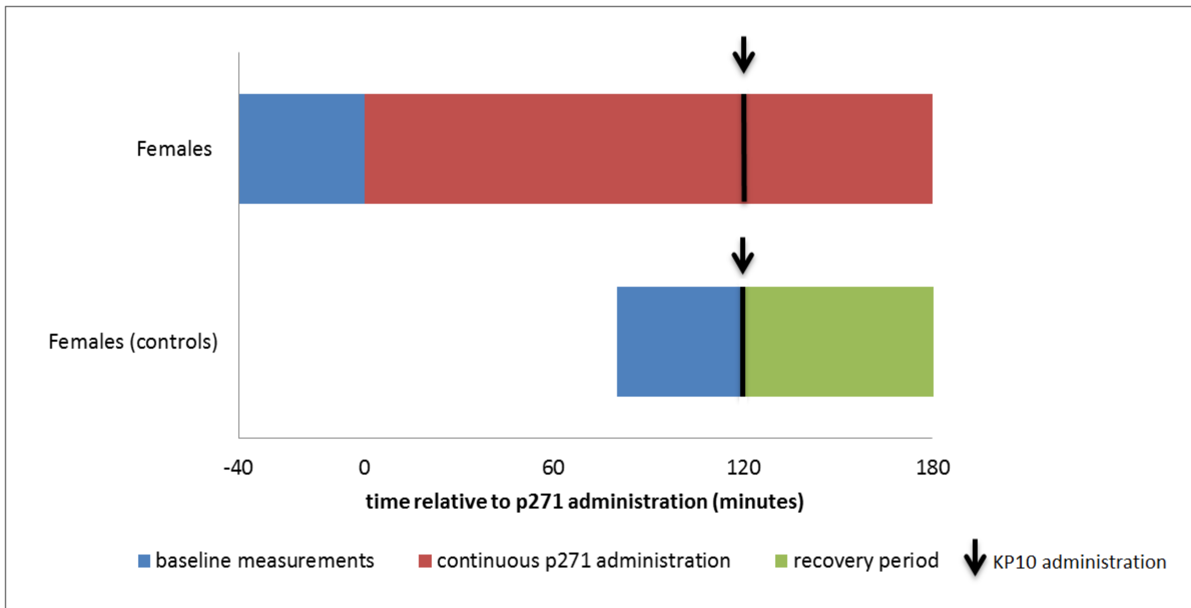

Figure 1. Schematic representation of the experimental design

2.2.4 Hormone determination

Plasma LH concentrations were determined using a heterologous radioimmunoassay (RIA) as described previously[25]. The intra-assay and inter-assay coefficients of variation for values above 0.5 µg/L were 2.3% and 10.5%, respectively. The lowest detectable amount of LH was 0.3 µg/L.

2.2.5 Statistical analyses

In this paper only the data of the follicular phase were analyzed because not all plasma samples of the other phases were assayed yet. Statistical analyses were performed with the program GraphPad Prism 5.03. A Kolmogorov-Smirnov test was used to check if the data was normally distributed. Because not all data were normally distributed, nonparametric tests were used. To see if LH concentrations increase after cKP-10 injection a Kruskal-Wallis test was performed followed by a post hoc Dunn's Multiple Comparison test for both control and test group independently. For the control group the average of the basal values t=-30 and t=0 was compared to t=10, 20, 40 and 60. For the group receiving p271 the average of basal values t=90 and t=120 was compared to t=130, 140, 160 and 180. These timepoints are congruent in both groups to the cKP-10 injection.
For the group receiving p271 another Kruskal-Wallis test was performed on the average of basal values before p271 administration (t=-40 and t=0) compared to t=30, 60, 90 and 120 to test if p271 caused a decrease in basal plasma LH concentrations.
The area under the curve (AUC) was calculated for each individual dog from time of cKP-10 injection to 60 minutes after cKP-10 injection minus basal values. Basal values were calculated for each individual dog and consist for the dogs in the control group of the average of t=30 and t=0. For the dogs treated with p271 basal values consisted of the average of t=90 and t=120. The AUC of control and test group was compared to see if there was a difference in KP-10 response between the groups using a two tailed t-test (data were normally distributed). The statistical significance level was set at $p<0.05$.

3. RESULTS

3.1 In vitro concentration response study with human and canine KP-10

In this study we examined the calcium response (fluorescence as in indicator for intracellular calcium) to various concentrations hKP-10 and cKP-10 using flow cytometry. The results of one experiment are presented in Figure 2 as a typical example of calcium flux caused by hKP-10. The figures show that as the concentration increased the number of cells which react with a calcium flux (high fluorescence) increased and the cells react within a shorter time. The same analysis and figures were made with cKP-10 concentrations and the same could be concluded. The difference of the mean fluorescence of gate P4 (basal fluorescence) and gate P5 (after the addition of the stimulus) was the response of the stimulus. Both human and canine KP-10 gave a typical sigmoidal concentration-response curve when concentration was set out against fluorescence response (difference of the mean fluorescence of gate P5 and P4) (Fig 3). In the hKP-10 concentration response study, the concentrations 10^{-8}, 10^{-7} and 10^{-6} were significant higher from the negative control and 10^{-7} and 10^{-6} were significantly higher than 10^{-8}. The concentration 10^{-8} was the lowest concentration which gave a significant response and therefore, this concentration was chosen to use in combination with p234 and p271 (see 3.2). In the cKP-10 concentration response study, the concentrations 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} were significant higher from the negative control and 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} were significantly higher than 10^{-9}.

3.2 In vitro study of the antagonists p234 and p271

Figure 4 shows the results of the competition binding study. There was no significant difference in calcium-flux between the negative control (hKP-10 only) and the various concentrations of p234 and p271 in combination with hKP-10. Peptides 234 and 271 gave both no intrinsic calcium stimulation.

3.3 P271 in the follicular phase of the estrous cycle of the dog

Figure 5 shows the results of the LH concentrations of all the dogs plotted against time (relative to cKP-10 injection in the control group and relative to the start of p271 infusion in the test group). The administration of cKP-10 to the control dogs caused a significant increase of [LH] and reached maximum concentration 10 minutes after the injection. Over the next 50 minutes [LH] gradually declined and from 20 minutes after the injection no statistical difference was found compared with basal values. The administration of cKP-10 to the dogs receiving p271 also caused a significant increase of [LH] and reached maximum concentration 10 minutes after the injection. No significant difference was found from 20 minutes after the cKP-10 injection compared to basal values. P271 did not cause a significant decrease in baseline LH concentrations. Figure 6 shows the LH response to cPK-10 and the integrated LH secretion (calculated as AUC) during 1 hour after cKP-10 injection for both groups. There was no significant difference between the AUC of the control dogs and the dogs receiving p271. These results indicate that p271 was not able to reduce the LH response to cKP-10.

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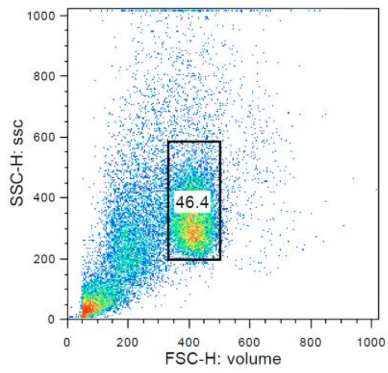

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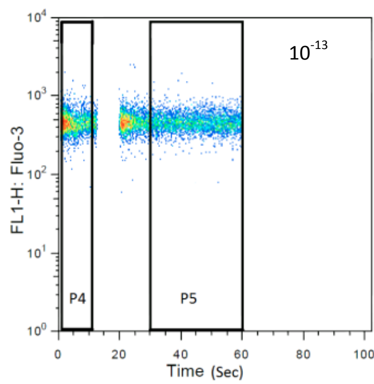

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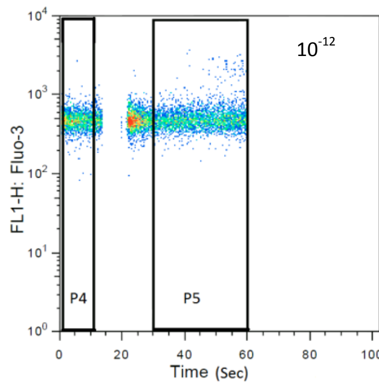

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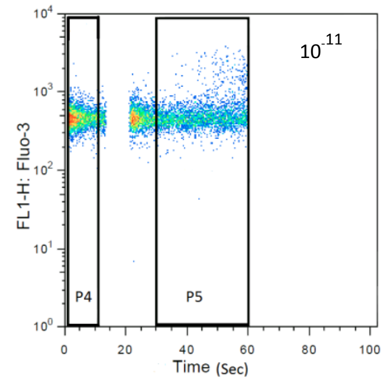

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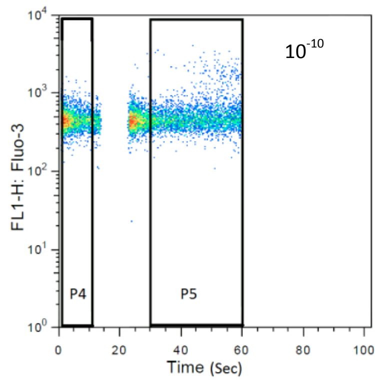

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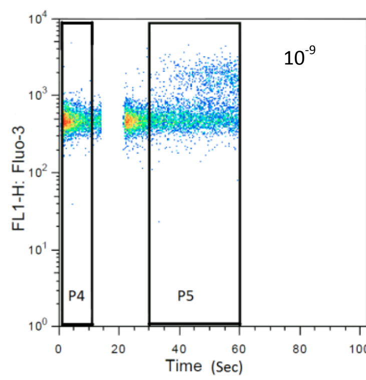

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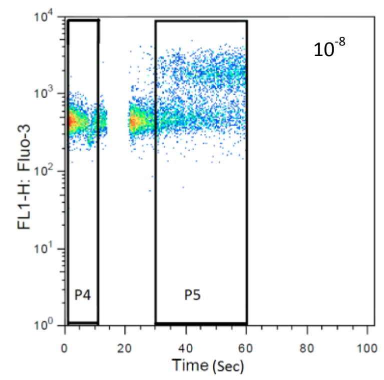

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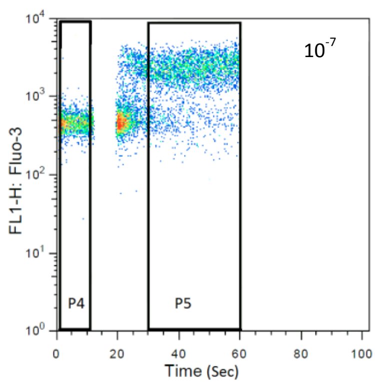

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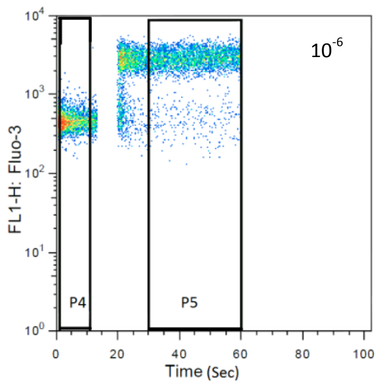

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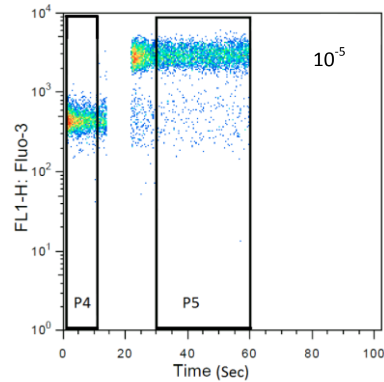


Figure 2. Results of one experiment with hKP-10. **A,** Fluo-3 AM labeled Chem-1-GPR54 cells on the FSC vs. SSC plot with the living cell population gated for further analysis. Every dot represents one cell. In figures **B-J** the fluorescence of the gated cell population is plotted versus time. Every dot represents the fluorescence of one single cell. The gap between approximately 15 and 20 seconds is caused by the short removal of the tube with cells for the addition of the stimulus. The difference of the mean fluorescence of gate P4 (basal fluorescence) and gate P5 (after the addition of the stimulus) was the response of the stimulus. **B,** Negative control. **C-J,** hKP-10 concentrations 10^{-13} to 10^{-6} M in increasing order.

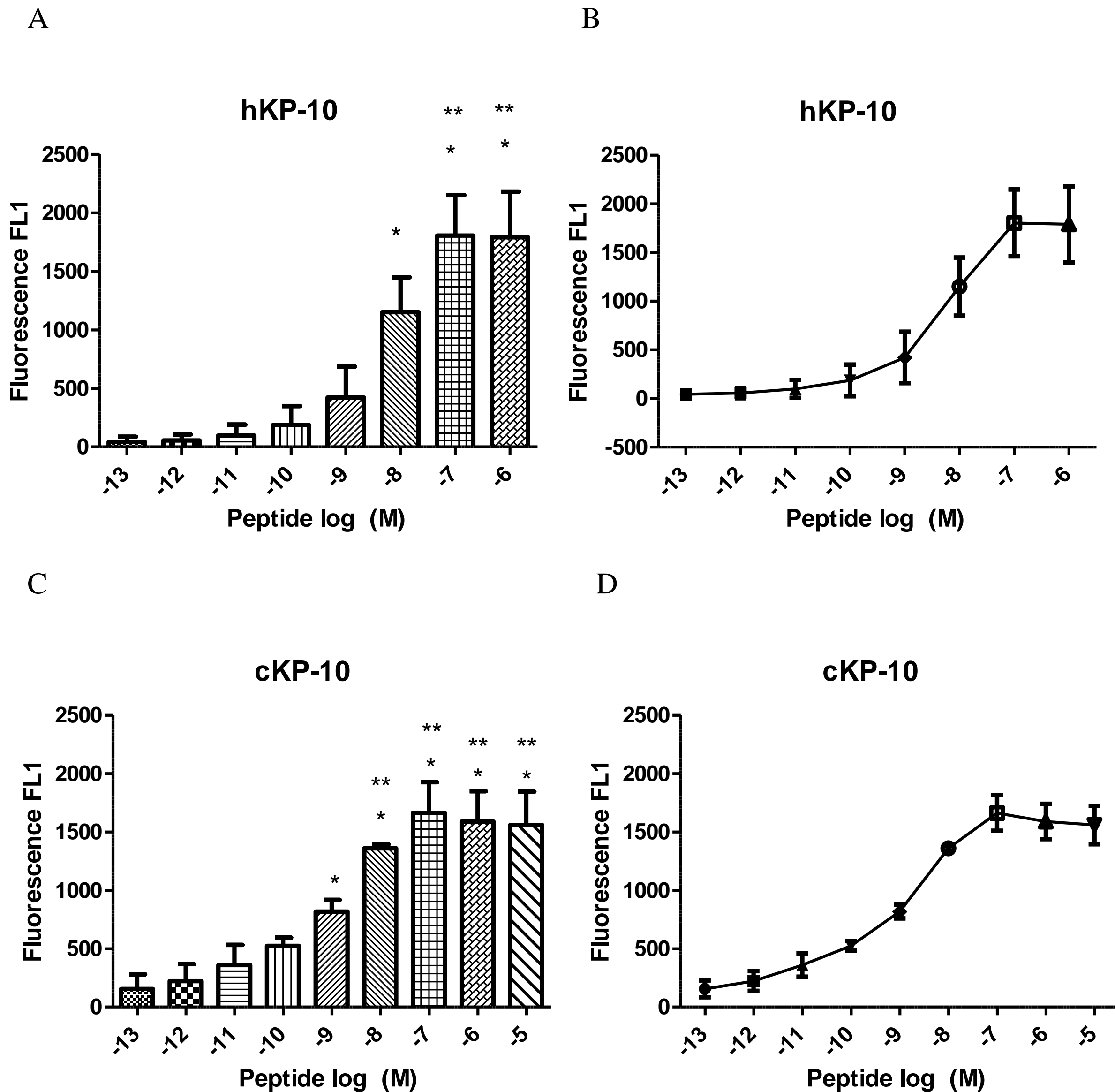
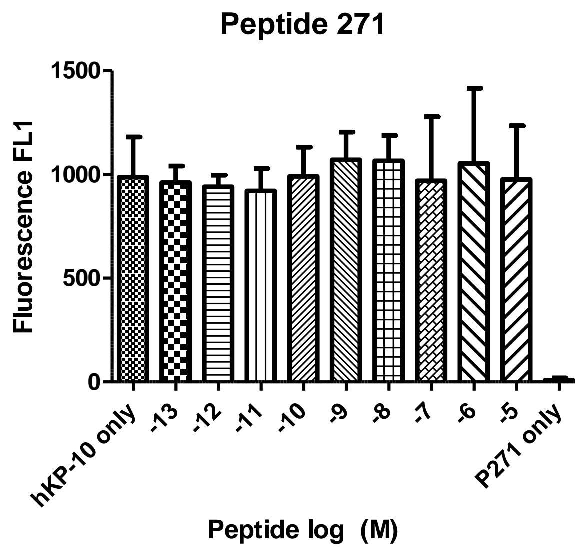


Figure 3. A, Diagram of the results of concentration response study with hKP-10. **B,** Concentration response curve with hKP-10. **C,** Diagram of the results of concentration response study with cKP-10. **D,** Concentration response curve with cKP-10. Both human and canine KP-10 gave a typical sigmoidal concentration response curve. In the diagrams the significance with the negative control is indicated with an asterisk (*) and the significance with 10^{-8} (hKP-10) and 10^{-9} (cKP-10) is indicated with double asterisk (**).

A



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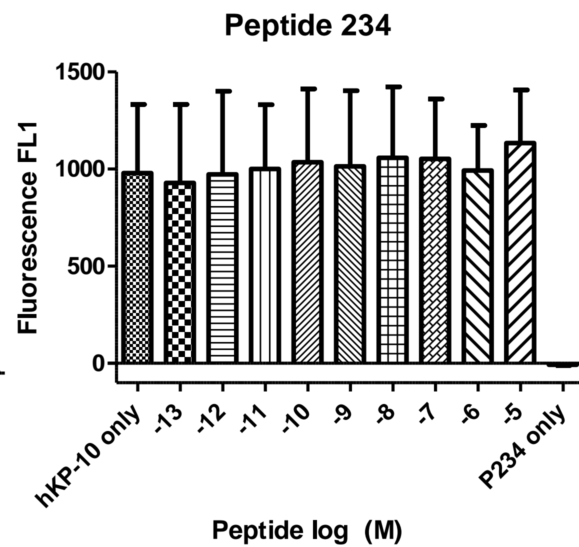
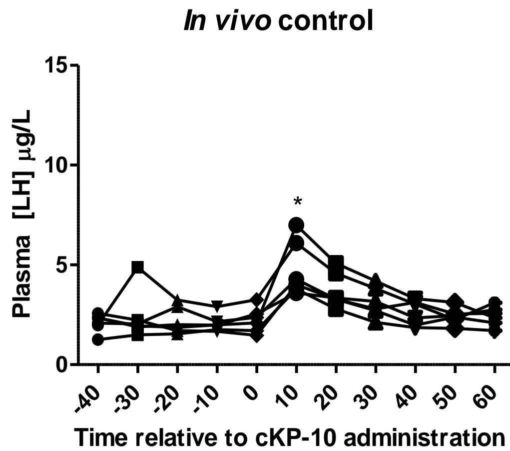


Figure 4. The effects of p271 and p234 in various concentrations on calcium flux caused by hKP-10 10^{-8} M. P234 and p271 could both not reduce the calcium flux caused by hKP-10 10^{-8} M. Both peptides alone had no intrinsic calcium flux (highest concentration of 10^{-5} M shown in this figures). **A,** P271 in various concentrations in competition with hKP-10 10^{-8} M. **B,** P234 in various concentrations in competition with hKP-10 10-8 M.

A



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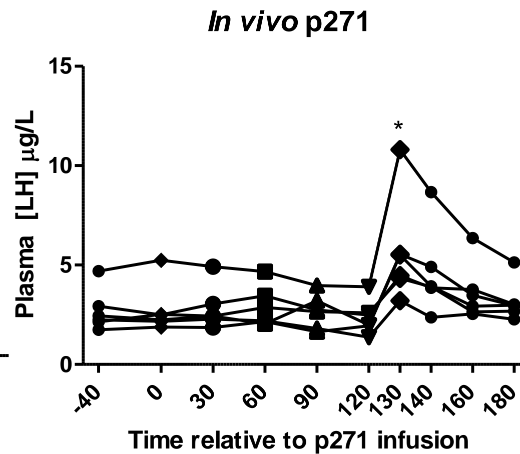
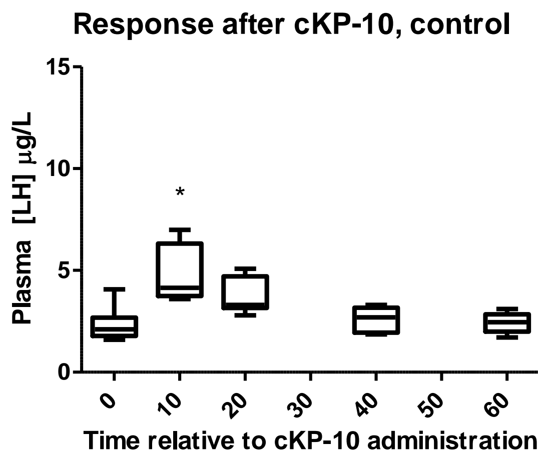
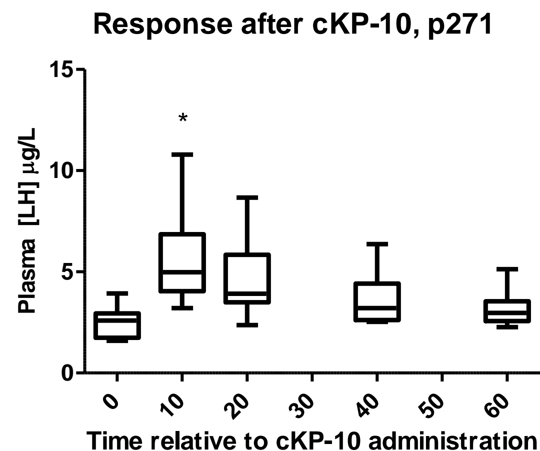


Figure 5. The results of experiments to the efficacy of the antagonist p271 in preventing KP-10 response in the follicular phase of the estrous cycle of the dog. Each line represents one single dog. The control group received one iv injection with cKP-10 (0,5 µg/kg). The dogs in the experimental group received the antagonist p271 (50 µg/kg) by constant iv infusion and one iv injection with cKP-10 (0,5 µg/kg) was administrated 2 hours later. **A,** LH significantly increased 10 minutes after cKP-10 administration in the control dogs and also significantly decreased over the next 50 minutes **B,** In the dogs receiving peptide 271, LH also significantly increased 10 minutes after cKP-10 injection and then gradually declined. Significance compared with basal values indicated with an asterisk (*).

A



B



C

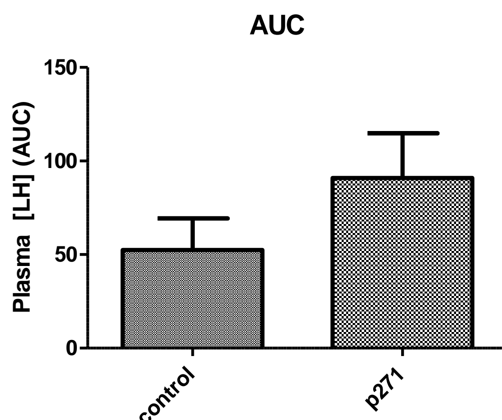


Figure 6. A, LH response after cKP-10 administration in the control group. Median is plotted alongside with the 0, 25, 75 and 100 percentiles. T=0 represents the average of t=-30 and t=0. **B,** LH response after cKP-10 administration in the group receiving p271. Median is plotted alongside with the 0, 25, 75 and 100 percentiles. T=0 represents the average of t=90 and t=120**.** Significance compared with basal values indicated with an asterisk (*).

C, AUC of the [LH] between cKP-10 injection to 60 minutes afterward minus the basal values (average of t=-30 and 0 for control, t=90 and t=120 for p271). No significant difference was found between the AUC of the controls and the AUC of the dogs receiving p271.

4. DISCUSSION

Finding an alternative to surgical contraception is necessary to solve the problem of canine overpopulation around the world. Because the neuropeptide kisspeptin and its receptor GPR54 play a crucial role in the reproductive system of mammals, finding an efficient kisspeptin antagonist could be the first step towards a new method for non-surgical contraception. In this report the peptides 271 and 234 were tested *in vitro* using flow-cytometry on a cell-line expressing the human GPR54 receptor. Besides, the antagonist p271 was tested *in vivo* in different stages of the estrous cycle of the bitch. The hypothesis was that both peptides would inhibit kisspeptin-stimulated intracellular calcium release *in vitro* and that p271 would suppress the HPG axis in different estrous stages of the bitch *in vivo*.

We were not able to find an antagonistic effect of p234 and p271 by flow cytometry. Both peptides could not inhibit the hKP-10 induced calcium release. Because p271 had our specific interest because of its ability to cross the blood brain barrier, we tested this peptide first on its antagonistic effects. After we found no antagonistic properties we decided to test p234, with the idea that p271 maybe was not able to bind to the receptor due to his penetratin tag. Unfortunately p234 also showed no antagonistic properties. These results are in contrast with previously published data. With the search for kisspeptin antagonists Roseweir *et al.*[4] used an inositol phosphate (IP) stimulation assay to evaluate the antagonistic properties *in vitro*. Activation of the GPR54 receptor results in an increase in IP_3 followed by the increase in intracellular calcium. IP_3 production is very rapid and transient before it is metabolized to IP_2, $IP_{(1)}$ and finally myo-inositol[26]. In the mentioned study lithium chloride (LiCl) is added to the cells to block the degradation of IP to myo-inositolin allowing it to accumulate in the cell, where it can be measured as a substitute for IP_3 (figure 7). In that assay, CHO/GPR-54 cell monolayers were stimulated with 10^{-8} M KP-10 alone or in combination with antagonists (10^{-10} M - 10^{-6} M) for 1 hour. P234 was found to be a potent kisspeptin antagonist as it inhibited KP-10 stimulated inositol phosphate and had no intrinsic IP production. P234 inhibited 10nM KP-10 stimulation of IP by 93% with an IC50 of 7nM[4]. In our experiments we used the same KP-10 concentration (10^{-8} M) in combination with a greater dilution series of p234 and p271 (10^{-13} –10^{-5}). We used a different cellline and instead of IP we used intracellular calcium as an indicator for receptor activation. Because IP_3 induces the mobilization of intracellular calcium after receptor activation it is expected that if p234 inhibits IP_3 it would also inhibit the calcium flux. Given the good results of the concentration response study flow-cytometry seems a good way of measuring the calcium flux. However, for the antagonist binding study we had no positive control. For future studies, the use of a positive control such as the PLC inhibitor U-73122 (a universal antagonist) in combination with KP-10 could be very useful. Roseweir *et al.*[4] stimulated the cells during one hour with both KP-10 and antagonists before measuring IP. We mixed KP-10 and the antagonist before adding that mixture to the cells and measurements started from the moment of addition to the cells. It is therefore possible that p234 must have longer time in order to properly bind to the receptor. However, Roseweir *et al.*[4] also investigated the affinity of p234 for the receptor and found that p234 bound with the same affinity as KP-10. When ^{125}I-labeled KP-10 was competed with either KP-10 or p234 they both bound with an affinity of 2nM[4]. When KP-10 and p234 have the same binding affinity for the receptor, longer incubation time of p234 with the cells should not be necessary since we found calcium flux a few seconds after the addition of KP-10 to the cells.

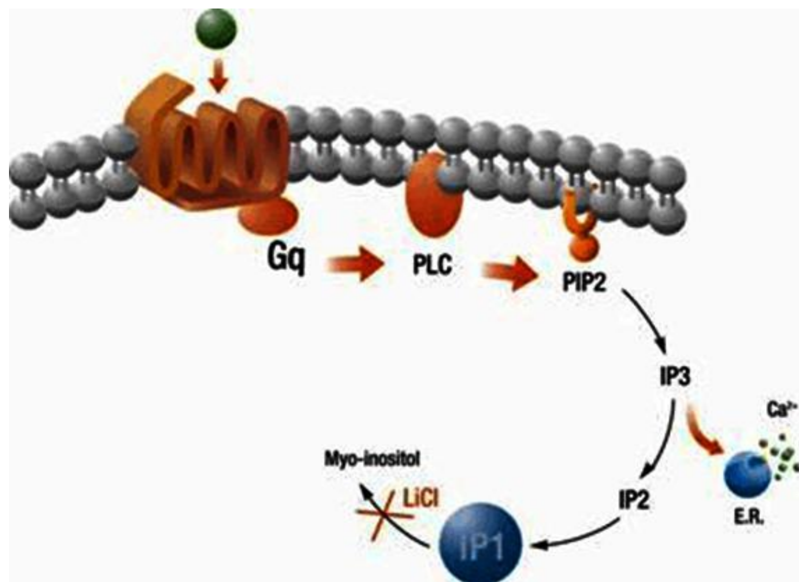


Figure 7. Cascade after receptor activation[26].

With regards to the *in vivo* study only the results of the follicular phase are discussed in this paper. We were not able to find an antagonistic effect of p271 in this phase of the estrous cycle. The AUC was not different for the group treated with p271, therefore we concluded that p271 was not able to reduce the LH response to cKP-10 in the bitch during the follicular phase. This is in contrast to previously published *in vivo* results finding antagonistic effects of p271 *in vivo*. There is only one study published in which p271 is systemically administered in contrast to other *in vivo* antagonist studies, which describe central peptide administration[21]. Intra-peritoneal injections of p271 inhibited the LH response to KP-10 in male rats following both i.c.v. and systemic administrations of KP-10[21]. Further, all published *in vivo* results of p234 and p271 are after central administration[4,21,27]. Central infusion of p271 resulted in a decrease in LH pulse frequency in OVX ewes. Besides, p271 inhibited E2-induced LH surge when administrated centrally to OVX ewes[27]. In our study the administration of p271 did not resulted in the lowering of basal LH, or a reduction of LH response to cKP-10. One explanation could be that an excess of cKP-10 is present, so that the antagonist is unable to bind to the receptor. However, we used the lowest optimal dose of KP-10 determined in preliminary studies. Another possibility could be that the dosage of p271 we used was too low. In the rat study three ip boluses of 5nmol were injected at 120, 60 and 0 minutes before KP-10 administration. This corresponds to approximately 25 µg/kg/hour. We use a dosage of 50 µg/kg/hour and therefore it is unlikely that the dosage of p271 was too low. It is possible that the reproductive mechanisms in the dog are different from that in rats. Neurokinin is a co-factor in the kisspeptin signaling pathway which seems to intensify the kisspeptin signal[28]. It is expected that this co-factor is more important in some animal species than it is in other species for reproductive function. The administration of a neurokinin receptor antagonist to male Beagle dogs caused a rapid decrease in reproductive hormones[29]. Switching off neurokinin apparently causes kisspeptins to work not properly anymore in the dog. In contrast, male and female rats administrated the same neurokinin antagonist did not exhibit similar findings when compared to the dog[29]. It seems likely that this system of co-factors in the kisspeptin signaling is less developed in rats but plays a substantial role in canine reproductive function.

Before drawing firm conclusions about p271, the results of the other cycle phases should be evaluated since LH response to KP-10 administration also differ between the phases of the

bitch (data not published), woman[30] and ewe[27]. Therefore it is also possible that the response to the antagonist varies between phases of the estrous cycle.

To date six possible kisspeptin antagonists have been developed: five peptide antagonists (peptides 230, 234, 271, 273 and 276) and one small molecule antagonist (compound 15a)[31]. In this study we tested only the peptide antagonist p234 (and its variant with a penetratin tag). A good method for calcium flux assay as a measure for receptor activation is necessary to test other kisspeptin antagonists. Given the good results of the concentration response study flow-cytometry seems a good way of measuring the calcium flux. It would be preferred to use a cellline expressing the canine GPR54 receptor, but this is not yet available.

5. CONCLUSION

P271 is probably not a good kisspeptin antagonist for the use in the dog. The results in the other phases of the estrous cycle should be evaluated in order to establish with certainty. Other kisspeptin antagonists should be further investigated and tested. Flow-cytometric calcium flux assays seems like a good method to investigate receptor activation *in vitro*.

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