Distribution of Kisspeptin and the Kisspeptin Receptor in the Canine Hypothalamus

Master research project Veterinary Medicine

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Abstract

Kisspeptin is a protein that plays an important role in the hypothalamic-pituitary-gonadal axis. The major populations of kisspeptin neurons in the hypothalamus are found in the arcuate nucleus and the preoptic area in different mammalian species. Kisspeptin is known to regulate the release of GnRH from GnRH neurons in the hypothalamus and in this way regulates the release of luteinising hormone and follicle stimulating hormone by the pituitary gland as well. Luteinising hormone and follicle stimulating hormone are important hormones for normal gonadal development and function. Hence, the kisspeptin signalling system is an interesting target to therapeutically modulate the reproductive cycle in the bitch, for example non-surgical oestrus prevention or induction of ovulation. We studied the distribution and localisation of kisspeptin and the kisspeptin receptor in the canine hypothalamus by performing immunohistochemical analysis of hypothalamic tissue sections. Previous research shows that the kisspeptin receptor is expressed on GnRH neurons, to confirm this we will also perform an immunohistochemical study using an antibody raised against GnRH. We found one population of kisspeptin receptor immunopositive neurons in the hypothalamus of the dog. These neurons were present in the ventromedial portion of the hypothalamic tissue on both sides of the third ventricle, we consider this area to be the arcuate nucleus. Kisspeptin immunopositive neurons were also present in the arcuate nucleus and also more dorsal in the ventromedial nucleus and the dorsomedial nucleus. Unfortunately, we did not find GnRH immunopositive cells in the canine hypothalamus. It is known from studies in other mammalian species that there are two major populations of kisspeptin and kisspeptin receptor expressing neurons: the arcuate nucleus and the preoptic area. In this study we only found one population of neurons expressing the kisspeptin receptor and kisspeptin, possibly because the POA was lost during dissection of the hypothalamus from the brain, or because kisspeptin and the kisspeptin receptor were downregulated in these areas because the bitch used in our study was in anoestrus. More research is necessary to demonstrate the localisation of GnRH neurons in the canine hypothalamus by using immunohistochemistry and eventually a double immunohistochemistry protocol should be performed to find out if the kisspeptin receptor is indeed present on GnRH neurons in the dog as well.

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1. Introduction

Kisspeptin (KP) is a protein involved in the regulation of the release of Gonadotropin Releasing Hormone (GnRH) by the hypothalamus.[1] GnRH stimulates the anterior lobe of the pituitary gland to release Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH). LH stimulates the cells of Leydig in the testis to produce testosterone and in females, it stimulates ovulation and the formation of corpora lutea in the ovary. FSH is involved in the function of the Sertoli cells in the testis and induces follicular development and oestradiol synthesis in the ovary.[2]

KP plays a key role in the hypothalamic-pituitary-gonadal (HPG) axis by mediating negative feedback (males and females) and positive feedback (females) of gonadal steroids on the hypothalamus. There are two populations of KP neurons found in the hypothalamus of different animal species, such as sheep, goats, pigs and horses: rostral in the preoptic area (POA) and caudal in the arcuate nucleus (ARC). These KP neurons express gonadal steroid receptors in contrast to GnRH neurons. Negative and positive feedback on the HPG axis by gonadal steroids is mediated by down- or up-regulation, respectively, of kiss1 mRNA in the distinct areas of the hypothalamus. Down-regulation and up-regulation of kiss1 in the ARC induces a pulsatile release of GnRH in both males and females. Up-regulation in the POA induces a GnRH surge leading to the preovulatory LH surge in females. [1]

Besides sex steroids, KP neurones can also be influenced by leptin (suggesting a link between nutrition and reproduction) and by photoperiod in seasonal breeding animals.[3]

The KP receptor is a G-protein coupled receptor also known as kiss1r or GPR54. This receptor can be activated by different C-terminal fragments of a protein encoded by the *KiSS1* gene; KP-54, KP-14, KP-13 and KP-10, the kisspeptins. The C-terminal sequence characteristic for this family of proteins is: Arg-Phe-Nh2 and every peptide binds and activates the KP receptor with the same affinity.[5,6]

Dysfunction of KP or the KP receptor leads to impaired or absence of fertility in both sexes. In humans and mice there is evidence that mutations in *KiSS1R* can lead to hypogonadotropic hypogonadism with impaired pubertal maturation and reproductive function.[3,4]

KP and its receptor are interesting targets for modulating reproduction in dogs because of their key role in the normal function of the HPG axis. In the future induction of ovulation and non-surgical oestrus prevention could be achieved by therapeutically influencing the KP signalling system in the dog.

The reproductive cycle of the bitch is very complex. It consists of an anoestrus phase of various length (2-9 months) followed by pro-oestrus during which the oestradiol level gradually increases. After the oestradiol peak the oestrus starts, both LH and FSH peak in the first days and ovulation is completed at about the third day of the oestrus. The luteal phase starts and takes approximately 75 days, during which progesterone rises and gradually decreases again. The long anoestrus period is responsible for a cyclic profile of about three cycles in two years (depending on the breed).[2]

It is already clear that the coding sequences for KP and the KP receptor are present in the canine genome.[5] Intravenous administration of canine KP-10 results in an increase in plasma LH, FSH and oestradiol concentation in the blood of anoestrus bitches.[5] These results demonstrate the existence of the kisspeptin signalling system in the dog.

KP and the KP receptor are mainly found in the ARC and in the POA in different mammalian species[7] and most GnRH neurons in adult mice and rats are known to express the kisspeptin receptor.[6,8,9] We expect to find a similar distribution of KP and the KP receptor in the hypothalamus of the dog and hopefully we will be able to confirm our findings with a double immunohistochemical staining using antibodies raised against both the KP receptor and GnRH.

In order to be able to visualize the predicted localisation of KP and its receptor and to interpret the results of the immunohistochemistry it is important to know more about the anatomy of the hypothalamus (which is shown in fig. 1).

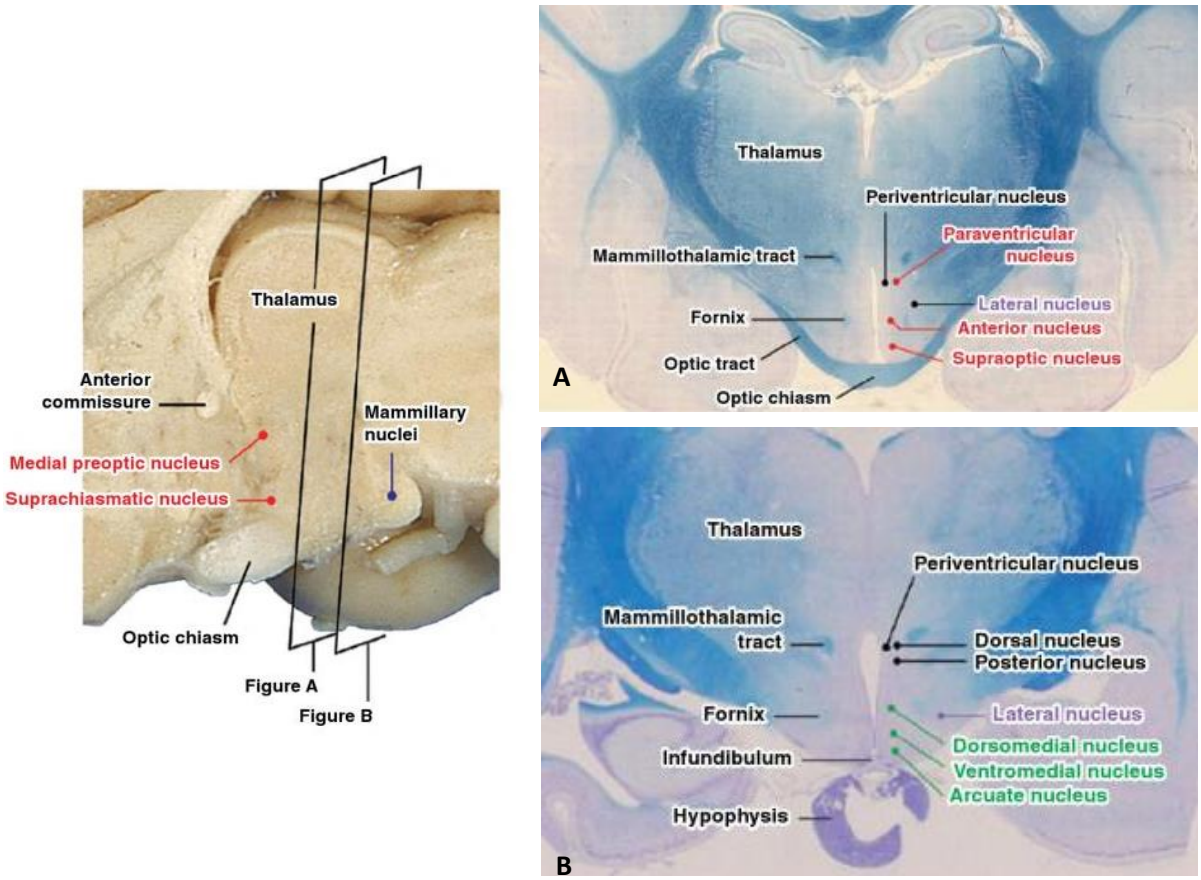

Fig. 1: Anatomy of the canine hypothalamus in coronal sections. A: section at the level of the caudal portion of the optic chiasm and optic tract. B: section at the level of the infundibular stalk.[10]

2. Aim of the study

The aim of this study is to determine the distribution and localisation of kisspeptin and the kisspeptin receptor in the hypothalamus of the dog. To do this we will perform immunohistochemistry on hypothalamic tissue sections using an antibody raised against KP and the KP receptor. An immunohistochemical study of GnRH will also be performed, to assess if the kisspeptin receptor is expressed on GnRH neurons in dogs as well.

3. Materials and methods

3.1 Hypothalamic tissue

The hypothalamus was obtained from a 13 year old Beagle bitch in anoestrus that was used for another study (DEC 2012.III.03.028). The bitch was euthanized because of age-related physical problems but these were not related to the study. The dog was euthanized and after bleeding a 4% solution of buffered formalin was perfused through the arteria carotis to fixate the brain as soon as possible. The hypothalamus was dissected from the brain and cryopreservation with 30% sucrose took place before 30 micrometer coronal sections were cut on a freezing microtome. The tissue slices were conserved in a 30% sucrose solution and stored at -20 °C for later use.

3.2 General protocol for the immunohistochemistry

The sections were thawed by putting them in a refrigerator (4°C) overnight and after that they were rinsed 3 times in PBS (0.1M) for 10 minutes each time to remove the sucrose. To reduce endogenous peroxidase activity, the sections were soaked in 1% hydrogen peroxide for 30 minutes. To remove the hydrogen peroxide the sections were rinsed in PBS-T (0.1M PBS + 0.05% Triton-X-100) 3 times for 10 minutes each time. A universal protein block (0.5% bovine serum albumin (BSA) in PBS-T) was applied for 20 minutes at room temperature. The primary antibody was diluted in PBS-T + BSA and incubated overnight at 4°C (during incubation the sections were mixed constantly with the solution by putting them on a moving surface). The next day we rinsed the tissue sections in PBS-T again 3 times for 10 minutes each time and we applied the secondary antibody (also diluted in PBS-T + BSA). The secondary antibody was incubated for at least 60 minutes at room temperature on a moving surface. The sections were then washed again in PBS-T for 3 times 10 minutes each time and the DAB-substrate (SIGMAFAST, 3,3'-Diaminobenzidine tablets, D4168) was applied for 10 minutes at room temperature. The tissue slices were washed in PBS 3 times for 10 minutes each time and after that they were mounted on Polysine-slides. The slides were dried 48 hours at 30 °C.
Counterstaining was performed by soaking the slides in 0,1% Cresyl Violet Acetate for 5 minutes (Nissle staining). This was followed by alcohol series increasing in concentration from 70% to 100% to dehydrate the tissue. The coupes were washed in xylene for two times and enclosed in DPX before applying a coverslip.

3.2.1 *Immunohistochemistry: kisspeptin receptor*

Antigen retrieval was accomplished by incubating the sections in 0.01M sodium citrate buffer (pH 6.0) at 80 °C for 20 minutes. As a primary antibody, we used the polyclonal rabbit antibody KiSS1R/GPR54 Antibody NLS1926 (Novus Biologicals, Littleton, CO). The primary antibody was diluted 1:200 in PBS-T + BSA. As a secondary antibody we used polyclonal goat anti-rabbit immunoglobulins/HPR P0448 (DAKO, Glostrup, Denmark) diluted 1:500 in PBS-T + BSA.
Negative control experiments were performed by excluding the primary antibody from the protocol and replaced it by normal rabbit serum. A positive control experiment was performed on CHEM-cells expressing the human KP receptor on their cell surface. These cells were fixated in 4% formaldehyde and for the IHC we used the same protocol as we used on the hypothalamic tissue.

3.2.2 *Immunohistochemistry: kisspeptin*

As a primary antibody we used the polyclonal rabbit antibody Anti-Kisspeptin Antibody AB9754 (Merckmillipore, Billerica, MA) and diluted it 1:1000 in PBS-T+BSA. As a secondary antibody we used polyclonal goat anti-rabbit immunoglobulins/HPR P0448 (DAKO, Glostrup, Denmark) diluted 1:500 in PBS-T + BSA. Negative control experiments were performed by excluding the primary antibody from the protocol.

3.2.3 *Immunohistochemistry: GnRH*

The primary antibody we used for this experiment was a monoclonal mouse antibody Anti-Gonadotropin-Releasing Hormone Antibody MAB5456 (Merckmillipore, Billerica, MA). The primary antibody was diluted 1:500 in PBS-T + BSA. The secondary antibody was a goat anti-mouse IgG + IgM 115-035-044 (Jackson Immunoresearch, West Grove, PA) also diluted 1:500 in PBS-T + BSA.
Negative control experiments were performed by excluding the primary antibody from the protocol. Instead of the primary antibody we used normal mouse serum 1:10 in PBS-T + BSA in the negative controls and incubated it overnight at 4 °C as well.

3.3 Delineation of hypothalamic nuclei

In order to determine the location of the immunopositive neurons in the coronal hypothalamic sections we used the atlas of the beagle brain by Palazzi[11] and Fundamentals of canine neuroanatomy and neurophysiology by Uemura[10].

3.4 *Ethics of experimentation*

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University (DEC 2012.III.03.028).

4. Results

4.1 Kisspeptin receptor

DAB precipitate was visible on coronal sections of the canine hypothalamus on neurons in the ventromedial area of the hypothalamus. On both sides of the third ventricle a symmetrical group of about 8-20 positive cells was detected (as is shown in figure 2A). Not all neurons in the particular region did express the KP receptor, only some of the largest neurons were positive. The immunopositive cells were distributed homogeneously between the neurons in the positive region (as is shown in figure 3A). The DAB precipitate was visible as individual dots on the cell surface of the neurons and only covered a part of the cell (as is shown in figure 4A-D). In some cells it was even possible to see the precipitate on the axon hillock. Other areas of the hypothalamus are free of precipitation and there is no DAB precipitation visible in the negative control sections (see figures 2B, 3B and 4E). Unfortunately, the positive control experiments using the CHEM-cells expressing the human KP receptor were completely negative.

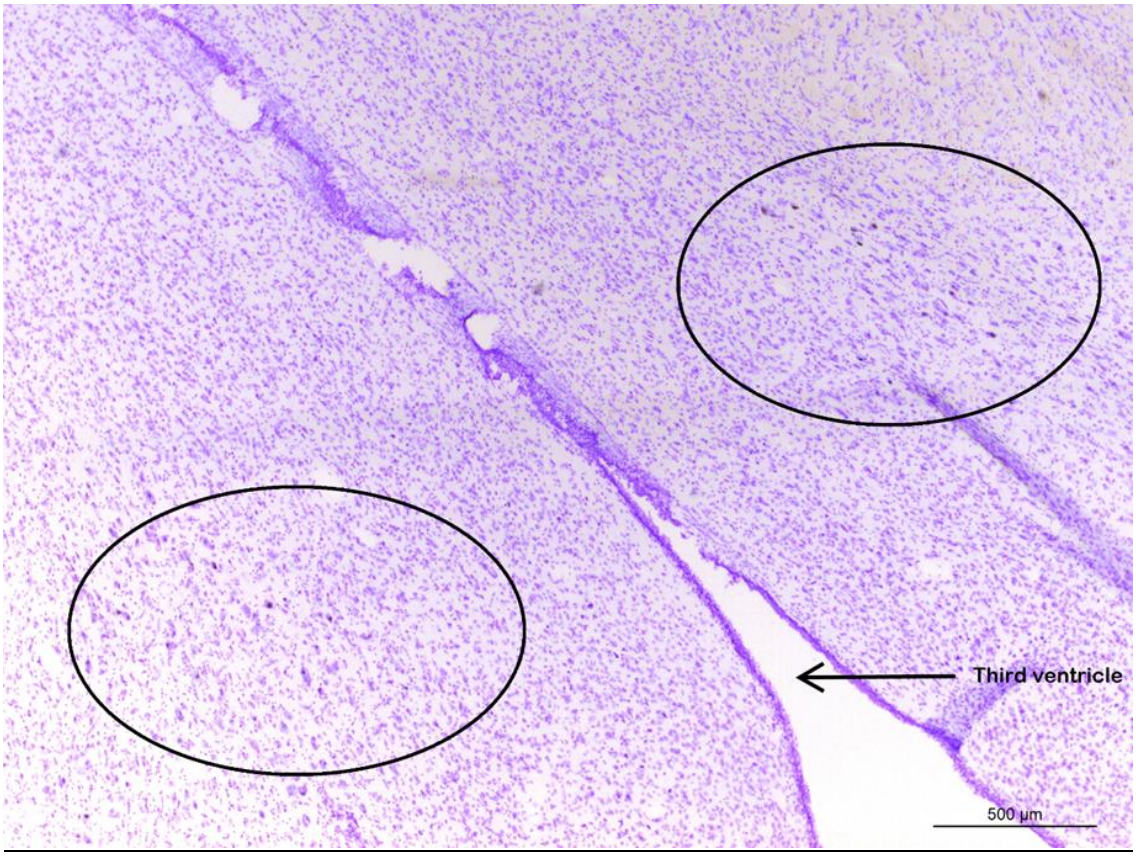

Fig. 2A: location of the KP receptor immunopositive neurons (within the ovals) in the arcuate nucleus on both sides of the third ventricle.

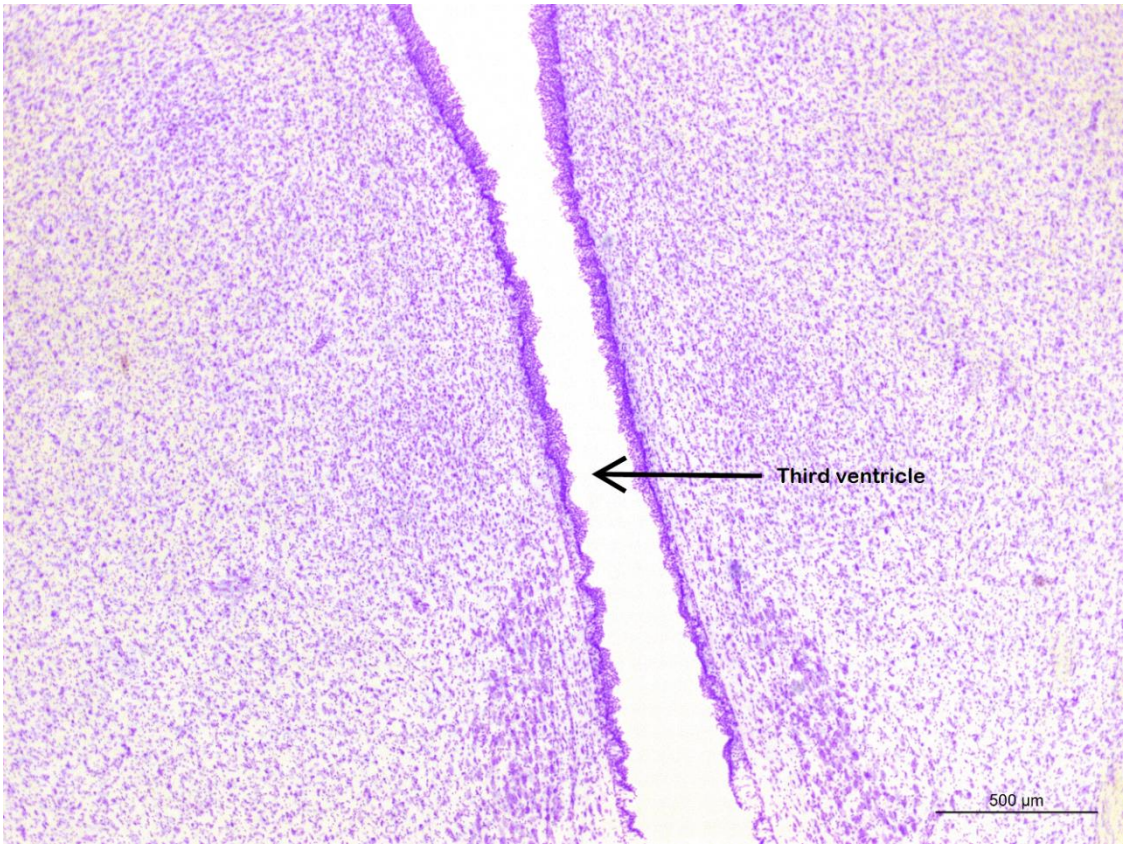

Fig. 2B: Negative control experiment of the same area of the hypothalamus as shown in Fig 2A.

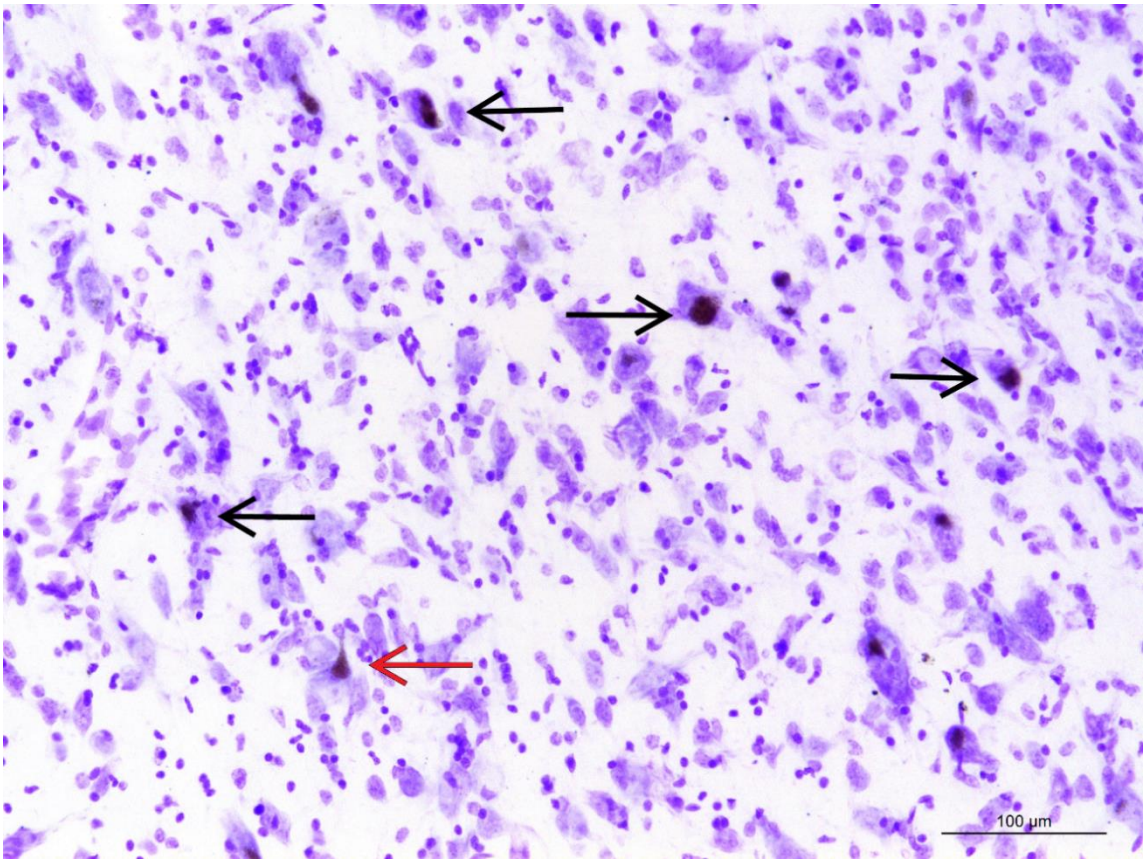

Fig. 3A: KP receptor immunopositive cells (arrows) in the arcuate nucleus. The triangular shape of DAB precipitate on the neuron with the red arrow indicates the presence of the KP receptor on the axon hillock.

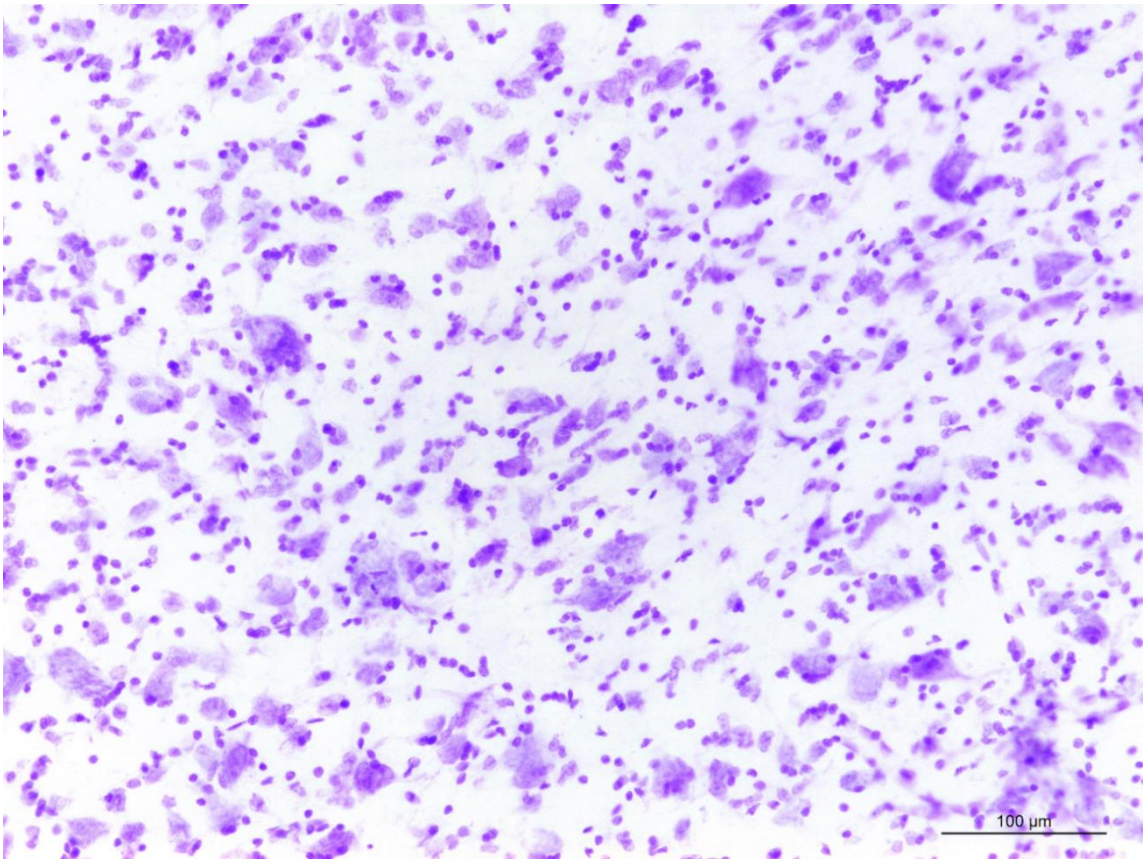

Fig. 3B: negative control experiment of the same area as shown in fig. 3A.

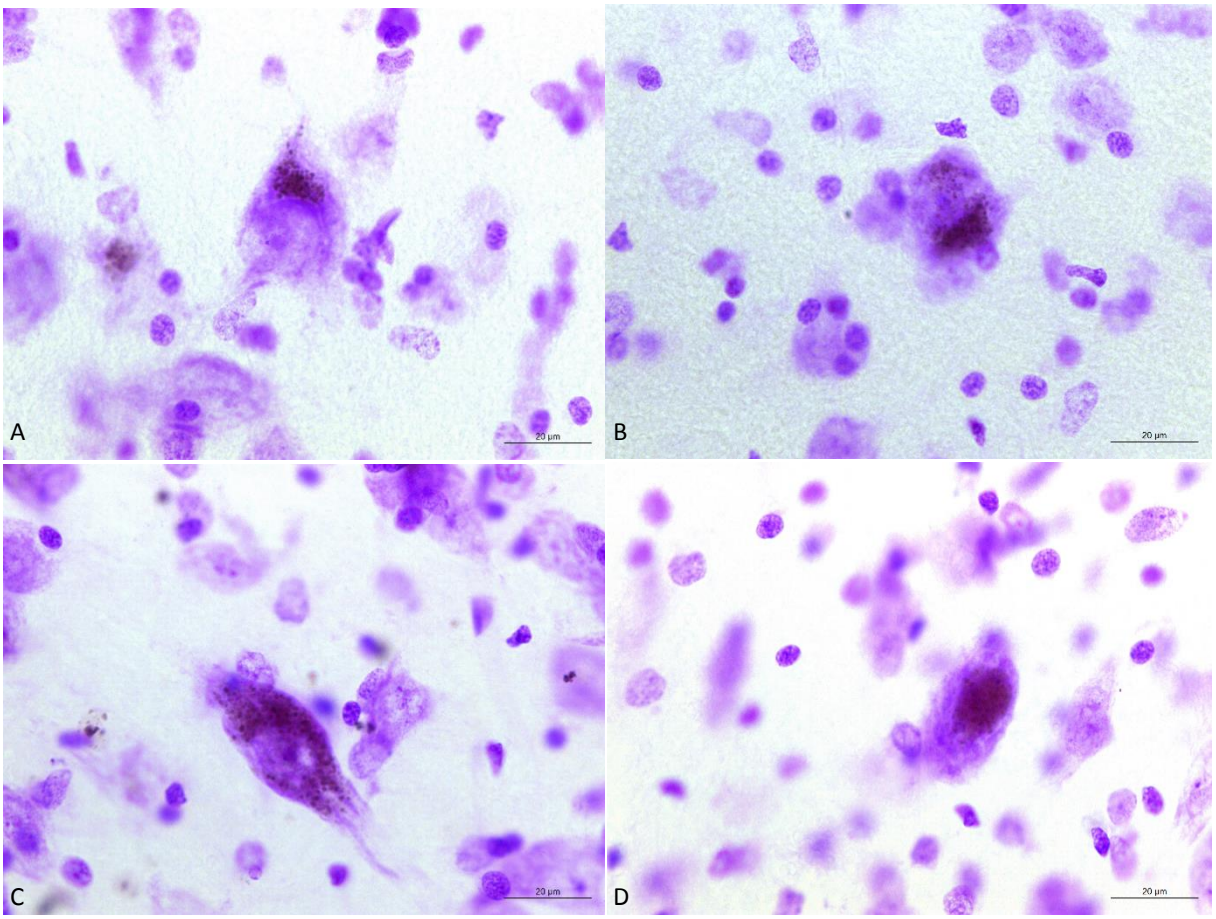

Fig. 4A-D: KP-receptor immunopositive neurons in the arcuate nucleus. (The scale bar indicates 20µm)

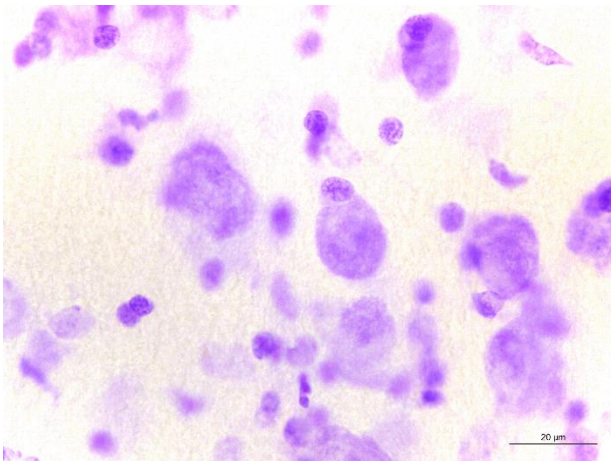

Fig. 4E: negative control experiment of the same area as fig. 4A-D. (The scale bar indicates 20µm)

Based on the anatomy of the hypothalamus obtained from figure 1[10] and the atlas of the beagle brain[11] we concluded that the area in which we observed the KP receptor immunopositive neurons is the arcuate nucleus.

4.2 Kisspeptin

Again in the ventromedial area of the coronal hypothalamic sections we observe DAB precipitate on both sides of the third ventricle (as is shown in figure 6A). In these positive areas we observed that not all neurons are KP immunopositive. In each area we find 20 – 30 KP immunopositive neurons, the cells are homogenously distributed in the particular region (as is shown in figure 7A). There are also some cells that are located more apart from each other dorsal to the positive area.
When observing the individual cells it is clear that the DAB precipitate dots are not homogenously distributed in the cells but that they are present in groups (see figure 8A - D).
Other areas of the hypothalamus are free of precipitation and there is no DAB precipitation visible in the negative control sections (as is shown in figure 6B, 7B and 8E).

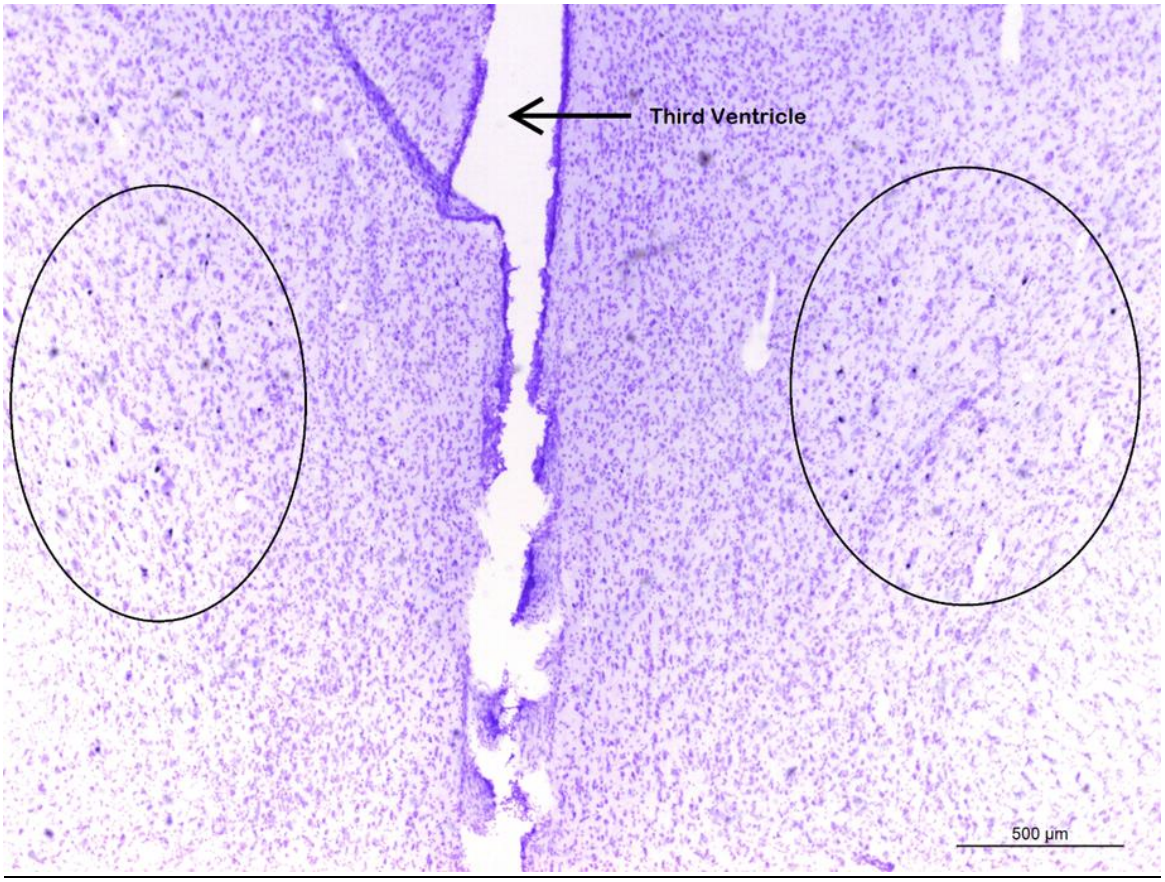

Fig. 6A: KP immunopositive cells (within the ovals) in the arcuate nucleus on both sides of the third ventricle.

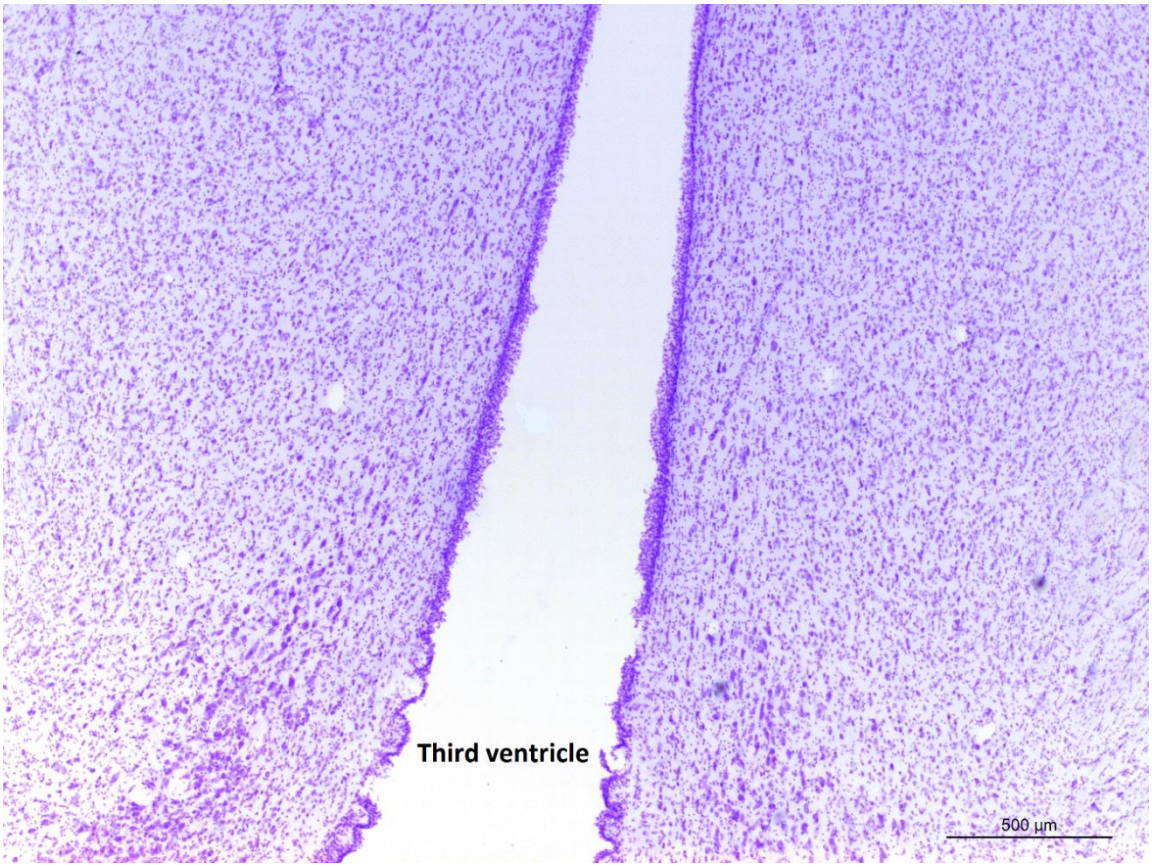

Fig. 6B: Negative control experiment of the same area as shown in Fig. 6A.

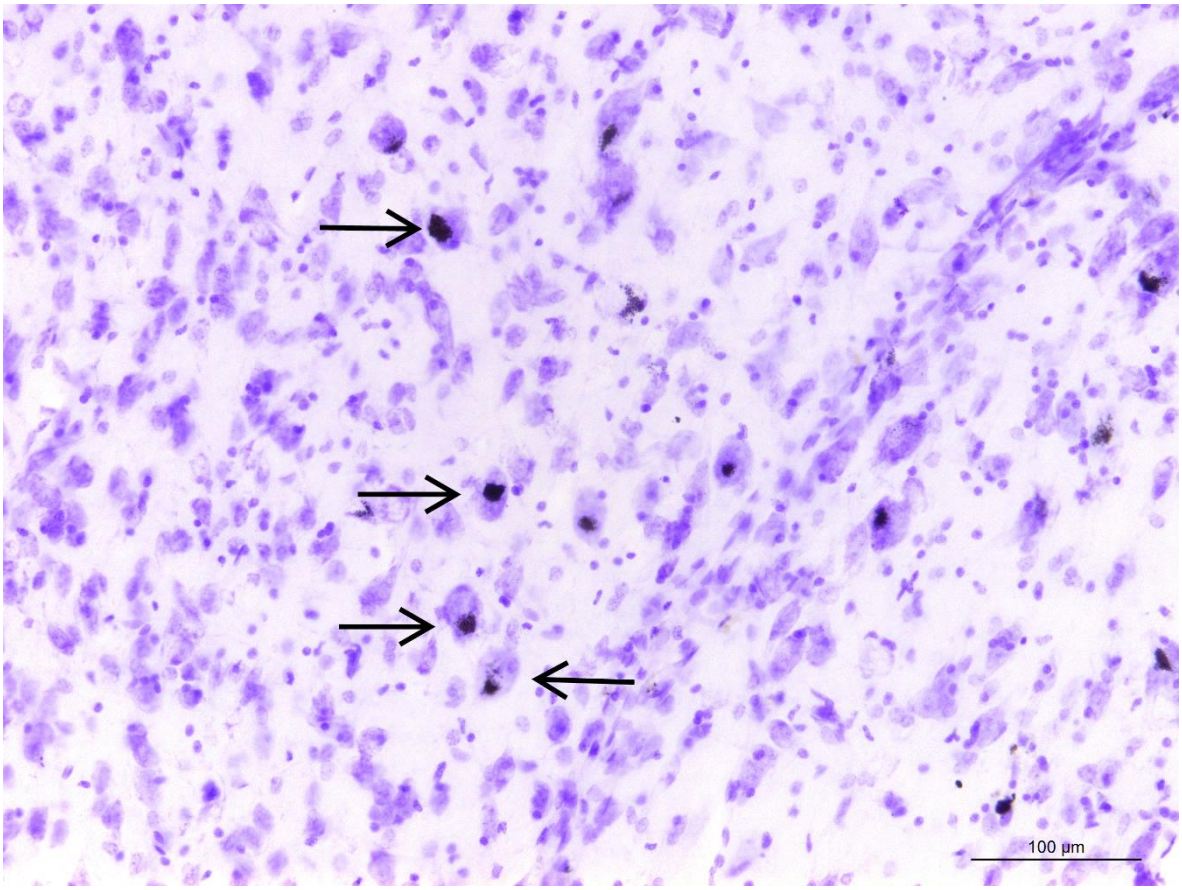

Fig. 7A: KP immunopositive cells (arrows) in the arcuate nucleus.

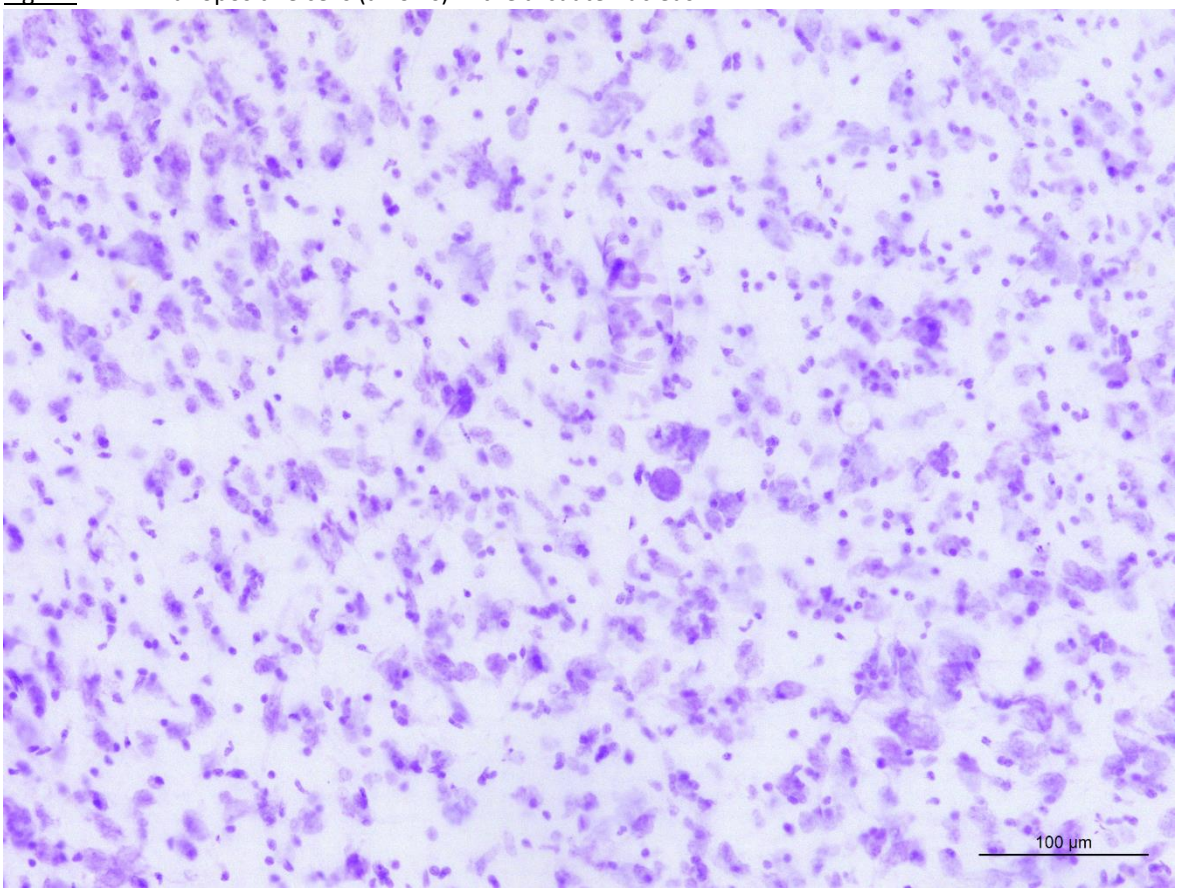

Fig. 7B: negative control experiment of the same area as is shown in fig. 7A.

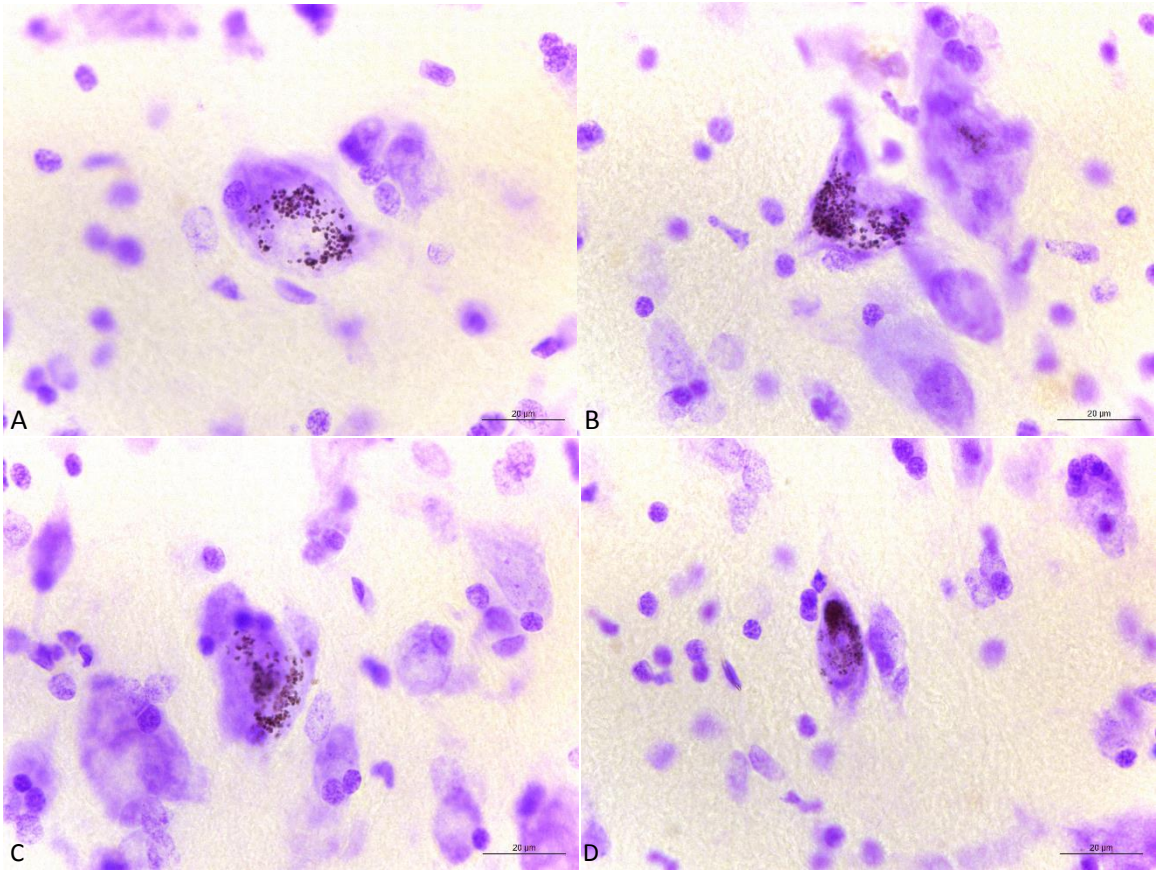

Fig. 8A-D: KP immunopositive cells in the arcuate nucleus. (The scale bar indicates 20µm)

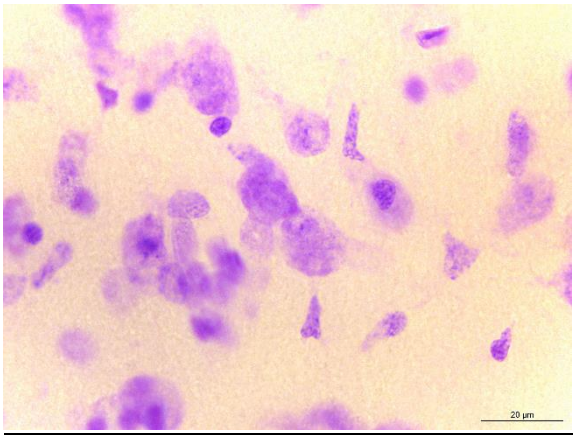

Fig. 8E: negative control experiment of the same area as is shown in fig. 8A-D. (The scale bar indicates 20µm)

Based on the anatomy of the hypothalamus as described in figure 1 and the atlas of the beagle brain[11] we concluded that the KP immunopositive cells are located in the ARC. The individual neurons we observed more dorsal are located in the VMN or even in the DMN.

4.3 GnRH

Unfortunately, our multiple attempts to find GnRH neurons in the canine hypothalamus did not produce reliable results. In some experiments all coupes were negative and during other experiments all coupes, including the negative control experiments, were positive and showed DAB precipitate.

5. Discussion

In this study, we demonstrated one population of neurons expressing the KP receptor. On both sides of the third ventricle we observed a population of KP receptor immunopositive neurons in the ventromedial corner of the hypothalamus. Based on the anatomy of the hypothalamus[10,11] we concluded that this area is the ARC. When observing individual cells we concluded that the KP receptor is expressed on a specific area of the cell surface. In some neurons it was even possible to see the triangular shape of the axon hillock in the DAB precipitate. The KP receptor is involved in the regulation of the release of GnRH so this specific location of the receptor on the neuron is very sufficient. Probably KP acts directly on the axon hillock, which is the place where all the input on the neuron is integrated and eventually the efferent action potential is started. Data about the distribution of the KP receptor in the canine hypothalamus is lacking and research in other species is very rare. Based on the few studies performed on other mammalian species there are other locations where the receptor can be found. A study in male rhesus monkeys shows that expression of kiss1r mRNA can be found in the medial and lateral ARC and in the ventral aspect of the ventromedial hypothalamus and another study also found kiss1r in the median eminence (ME).[12,13] The kiss1r is also found in the medial basal hypothalamus (MBH) and in the POA in castrated male and female Rhesus Monkeys.[14,15] In female rats the expression of kiss1r is mainly seen in the ARC, rostral periventricular area of the third ventricle (RP3V), the medial septum and rostral preoptic area (MS/rPOA).[16] Other studies in female rats found kiss1r only in the ARC and the (m)POA[17,18] and in the anteroventral periventricular nucleus(AVPV).[19] A study in mice (using transgenic Gpr54 LacZ knock-in mice, which makes it possible to visualize expression of kiss1r by histochemical staining) found no evidence for kiss1r expression in the ARC and RP3V but did find expression of the kiss1r in the periventricular region of the posterior hypothalamus, in the lateral and medial septum(LS/MS) and in the rPOA.[20] Because most of these studies used RT-PCR of mRNA extracted from blocks of dissected brain tissue it is difficult to estimate the exact location of the KP receptor.

A study in sows showed that the expression of the kiss1r mRNA is dependent on the different oestrous phases, with an increase from the ovulation phase to luteal phase and a decrease during the follicular phase.[21] It is possible that the quantity of kiss1r expression in the dog hypothalamus is also dependent on the oestrous phase. The hypothalamus we used in this study was obtained from a bitch in anoestrus, which could be the reason why we found only one population of KP receptor immunopositive neurons in the hypothalamus. In rats there is evidence that the expression of kiss1r could be downregulated by stress.[18] Because the dog was old and had some physical problems it is possible that she was exposed to some stress before she was euthanized, which could have led to a downregulation of the expression of kiss1r.

Another possible reason why we only found one nucleus of KP receptor expressing cells is that the other nuclei were not enclosed in the sample of hypothalamic tissue. The borders between the hypothalamus and the neighbouring brain tissue are not very clear at some points. Thus, it is possible that during dissection not all the hypothalamic tissue is taken out of the brain and, for example the POA (which is located in the very rostral part of the hypothalamus) was not included at all.

Unfortunately, we did not manage to get positive results out of our positive control experiment using the cytospin CHEM cells expressing the human KP receptor. We used the same protocol as we did on the hypothalamic tissue. Probably these cells needed another IHC protocol to confirm the presence of the receptor. The antibody we used was raised against synthetic 17 amino acid peptide from 2nd extracellular domain of the human receptor. Maybe this domain is not expressed the same way in the CHEM cells as it is in real hypothalamic tissue.

When studying the results of the immunohistochemistry of kisspeptin, KP immunopositive neurons in the ARC, but in contrast to the KP receptor IHC in some sections it seems like there is a second population of KP expressing neurons more dorsal to the ARC (possibly in the VMN or DMN). Data

about the neuroanatomical localisation of KP secreting neurons in the dog is also lacking.
An immunohistochemistry study in human hypothalamus tissue showed that the largest density of KP neurons in was found in the ARC (also called the infundibular nucleus, in humans) and also some KP neurons were found in the POA and ME. Another important finding in this study are the neuronal contacts between the kisspeptin immunoreactive fibers and the GnRH cells, suggesting the influence of KP on GnRH-release.[22] Similar to humans most KP neurons in Rhesus monkeys are found in de ARC and to a lesser extent, also in the POA and median eminence (ME).[13] In the hypothalamus of female mice the largest population of KP neurons, detected by immunohistochemistry, was found in the RP3V, the second largest population in the ARC and also some KP neurons were found in the dorsomedial nucleus(DMN).[23] On the other hand, a study in pro-oestrus rats only showed immunopositive cellbodies in the ARC, while immunopositive nerve fibres are more widespread throughout the brain.[24] Yet another study in female rats found KP neurons in the ARC as well as in the RP3V.[25] In intact male goats, kisspeptin immunoreactive neurons were found in the POA, ARC and ME while in castrated goats these neurons were only found in the ARC and ME. These findings suggest that gonadal steroids influence the location of expression of KP.[26] In female horses the major population of kisspeptin immunoreactive neurons is found in the ARC (2-4 hours after ovulation).[27] In another study in oestrous mares, KP neurons were found in the ARC, the DMN and the preoptic periventricular zone.[28]
As described above it is possible that we did not demonstrate other populations of KP neurons in this hypothalamus because the other nuclei have been lost during the dissecting process. Because the bitch was in anoestrus it is possible that the expression of kisspeptin in other areas of the hypothalamus is downregulated (such as is found in intact versus castrated male goats[26]).
We were not able to perform a positive control experiment with cells known to express kisspeptin. KP nerve fibres have been found in close proximity to GnRH neurons in different animal species like humans[22], horses[27,28], goats[26] and rats[25]. It is clear from studies in other animals that the KP receptor is expressed on GnRH neurons.[7] Two studies in ewes show that the GnRH neurons in de POA also express *Kiss1r* mRNA.[29,30] In male mice >90% of the GnRH neurons are found to express the KP receptor[8] and in the POA all of the kisspeptin receptor expressing cells are GnRH neurons.[6,20] Analyses in mice also showed that the expression of the KP receptor on GnRH neurons increases gradually while growing up as adults.[20]
Because peripherally administered KP gives an increase in LH, FSH and oestradiol release it is likely that GnRH neurons in the hypothalamus of the dog will also express the kisspeptin receptor. Alternatively, there could be a cell or neuron that forms a link between the KP neuron to the GnRH neuron. Because it is known from the literature that the KP receptor is present on GnRH secreting neurons we assumed it would be interesting to perform IHC with an antibody raised against GnRH as well. Unfortunately, we were not able to find GnRH neurons using IHC. The antibody we used was tested on rat, mouse, hamster, sheep and monkey GnRH, maybe it did not work on canine GnRH, or maybe our protocol was not suitable for this type of antibody.
To confirm the presence of the kisspeptin receptor on GnRH neurons we planned to do a double immunohistochemistry protocol with antibodies raised against both the KP receptor and GnRH. Unfortunately the immunohistochemistry of GnRH did not work out. In the future, more attempts should be made to demonstrate the localisation of GnRH in the canine hypothalamus and eventually double immunohistochemistry could be performed to confirm our findings of the KP receptor in the arcuate nucleus in the dog. For these studies we should use the hypothalamus of another (or more) dogs and it would be interesting to take the hypothalamus from dogs in different phases of the reproductive cycle.

6. Conclusion

Because of its important role in the hypothalamus-pituitary-gonadal axis, kisspeptin could be an interesting target for therapeutically influencing the reproductive cycle of the female dog. The aim of our study was to evaluate the distribution and localisation of kisspeptin and the kisspeptin receptor in the canine hypothalamus. We observed the presence of kisspeptin and the kisspeptin receptor in the arcuate nucleus of the hypothalamus of a female dog in anoestrus. In the future, more research is necessary to find out if other nuclei in the hypothalamus also express kisspeptin and its receptor, for example the POA. To do this the hypothalamus should be dissected with wider limits and maybe it is interesting to use the hypothalamus of a bitch in another cyclic phase to compare that with our findings in an anoestrus bitch. To confirm our findings it is important to perform a double immunohistochemistry protocol to find out if the kisspeptin receptor is indeed expressed on GnRH neurons in dogs.

7. References

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