KISSPEPTIN *IN VITRO*: A CONCENTRATION RESPONSE STUDY AND TESTING OF KISSPEPTIN ANTAGONIST P271

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ABSTRACT

Kisspeptin is an important regulator of the hypothalamic-pituitary-gonadal axis in mammals. It acts on the GnRH neurons trough the GPR54 receptor initiating GnRH release. The aim of this study is to determine the optimal concentration of kisspeptin-10 to activate the GPR54 receptor *in vitro*. This data is then used for *in vitro* testing of the kisspeptin antagonist P271. The GPR54 receptor is a G-protein coupled receptor, which when activated raises the intracellular calcium level. We used a single-cell fluorescence microscopy on Chem-1 cells (expressing the human GPR54 receptor) loaded with the calcium-sensitive fluorescent dye Fura-2. We were unable to create a reproducible concentration-response study, however we found two concentrations of kisspeptin-10 that gave a reproducible peak (10^{-6}M and 10^{-7}M). These concentrations were used to test the antagonist P271. In this study peptide P271 showed agonistic properties and no antagonistic properties. This could be because of the method used in these experiments.

1. INTRODUCTION

For many reasons there is a need for a non-surgical contraceptive for dogs and cats. In developing countries, but also in the United States and in parts of Europe stray dogs and cats are a huge problem. They can be a reservoir for transmissible diseases and form a danger for humans and domesticated animals.[1]To limit pet reproduction spaying and neutering of stray animals and also of animals not meant for breeding is advised. For stray animals time, money and logistics are an issue (e.g. stray animals need to be caught first). But also pet owners can be reluctant to spay or neuter their animal, for several reasons. Pet owners tend to feel compassionate with their animals, feel it to be an unnecessary surgery/procedure and costs (especially ovariohysterectomy) are a common complaint.[1] This although spayed and neutered dogs have a significantly longer lifespan; 26,3% in females and 13,8% in males. This might be because of increased health benefits. In spayed dogs uterine pathology (e.g. pyometra) is virtually non-existent. Also spaying is an important part of treatment in case of diabetes mellitus in intact females. In males neutering is as well a therapeutic as preventive measure for conditions like benign prostate hyperplasia, chronic prostatitis, perineal adenomas and perineal hernias.[2] Sterilized dogs are less likely to die of infectious disease, trauma, vascular and degenerative disease, and more likely to

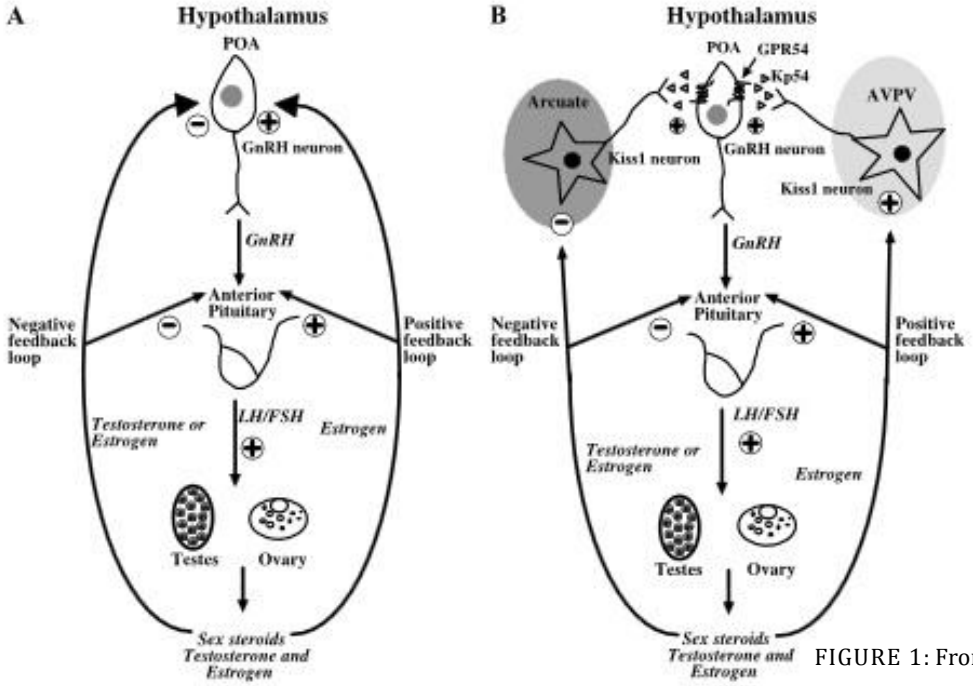

FIGURE 1: From Colledge (2008)[6]

die from neoplasia and immune-mediated disease.[3] All these reasons contribute to the need for a non-surgical (reversible) way of contraception in our companion animals.[1, 4]

The mammalian reproductive system is regulated by hormones in a feedback system called the hypothalamic-pituitary-gonadal axis (Fig 1.A). Kisspeptins and the GPR54 receptor have a significant role in the feedback system (Fig 1.B). Kisspeptins act as a key mediator of GnRH release in the hypothalamus. They act directly on the GnRH neurons in the hypothalamus via the GPR54 receptor to stimulate GnRH release. Negative feedback of sex steroids is provided via down regulation of *kiss1* mRNA levels in the arcuate nucleus of the hypothalamus, whilst positive feedback (e.g. oestrogen) is provided by up regulation of *kiss1* in the AVPV region (in rodents) and ARC region (in sheep and primates) of the hypothalamus.[4, 5]

The *kiss1*-gene encodes a polypeptide consisting of 145 amino acids. This peptide is a precursor peptide which gives rise to a second precursor peptide of 126 amino acids which is then proteolytically cleaved and modified to form a C-terminal amide of 54 amino acids. This is the first active form of the peptide KP-54. KP-54 can be cleaved even further, into fragments of 14, 13 and 10 amino acids in lenght. All these fragments are biologically active.[5]

The GPR54 receptor has an important role in human fertility; loss of function of the GPR54 receptor (due to mutations in the kisspeptin receptor gene) is found in patients suffering from IHH (idiopathic hypogonadotropic hypogonadism). Also in other mammals it is confirmed that the GPR54 receptor is of significant value. Mice with a mutation on the GPR54 gene failed to develop pubertal sexual development and remain sterile. In mice with a mutation of the *kiss1* – gene similar symptoms develop but these mice remain able to produce gonadotropic hormones after kisspeptin injections.[6]

All of this indicates that kisspeptin and the GPR54 receptor play a center part in supporting mammal fertility.

The GPR54 receptor is a G-protein coupled receptor, and works via the phospholipase C pathway. The G-protein

is in this case activated by kisspeptins on the GPR54 receptor and the membrane bound phospholipase C is then activated. Phospholipase C then cleaves inositol phospholipid (a component of the cell membrane, cytosol side) in two messenger molecules; inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses into the cytosol where it will encounter the endoplasmic reticulum and bind to and open Ca^{2+} channels. This causes a sharp rise in the cystosolic Ca^{2+} concentration. The free Ca^{2+} in the cytosol together with DAG (which is still on the cytosol side of the cell membrane) recruit an enzyme called protein kinase C. Active protein kinase C phosphorylates a set of intracellular proteins depending on the cell type.[7] In case of the GnRH-1 neurons this cascade causes depolarization of the neuron, increasing the frequency of GnRH release.[8]

By measuring the intracellular Ca^{2+} concentration we have a reliable method for measuring the activation of the GPR54 receptor. By this means we have an *in vitro* method of determining activation of the GPR54 receptor. This way *in vitro* testing of antagonists can be commenced.

Because of the problems with surgical contraceptives (as mentioned above) it would be a breakthrough to be able to peripherally block the GPR54 receptor. If blocking the GPR54 receptor leads to a complete block of the HPG – axis, then a kisspeptin antagonist would be a perfect candidate for a non surgical contraceptive in our companion animals. To date five possible kisspeptin antagonists have been developed: four peptide antagonists (peptides 230, 234, 273 and 276) and one small molecule antagonist (compound 15a).[5]

However, to date there is no data available about kisspeptin signaling in the dog. Preliminary studies indicated the presence of the kisspeptin system in the dog (manuscript under construction), but more studies are needed to explore the role of kisspeptin in the canine reproductive system.

The aim of study is to examine kisspeptin receptor activation *in vitro* to create a reliable experimental setting to test kisspeptin antagonists, eventually on cells that express the canine GPR54 receptor.

2. MATERIALS AND METHODS

2.1 Peptides

Human Kisspeptin -10 Metastin (45-54) produced by the American peptide company. Sequence: YNWNSFGLRF-NH2.[5]

Kisspeptin antagonist P271 (P234 with a penetratin tag) produced by the American peptide company. The chemical structure is RRMKWKKY(DA)NWNGFG(D-W)RF-NH2.[5]

Peptide solutions were prepared daily. Kisspeptin was first dissolved in deionized water (Milli-Q) to create a stock solution of 0,5 mM. Further dilutions were made in extern medium. P271 was first dissolved in DMSO (stock solutions of 1 mM) and also diluted in extern medium.

2.2 Chemicals

Fura-2-AM (obtained from Molecular Probes, Invitrogen, Breda, The Netherlands).

Dimethyl sulfoxide (DMSO), ionomycin and EDTA (obtainded from Sigma Aldrich, Zwijndrecht, The Netherlands).

Extern medium: saline solution containing (in mM) 125 NaCl, 5.5 KCl, 2 CaCl2, 0.8 MgCl2, 10 HEPES, 24 glucose, and 36.5 sucrose (pH 7.3), were prepared with deionized water (Milli-Q; resistivity > 18 MΩ·cm).

Solutions of ionomycin (in DMSO) and EDTA (in extern medium) and the prepared extern medium were kept at -20°C and thawed on the day of the experiment.

2.3 Cells

Transfected Chem-1 cells with full length human GPR54 cDNA obtained from Chemicon international. Before starting the experiment cells are removed from the liquid nitrogen and immediately placed in warm water bath (37°C). After thawing cells are transferred into a T75 flask containing 20 mL growth media and placed in an incubator (humidified, 37°C and 5% CO_2) overnight. The cell will have adhered to the flask. One day before testing cells are treated with trypsin (0.05%) for 5 – 10 minutes (at 37°C) to dislodge the cells. Trypsin is neutralized by adding 4 volumes of growth medium (4% or 10% fetal calf serum). After that cells are placed on glass-bottom dishes for Ca^{2+} imaging experiments and incubated overnight(humidified, 37°C and 5% CO_2) to allow the cells to adhere.

2.4 Ca^{2+} imaging microscopy

Changes in the intracellular calcium concentration were measured on a single cell level using the Ca^{2+} - sensitive fluorescent ratio dye Fura-2 AM. Before each experiment cells are loaded with Fura-2 AM. After this cells are placed on the stage of an Axio observer A1 inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmBH, Gräfelfing, Germany). Fluorescent images are collected every 6 seconds evoked by 340 and 380nm wavelengths with an Image SensiCam digital camera (TILL Photonics GmBH). Cells were continuously superfused with extern medium (with added kisspeptin or antagonist) at a rate of ~0.6 ml/min using a ValveLink 8.2 perfusion system (AutoMate Scientific, Berkeley, CA).[9]

2.5 Concentration response study

Different concentrations of kisspeptin have been tested. A set of pilot data (not shown) suggested that the optimal concentration would be around 10^{-8} M and 10^{-10} M. Concentrations kisspeptin-10 between 10^{-6} M and 10^{-12} M were used (diluted with extern medium),

while starting each day of testing witch a 10^{-8} M as a positive control.

At the start of each experiment cells were loaded with 5µM Fura-2 AM (for 30 minutes at room temperature, in the dark), followed by 15 minutes of de-esterification (room temperature, in the dark). Cells are placed on the microscope and measurement is started. After 10 minutes of baseline recording in which cells are only exposed to perfusion with extern medium cells were exposed to different concentrations of Kisspeptin -10 during 18 seconds. Then came another 20 minutes of basal recording (perfusion with extern medium). At 30 minutes perfusion is stopped and minimum and maximum ratios (R_{min} and R_{max}) were determined by adding ionomycin (5µM) and at 32,5 minutes EDTA (17mM).

2.6 Kisspeptin antagonist experiments

In a separate experiment set the antagonist P271 was tested. Cells were loaded with Fura-2 AM and de-esterificated in the same manner. Again the experiments were started by a 10 minute basal recording. At 10 minutes perfusion was switched from extern medium to extern medium with added P271 (at 10^{-6} M). After another 10 minutes of perfusion, a Kisspeptin-10 stimulus of 18 seconds was administered (extern medium with added P271 (10^{-6}M) and Kisspeptin-10 (10^{-6}M). This was repeated with a 20-minute gap instead of a 10-minute gap between the start of the perfusion with antagonist and the kisspeptin stimulus.

To compare different concentrations we did the same set of experiments only with a lower kisspeptin-10 concentration (10^{-7}M).

For comparison reasons a double kisspeptin 10^{-6} M stimulus was done after 10 minutes baseline recording and a 20 minute gap between the two stimuli.

2.7 Control experiment

Because of inconsistent results during these experiments it was decided to add an control study. The experiment used the same experimental setup as previously described. At T=10 the perfusion was switched from the extern medium vial to a smaller vial (which would normally contain the antagonist and the kisspeptin) also containing extern medium.

3.1 Concentration response study

Ideally the cells should be dark until the kisspeptin-10 stimulus is administered at T=10. The height and width of the peak determine the GPR54 receptor activation. Stimulation of the cells leads to an increase in fluorescence, correlating to an increase in intracellular calcium concentration. In almost all the tests the cells show some rise in intracellular calcium when the stimulus is administered. Unfortunately the tests with the lower concentrations (10^{-8} M and lower) were inconsistent. Only the 10^{-6} M (and 10^{-7} M) gave consistent results (see figure 2).

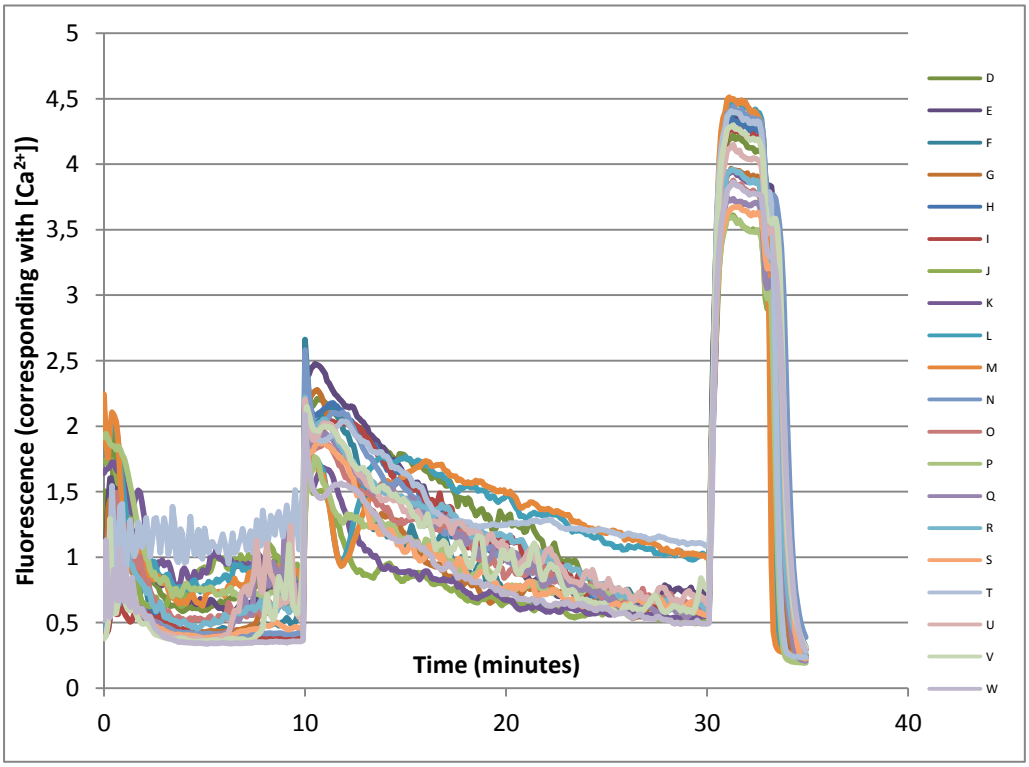

FIGURE 2: An example recording of a 10^{-6} M kisspeptin-10 stimulus (at T=10). Each line represents the intracellular calcium concentration in one cell, X represents time in minutes and Y represents the intracellular [Ca^{2+}] measured by the fluorescense of fura-2. At T=30 ionomycin is added and at T=32,5 EDTA . As stated some cells oscillate, even before a stimulus is administered. In this concentration you can see that all the cells, oscillating or ot, respond well on the stimulus.

Also sometimes the cells oscillated before a stimulus was administered (see figure 3). When the cells oscillated they responded only to the higher concentrations of kisspeptin-10 (10^{-7}M and 10^{-6}M). Due to the oscillating cells and the fact that in the lower concentrations of kisspeptin-10 the results were not reproducible (no consistent peak) it was decided to temporarily stop the concentration – response part of this study. Since 10^{-6} M and 10^{-7} M kisspeptin-10 were the only concentrations that gave reliable results it was decided to test the P271 antagonist with these concentrations.

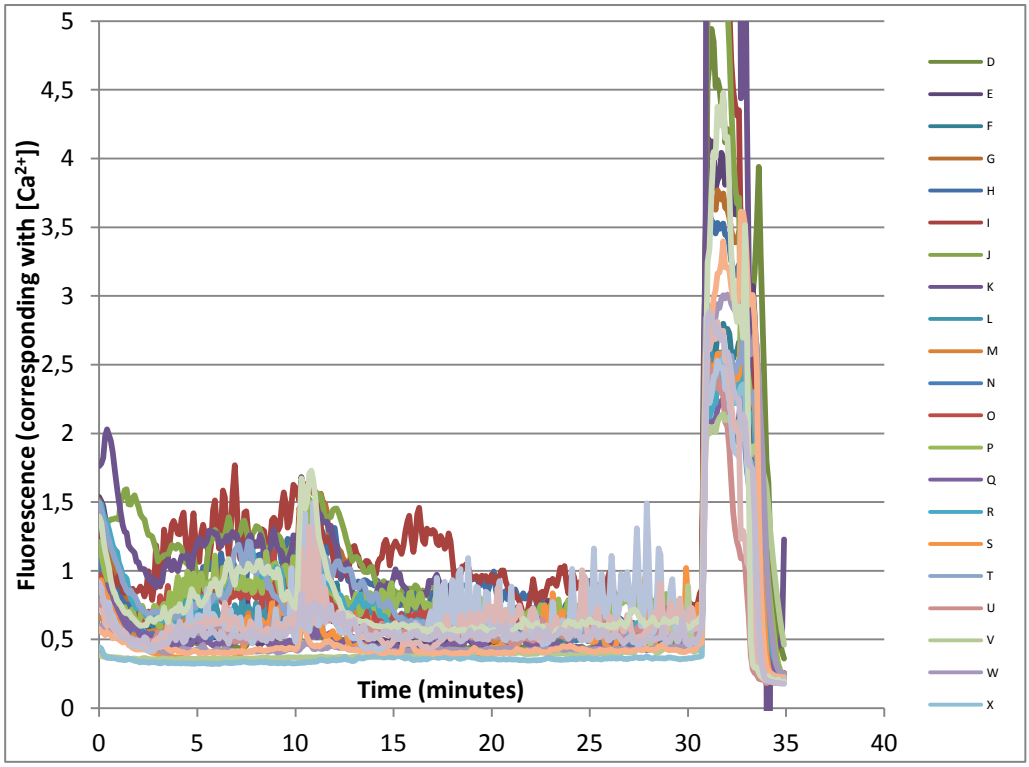

FIGURE 3: An example recording of a 10^{-8} M kisspeptin-10 stimulus (at t=10). In this image the oscillating cells can clearly be seen. Also this image shows that the cells that oscillate before the stimulus is given do not respond as well to the stimulus as the non oscillating cells.

3.2 P271 antagonist

There was a substantial rise in the intracellular calcium concentration when the perfusion was switched to P271. After the switch it took more than 10 minutes for the intracellular calcium to return to basal levels. In this setup the intracellular calcium concentration was not back to basal levels when at T=20 the kisspeptin-10 stimulus was administered. Even though the cells gave a clear response to the kisspeptin-10 stimulus, they hadn't returned to basal calcium levels (see figure 4); so the experiment was repeated with a longer pause between the start of the perfusion and the kisspeptin-10 stimulus (see figure 5).

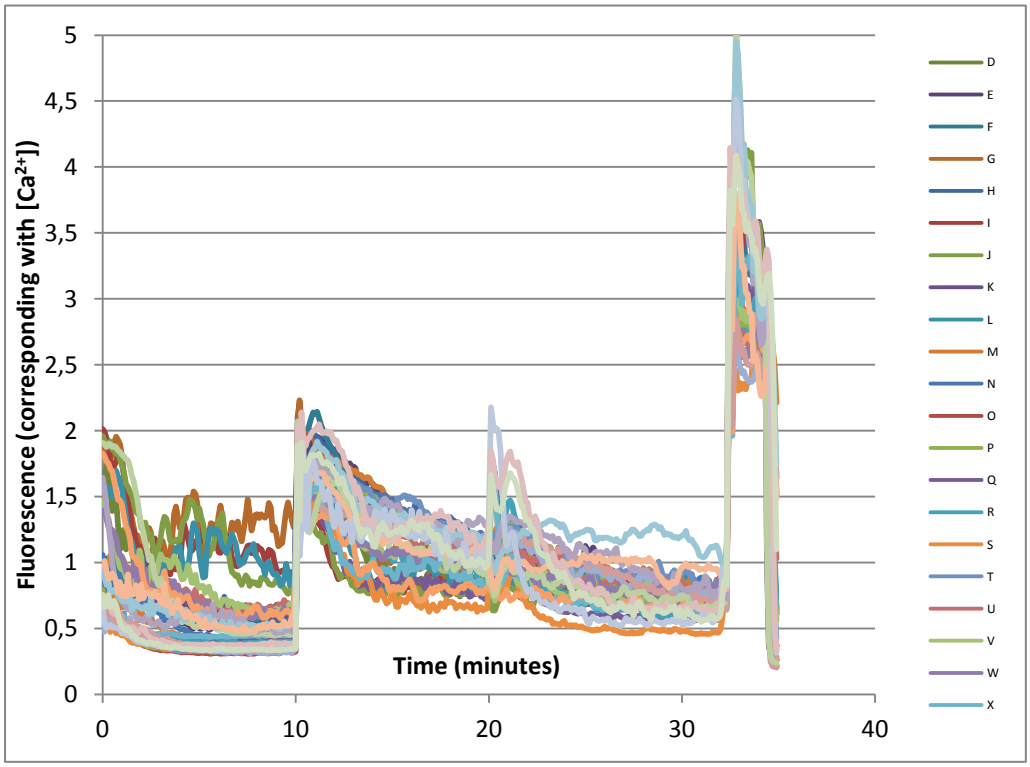

FIGURE 4: An example recording of the testing of P271. Each line represents the intracellular calcium concentration of an individual cell, X represents time (minutes) and Y the intracellular calcium concentration (same as before). At T=10 perfusion with P271 10^{-6} M is started and at T=20 the kisspeptin-10 10^{-6} M stimulus is given.

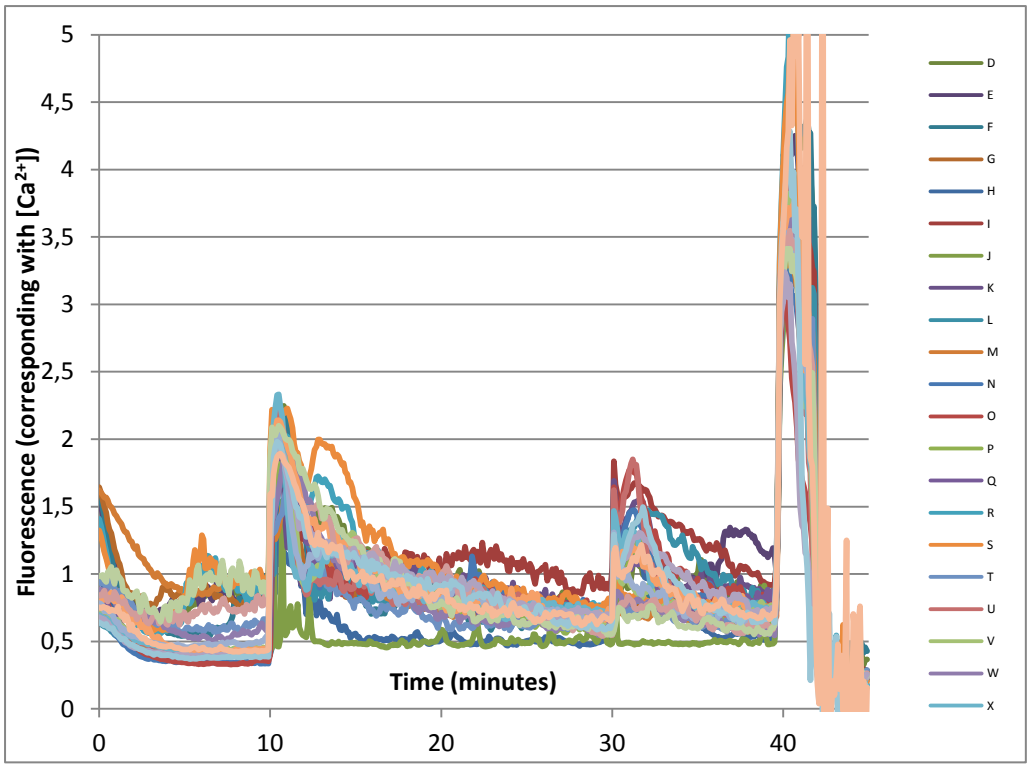

FIGURE 5: Executed the same as figure 4; only kisspeptin-10 10^{-6} M stimulus was not given until T=30.

To see whether the concentration kisspeptin-10 versus P271 was of influence for the effectiveness of the antagonist we did an experiment in which the kisspeptin-10 concentration was lowered to 10^{-7} M. Even then the kisspeptin-10 stimulus is clearly visible (figure 6). To compare the heights of these two peaks a double kisspeptin-10 10^{-6} stimulus was done in two settings, one with a stimulus at T=10 and T=20 (data not shown). The other with the kisspeptin-10 stimulus at T=10 and T=30 (see figure 7).

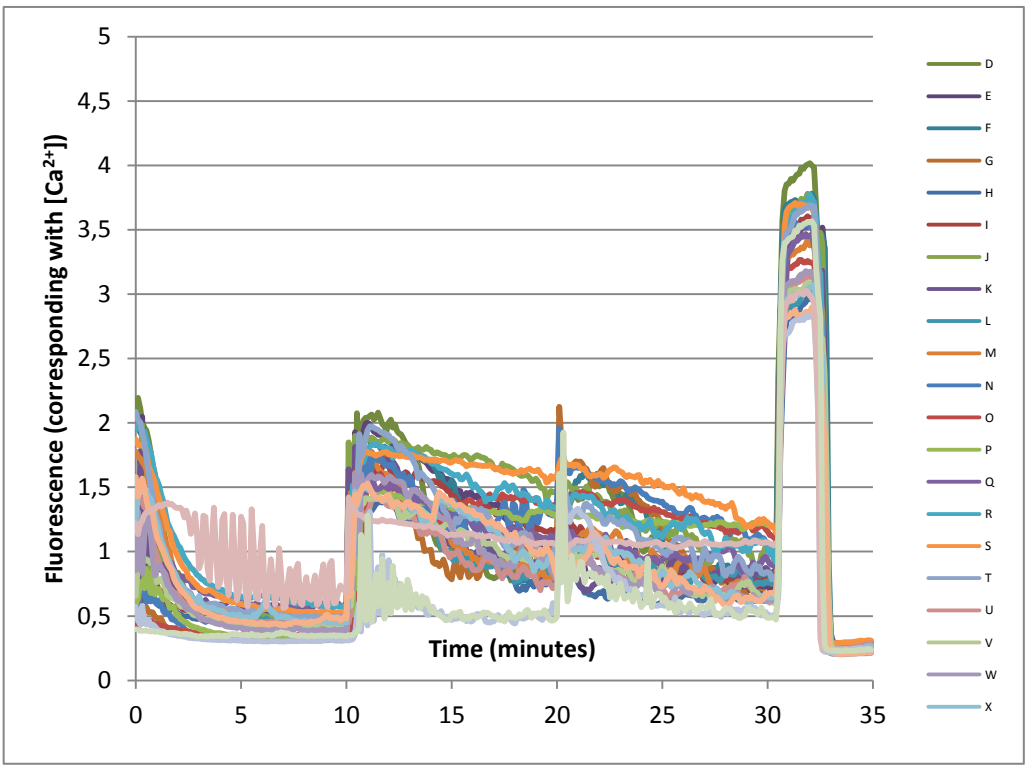

FIGURE 6: Testing of P271 10^{-6} M with a kisspeptin 10 10^{-7} M stimulus (at T= 20).

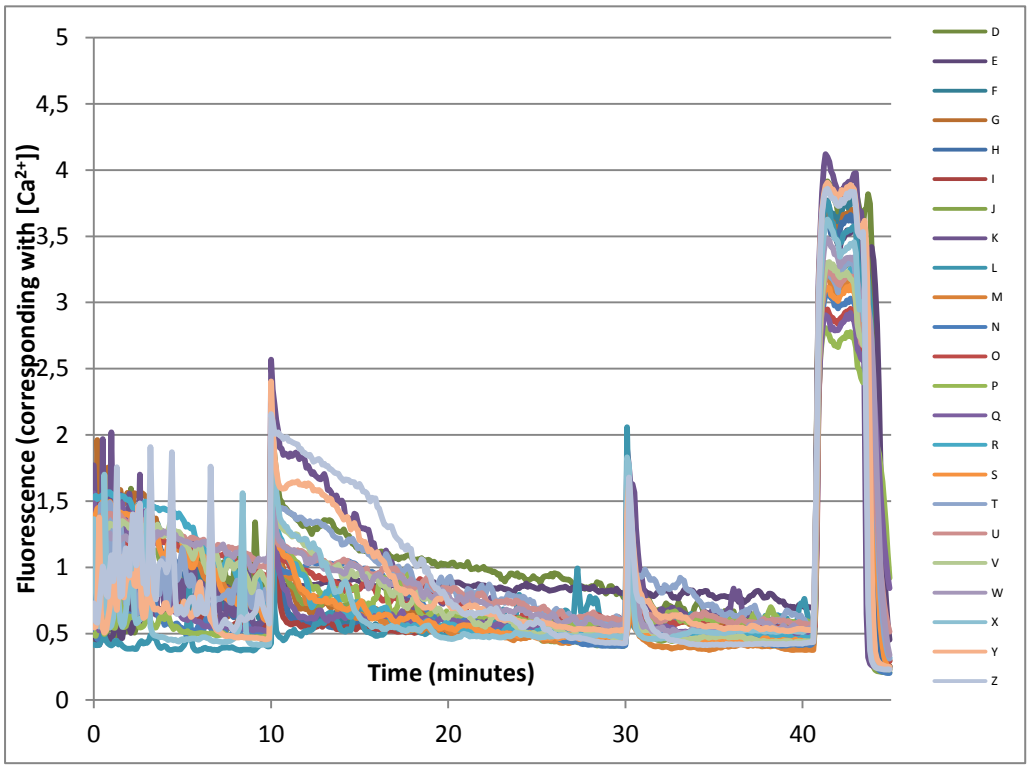

FIGURE 7: Double kisspeptin-10 10^{-6} M stimulus at T=10 and T=30. In this image the oscilating cells (between T=0 and T=10) are again clearly visible. The second kisspeptin-10 peak at T=30 is a bit less than the first peak at T=10.

3.3 Control experiment

Trying to exclude the perfusion as an influence of the inconsistent results in the concentration response part of the study an medium control was added to the experiments. At first glance nothing seems to happen (perfusion was switched at T=10) (see figure 8). But when the oscillating cells are removed there is a small peak visible at T=10 (see figure 9).

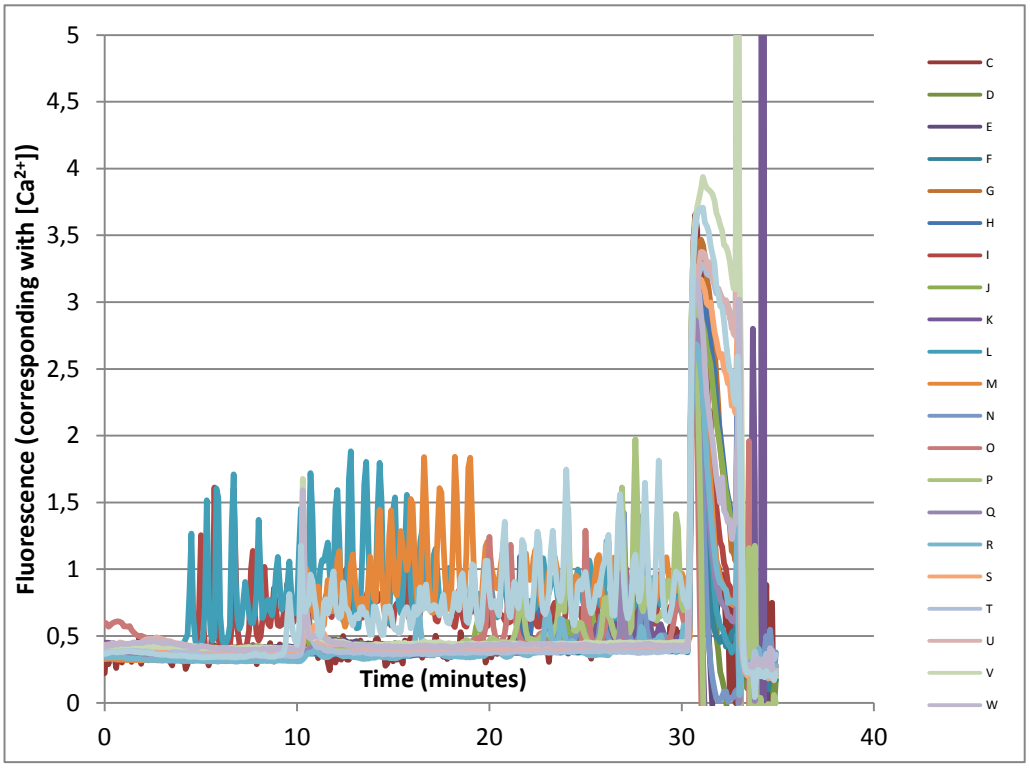

FIGURE 8: Medium control. Instead of switching to kisspeptin-10, the perfusion was switched to another tube containing the same medium (at T=10).

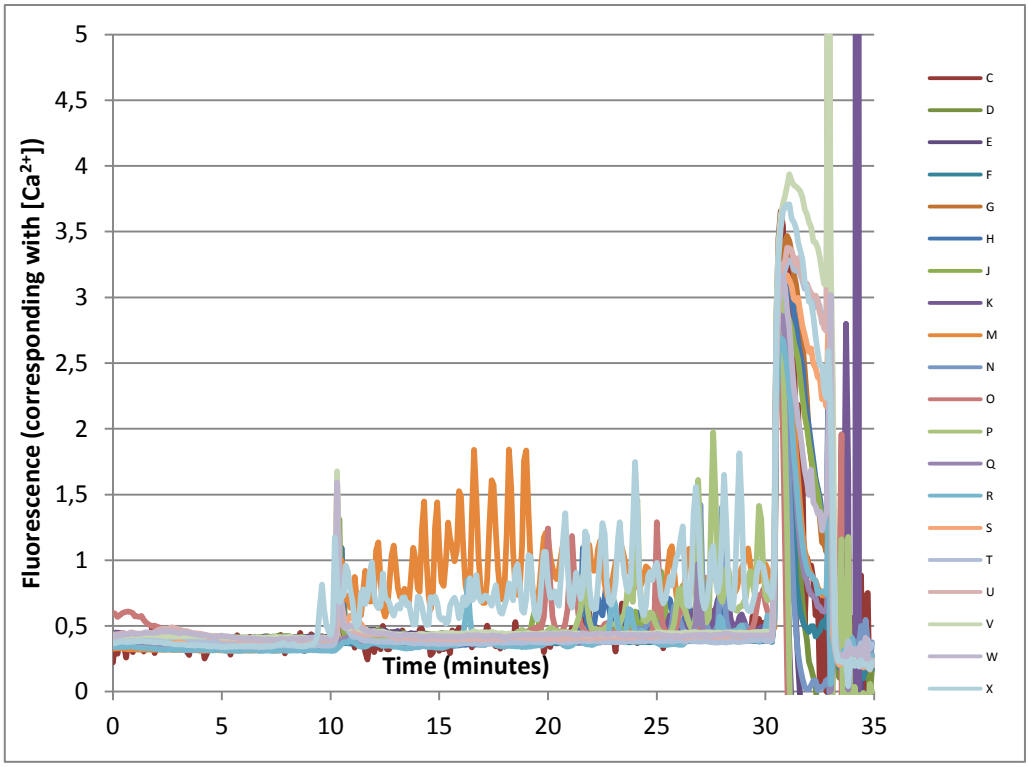

FIGURE 9: Same image as figure 8, only the two cells that oscillated before T=10 are removed. A small peak is now visible at T=10.

4. DISCUSSION

One of the aims of this study was to do an accurate receptor activity study for kisspeptin-10 *in vitro*, to be able to use that data for a receptor activity study for possible kisspeptin antagonists. Unfortunately we encountered some problems during the concentration response study. It seems that there is an unknown variable in this experimental setting that causes the cells to oscillate, and makes the results badly reproducible. The pilot data showed that the 10^{-8} M kisspeptin-10 should give a consistent peak. Unfortunately when we started the study this was not the case. At first we had some trouble with dissolving kisspeptin-10 and also its shelf life was questionable. Because of this it was decided to make fresh solutions on each experimental day. Even then there were continuous problems with the oscillating cells. We tried different densities of cells on the dishes, to exclude cell to cell contact as a reason for them to oscillate. Unfortunately this did not solve the problems with oscillations.

It is also a possibility that the cells used in this experiment are not well suited for this type of experiment (e.g. the cells might be sensitive to the pressure of the perfusion). In the medium control study some cells responded to the perfusion switch. The peak is not as obvious as with the higher concentrations of kisspeptin-10, but cannot be distinguished from the lower concentrations of kisspeptin-10 (e.g. 10^{-10} M and 10^{-12} M). Further research with this method is necessary to see if this method is a viable option for a concentration response study.

In the end only two of the concentrations kisspeptin-10 tested (10^{-6} M and 10^{-7} M) gave reproducible results.

The kisspeptin antagonist P271 was therefore tested with these concentrations of kisspeptin-10. At T=10 when perfusion is switched to the antagonist there was consistently a high peak in the intracellular calcium concentration. High enough for even oscillating cells to respond to it. After a 10 minute exposure of the antagonist a kisspeptin-10 stimulus was given, but in none of the experiments the stimulus was blocked. After 10 minutes of exposure with the antagonist, the cells were not returned to their basal levels of intracellular calcium. Therefore we repeated the experiments with 20 minutes exposure to the antagonist before administering the kisspeptin stimulus. These experiments also gave a peak as a response to the kisspeptin-10. The peaks of the kisspepin (at T=20 or T=30) are a bit lower than the peaks the antagonist show (at T=10). For comparison a double kisspeptin-10 stimulus was done (as seen in figure 7). The second kisspeptin peak is also considerably lower than the first. Unfortunately this meant that the tests with the kisspeptin antagonist P271 showed agonistic properties but no antagonistic properties.

During the development the kisspeptin antagonist P234 showed no inositol phosphate production and no intracellular Ca^{2+} release *in vitro*.[5] However a different test method was used. Roseweir et al[10] used an inositol phosphate stimulation assay to evaluate the antagonistic properties *in vitro*. Cells were stimulated with kisspeptin and antagonist for 1h, in a calcium and magnesium free medium. Inositol phoshate was bound and measured by radioactivity on a beta counter. This was compared to stimulus with only kisspeptin (see figure 10).[10] They tested different concentrations of the P234 antagonist against a fixed concentration of kisspeptin (10nM).

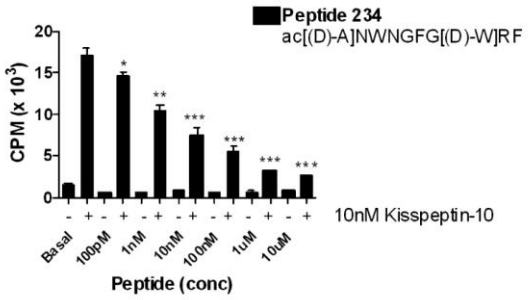

FIGURE 10: From Roseweir et al[10]: Peptide 234 is a potent inhibitor of kisspeptin-10 stimulation of IP. Substitution of Leu8 with D-Trp in combination with Ser5 substitution with Gly created potent antagonists. Additional substitution of Tyr1 with D-Ala (234 shown here) enhanced this (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Peptide 234 alone had no intrinsic IP stimulation. Bars show mean ± SEM of five experiments.[10]

This image suggests that there is antagonistic activity, but not a 100% blockage of the GPR54 receptor. We tested the antagonist and kisspeptin 1 µM, and a factor ten apart (1 µM antagonist P271 and 0,1 µM kisspeptin). Possibly in our experiments the concentration kisspeptin used is too high (in comparison to the antagonist) to see an antagonistic effect.

Various *in vivo* studies have been done with the P271 and the P234 antagonists. The results of these studies tend to be positive (e.g. the antagonist seems to be working).[10, 11] In one study they administered the antagonist to mice chronically, so it is unsure whether the effect seen was due to down regulation of the reproductive axis (preventing a rise in estrogen) or if it actually blocks the positive feedback of estrogen on GnRH release.[11, 12] In another *in vivo* study with P271 it was found to be antagonistic; it blocked the positive feedback of endogenous kisspeptin (it blocked pulsatile LH secretion). The antagonist was administered via intracerebroventricular infusion.[11]

In all these *in vivo* studies the antagonist was tested against endogenous kisspeptin or a suboptimal dose of kisspeptin-10.[10] It is unclear in which concentration endogenous kisspeptins work and very low dosages of kisspeptin-10 have been found able to stimulate GnRH secretion *in vivo* (1 fmol i.c.v.).[5]

It is possible that P234 and P271 actually do have an antagonistic effect on the GPR54 receptor, but only when there is a severe excess of the antagonistic peptide. These circumstances we did not test in this experimental setup (partially because of the poor results in the concentration response study). For future experiments these conditions need to be taken into consideration.

In conclusion; kisspeptin antagonist P271 showed agonistic properties and no antagonistic properties. But this experimental setup needs to be reviewed before it can be used as a reliable method for testing kisspeptin antagonists *in vitro*.

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