Localization of GPR54 and gonadotropin-releasing hormone in neurons in the hypothalamus of the dog

Master research project Veterinary Medicine

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A B S T R A C T

The KISS1 gene encodes a family of peptides called kisspeptins, which bind to the G protein coupled receptor GPR54. Dysfunction of GPR54 leads to an insufficient release of gonadotropins and a failure to enter puberty, which implies that KISS1/GPR54 signaling plays an essential role in reproduction. Kisspeptin directly stimulates the release of GnRH from the forebrain after activation of GPR54, which is expressed by GnRH neurons. There are two major hypothalamic kisspeptin populations, with one in the arcuate nucleus (ARC) and one in the preoptic area (POA) or anteroventricular periventricular nucleus (AVPV), depending on the species. KISS1 gene expression is regulated by the presence or absence of sex steroids. Kisspeptin neurons in the ARC have been implicated in mediating the negative feedback of sex steroids on GnRH secretion while the kisspeptin neurons in the POA / AVPV are positively regulated by sex steroids. In other words, ARC kisspeptin neurons are involved in controlling the tonic pulsatile release of GnRH, whereas kisspeptin neurons in the POA / AVPV are responsible for the induction of the pre-ovulatory LH surge. Although KISS1/GPR54 signaling could lead to new therapeutic opportunities such as non-surgical contraception, estrus prevention and ovulation induction, it has not yet been studied in the dog. The aim of this research was to identify the location of GPR54 containing neurons and GnRH neurons in the canine hypothalamus. This report has demonstrated that GnRH neurons and GPR54 containing neurons were present on both sides of the third ventricle in the arcuate nucleus (ARC) region and slightly more dorsal and lateral, which corresponds to the preoptic area (POA) in the hypothalamus of the Beagle bitch and shows theoretical co-localization of GnRH neurons and GPR54 in the ARC and POA, where GnRH neurons and GPR54 were separately detected by immunohistochemistry. Because only one dog was used in this research and the negative controls showed immuno-positive results, the results of this report should be interpreted with caution.

Keywords: HPG-axis, GnRH, GPR54, KISS1, kisspeptin, ARC, POA

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1. INTRODUCTION

1.1 The hypothalamus-pituitary gonadal (HPG) axis

Regulation of reproduction is among others organized in three levels: the hypothalamus, the pituitary and the gonads (the ovary and the testis). This system is called the hypothalamic-pituitary gonadal (HPG) axis. Hypothalamic neurons secrete gonadotropin releasing hormone (GnRH) into the portal blood system, which has the ability to induce self-priming in the pituitary and which stimulates gonadotropin secretion from the anterior pituitary. The gonadotropins Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) stimulate the production of sex steroids (estradiol, testosterone, progesterone), which are required for optimal spermatogenesis, oogenesis, and pregnancy. (Tena-Sempere and Huhtaniemi 2003; Colledge 2004; Roa et al. 2008; 2010). These hormones participate through negative and positive feedback loops in the dynamic regulation of the HPG-axis and thus in the regulation of mammalian reproduction (Colledge 2004; Roa and Tena-Sempere 2007; Roa et al. 2008).

It has been known that interactions between the hypothalamus and the anterior pituitary are crucial for the onset of puberty and the maintenance of normal adult sexual function (Colledge 2004). Puberty is initiated when gonadotropin-releasing hormone is secreted in sufficient levels by GnRH neurons to induce optimal gonadotropin release from the pituitary (Seminara, et al. 2003) to support spermatogenesis or oogenesis. (Ebling and Cronin 2000; Plant and Barker-Gibb 2004; Shahab et al. 2005; Gottsch et al. 2006).

Although the main components of the HPG-axis have been well described for many different mammalian species, the factors in the forebrain that initiate the onset of puberty and lead to sexual maturation are still not completely elucidated. Activation of pulsatile hypothalamic GnRH secretion is known to be a key event in the onset of puberty (Ebling and Cronin 2000), but there are several factors that are considered to be triggers of the GnRH neuron, i.e. GABA and neuropeptide Y (NPY), and peripheral hormones, such as leptin (Colledge 2004; Watanabe 2014).

Recently, it was shown that a dysfunction of the kisspeptin receptor GPR54 induces isolated hypogonadotropic hypogonadism (IHH) in humans and mice, which is characterized by an insufficient release of LH and FSH and a failure to enter puberty (Funes et al. 2003; De Roux et al. 2003; Seminara et al. 2003). This finding suggested that signaling through GPR54 is essential for sexual maturation and thus fertility in mammalian species.

1.2 The KISS1/GPR54 system

The KISS1/GPR54 system is a ligand-receptor system (Roa and Tena-Sempere 2007; Roa et al. 2008) comprising a number of structurally-related peptides encoded by the KISS1 gene with the ability to bind and activate the G protein coupled receptor GPR54 (Mikkelsen and Simmoneaux 2008; Roa et al. 2008; 2011; Tena-Sempere 2010), also known as OT7T175 or AXOR12 (Kotani et al. 2001; Muir et al. 2001; Othaki et al. 2001). The product of the KISS1 gene is a 145-amino-acid peptide that is enzymatically cleaved into a 54-amino-acid peptide, kisspeptin-54 (Popa et al. 2008).* Since kisspeptin-54 was found to suppress metastasis of melanoma and breast cancer cells, it was named 'metastatin' (Gottsch et al. 2006). The KISS1 peptides, all derived from the common precursor of 145 amino acids, included not only metastatin but also shorter fragments of this molecule that share a common C-terminal RF-amide (Kotani et al. 2001; Roa and Tena-Sempere 2007; Popa et al. 2008). Further cleavage of metastatin (kisspeptin-54) gives rise to these shorter fragments: kisspeptin-10 (KP-10), KP-13 and KP-14, all of which act with a similar affinity at GPR54 (Kotani et al. 2001; Gottsch et al. 2006). This group of peptides is collectively called kisspeptins, also termed RF-amide peptide super-family (Roa et al. 2008).

Although kisspeptins and GPR54 were first described in relation to suppressing cancer metastasis, they were subsequently shown to play an essential role in the control of the HPG-axis via regulation of GnRH secretion (Roseweir and Millar 2009). Both KISS1 mRNA and GPR54 mRNA expression in the hypothalamus increase in male and female rats at puberty (Navarro et al. 2004c). In monkeys, KISS1 mRNA also increases during puberty (Shahab et al. 2005). Dysfunction of the kisspeptin receptor GPR54 by inactivating mutations or targeted deletions produce isolated hypogonadotropic hypogonadism (IHH) in humans and mice, which is characterized by an insufficient release of LH and FSH by the pituitary and a failure to enter puberty, suggesting that signaling through GPR54 is a prerequisite for sexual maturation and thus fertility (De Roux et al. 2003; Funes et al. 2003; Seminara et al. 2003; Gottsch et al. 2004; Matsui et al. 2004; Gottsch et al. 2006; Kauffman et al. 2007; Ramaswamy et al. 2008; Roseweir and Millar 2009; Clarkson et al. 2010; Merkley 2013). An activating mutation, R386P, induced prolonged signaling of the receptor causing precocious puberty in a 8 year old girl. (Teles et al. 2008) Moreover, exogenous administered kisspeptin to pre-pubertal rodents and monkeys initiates various aspects of precocious puberty (such as LH secretion or advanced vaginal opening) (Navarro et al. 2004a; Han et al. 2005; Shahab et al. 2005; Kauffman et al. 2007).

** In rodents, the kisspeptin precursor is post-translationally spliced into a 52 amino-acid peptide (kisspeptin-52) (Mikkelsen, et al. 2009).*

1.3 Activation of GnRH neurons by kisspeptins

Additional studies have shown that kisspeptin-GPR54 signaling may have a regulatory function in the neuroendocrine reproductive axis- beyond acting as a simple 'gate' for the onset of puberty (Funes et al. 2003; De Roux et al. 2003; Seminara et al. 2003).

Administration of kisspeptin stimulates GnRH and gonadotropin release in rodents, sheep, cows, primates, dogs and humans (Gottsch et al. 2004; Irwig et al. 2004; Matsui et al. 2004; Thompson et al. 2004; Messager et al. 2005; Shahab et al. 2005; Kauffman et al. 2007; Mikkelsen and Simmoneaux 2008; Albers-Wolthers et al. 2014;) in a dose dependent manner. Doses as low as 1 fmol are effective for eliciting LH secretion (Gottsch et al. 2004; Navarro et al. 2005b). Based on studies of single effective doses of other neuropeptides in a variety of rodent species, it appears that kisspeptin is the most potent secretagogue for LH, making kisspeptin the most potent stimulator of LH known to date (Gottsch et al. 2004; Roseweir and Millar 2009; Merkley 2013). Remarkably, kisspeptins trigger the HPG-axis and induce LH release regardless of the route of administration (Gottsch et al. 2006). Low doses have been shown to be effective both centrally (I.C.V.) and systematically (I.P. and I.V.) at similar levels in rodents (Navarro et al. 2005a,b; Thompson et al. 2004). Kisspeptin has no effect on LH and FSH secretion in mice lacking a functional GPR54 gene, even though the GnRH neurons in these mice are phenotypically normal (Messager et al. 2005), suggesting that GPR54 is the only receptor for kisspeptin on GnRH neurons and that its main physiological function is to support GnRH secretion (Gottsch et al. 2006; Popa 2008). Kisspeptin neurons most likely reside within either the brain or pituitary, both of which have been shown to express GPR54 (Lee et al. 1999; Muir et al. 2001; Navarro et al. 2004c; Kinoshita et al. 2005).

Whether gonadotropins from the pituitary are also targets for kisspeptin is still unclear (Kauffman, et al. 2007). GPR54 is expressed in the human pituitary (Kotani et al. 2001; Muir et al. 2001), and kisspeptin stimulates gonadotropin release *in vitro* from cultured rat, pig, ovine and bovine primary pituitary cells (Navarro 2005b; Gutiérrez-Pascual et al. 2007; Okamura et al. 2013). It is therefore possible that peripherally administered kisspeptin also acts directly on the pituitary to stimulate the release of LH and FSH (Thompson et al. 2004). However, other studies have shown no apparent effect of kisspeptins on *in vitro* LH or FSH secretion in cultured primary rat pituitary cells or anterior pituitary fragments (Matsui et al. 2004; Thompson et al. 2004). Thompson et al. (2004) showed that kisspeptin has the ability to induce the release of GnRH from hypothalamic rat explants. Other than that, GnRH antagonists (acyline or cetrorelix) have the ability to inhibit the rise in LH and FSH from the pituitary before the administration of kisspeptin (Gottsch et al. 2004; Matsui et al. 2004; Mikkelsen and Simmoneaux 2008; Mikkelsen et al. 2009), suggesting that the stimulatory effect of kisspeptin on gonadotropin secretion is mediated by GnRH and that the direct stimulation of the pituitary by kisspeptin neurons is not a primary mechanism of gonadotropin secretion (Gottsch et al. 2004; Navarro, et al. 2004a,b; 2005b; Matsui et al. 2004). Furthermore, central administration of kisspeptin in rats induces Fos expression in GnRH neurons, which is indicative of neuronal stimulation (Irwig et al. 2004),

supporting the argument that GnRH neurons in the hypothalamus are the primary targets for the action of kisspeptin in the HPG-axis (Gottsch et al. 2004; Navarro et al. 2005b; Popa et al. 2008) and that GnRH is required for the stimulatory effect of kisspeptin on gonadotropin secretion (Gottsch et al. 2004; Kauffman et al. 2007).

1.4 KISS1/GPR54 expression

GnRH plays an essential role in controlling reproductive functions via two ways of secretion in females (Hassaneen et al. 2016). One is pulsatile secretion, which stimulates gonadotropin pulses, which in turn stimulates follicular development and the production of sex steroids. The other is surge secretion, which induces a LH surge followed by ovulation (Senger 2012). Kisspeptin neurons are thought to be involved in the regulation of both types of GnRH release (Messager et al. 2005; Okamura et al. 2013).

Kisspeptin neuronal cell bodies are located in two major hypothalamic areas in a variety of mammalian species (Oakley et al. 2009). One population resides in the hypothalamic arcuate nucleus (ARC) in humans, monkeys, sheep, rats, mice, hamsters, goats, pigs, and horses.

The other kisspeptin neuronal population is located more rostrally, in the preoptic area (POA) of sheep, fish and primates, and in the anteroventricular periventricular nucleus (AVPV) of rodents or the periventricular nucleus (PeN) of pigs (Tomikawa, et al. 2010) (Table 1).

Table 1 - Distribution of KISS1 cells in the mammalian nervous system.

SPECIES	ARC	PREOPTIC REGION		PeN
		POA	AVPV	
Fish		✓		
Hamster	✓		✓	
Mouse	✓		✓	
Rat	✓		✓	
Goat	✓			
Pig	✓			✓
Sheep	✓	✓		
Horse	✓			
Monkey	✓	✓		
Human	✓	✓		

References Table 1.

- Fish: Kim et al. (2014)
- Hamster: Mason et al. (2007)
- Mouse: Gottsch et al. (2004); Han et al. (2005); Kinoshita et al. (2005); Smith et al. (2005, b); Kauffman et al. (2007)
- Rat: Irwig et al. (2004); Kinoshita et al. (2005); Smith et al. (2006); Kauffman et al. (2007)
- Goat: Ohkura et al. (2009)
- Pig: Tomikawa et al. (2010)
- Sheep: Franceschini et al. (2006); Pompolo et al. (2006); Smith et al. (2007)
- Horse: Decourt et al. (2008); Smith et al. (2008; 2009)
- Monkey: Shahab et al. (2005); Rometo et al. (2007); Ramaswamy et al. (2008)
- Human: Hbrabovszky et al. (2010)

ARC kisspeptin neurons differ from those in the other population because of their extensive reciprocal connections with each other, and their co-expression of two other neuropeptides, neurokinin B (NKB) and dynorphin (Dyn). Due to this co-expression, the ARC kisspeptin neurons have been termed KNDy (Kisspeptin/NKB/Dynorphin) neurons (Merkley 2013). KNDy neurons are known to be responsible for the tonic pulsatile release of GnRH, whereas kisspeptin neurons in the POA / AVPV are involved in the pre-ovulatory LH surge in females (d'Anglemont de Tassigny 2010).

GPR54 was found to be mainly expressed in the brain, pituitary, and placenta (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). In humans, the highest expression of GPR54 mRNA was seen in the pancreas and placenta with lower levels in the spleen, lymph nodes, and brain (Ohtaki et al. 2001). The low expression of GPR54 mRNA in the brain probably reflects the restricted distribution of transcript to certain sites in the brain. In the brain, the highest level of expression is observed in the hypothalamus and amygdala (Thompson et al. 2004). GPR54 is highly concentrated in the midline hypothalamus, including the ARC, the PeN, and the dorsomedial hypothalamic area (Thompson et al. 2004).

It has been shown that the great majority of GnRH neurons co-express GPR54 and GnRH in single neurons isolated by laser capture from the cichlid fish *Oreochromis niloticus* (Parhar et al. 2004), in rats (Irwig et al. 2004), and in mice (Han et al. 2005; Messager et al. 2005). For example, Irwig et al. (2004) showed that in the rat 77% of GnRH neurons co-express GPR54 mRNA and that kisspeptin efficiently induced c-fos expression in > 85% of GnRH neurons. GnRH neurons in monkeys (Shibata et al. 2005) and horses (Decourt et al. 2008) also co-express GPR54. The highest number of GnRH neurons is located in the preoptic area (POA) (Matsui et al. 2004). GPR54 co-localizing with GnRH neurons in the POA and the immediate-early gene product Fos occurring in these neurons after central administration of kisspeptin suggests that kisspeptins act directly on GnRH neurons by binding to GPR54 expressed on these GnRH neurons (Irwig et al. 2004; Matsui et al. 2004; Popa et al. 2005).

1.5 The effects of sex steroids on KISS1/GPR54

GnRH neurons expressing GPR54 indicates that kisspeptins play an essential role in the regulation of reproduction. Sex steroids have already been established as important players in the functioning of the HPG-axis. Estradiol (E) and testosterone (T) are crucial because of their feedback to the brain in order to control the output from the hypothalamus and pituitary. It might be plausible that this feedback occurs directly at the level of GnRH neuron, however, this seems to not be the case, since GnRH neurons only express estrogen receptor beta (ERβ), and not ERα (Beymer et al. 2016).

Both populations of hypothalamic kisspeptin neurons have been shown to express both ERα and ERβ in all mammalian species studied. KISS1 gene expression is regulated by the presence or absence of sex steroids (Beymer et al. 2016). Kisspeptin neurons in the ARC have been implicated in mediating negative feedback of sex steroids on GnRH secretion. Estradiol has an inhibitory effect on KISS1 expression in the female rat (Navarro et al. 2004c) and

reduction of circulating levels of sex steroids by gonadectomy increases the number of KISS1 mRNA expressing neurons as well as the content of KISS1 mRNA per cell in the ARC (Smith et al. 2005a,b). The inhibition of kisspeptin activity by sex steroids in the ARC appears to be mediated by estrogen receptors (ER) in the female. Although kisspeptin neurons in the ARC are involved in the negative feedback of sex steroids, the more rostral nuclei of the forebrain act differently. The expression of KISS1 mRNA in the POA / AVPV and PeN of female mice is positively regulated by sex steroids (Smith et al. 2005b). Gonadectomy produces a dramatic decrease in the number of KISS1 mRNA expressing cells in the AVPV. The complex effects of estrogen and testosterone are also mediated by the androgen receptor (AR) (Gottsch et al. 2006). The differential expression of ER and AR in kisspeptin neurons (Smith et al 2005a, Kauffman et al. 2007) between the ARC and the AVPV may explain the opposite effect of sex steroids on the regulation of KISS1 gene expression in these two regions. Roa et al. (2008) found that in rodents the neuronal kisspeptin population in the AVPV expresses ERα, which mediates the positive feedback effects of estradiol. However, the exact distribution of sex steroids receptors remains to be further investigated in different mammalian species.

The POA / AVPV kisspeptin population is the site of estrogen positive feedback and the kisspeptin population in the ARC is the primary site of negative feedback (Smith et al. 2005a,b). This finding suggests that the ARC kisspeptin neurons regulate the tonic pulsatile release of GnRH which is important for proper ovarian maintenance including growth and maturation of developing follicles (Li et al. 2009), while the kisspeptin neurons in the POA / AVPV are involved in the estradiol-induced pre-ovulatory surge of GnRH and LH which occurs during the pro-estrus stage of the oestrous cycle (Smith et al. 2006; Clarkson et al. 2010; Dror et al. 2013; Beymer et al. 2016).

Interfering with KISS1/GPR54 signaling could result in new possibilities for breeding management and therapeutic interventions in dogs. The domestic bitch *(Canis lupus familiaris)* is a mono-estrous, non-seasonal, polytocous and spontaneous ovulator (Concannon 2011). The duration of the estrous cycle of the bitch is unique regarding to its length (De Gier et al. 2008). The cycle is divided into four phases: a 5-20 day pro-estrus, 5-15 day estrus, 50-80 day met-estrus (irrespective of pregnancy), and a non-seasonal anestrus typically lasting 80-240 days (Concannon 2011). New therapeutic opportunities such as non-surgical contraception, estrus prevention and ovulation induction could be achieved by manipulation of the KISS1/GPR54 system, but the role of KISS1/GPR54 signaling in the HPG-axis in the dog has not yet been fully elucidated. The aim of this research was to determine whether GPR54 containing neurons are expressed in the hypothalamus of the bitch and to identify the location of GPR54 containing neurons and GnRH neurons in the hypothalamus, in order to obtain a better understanding of this hormonal signaling system. To do this, a localization study using antibodies against GnRH and GPR54 on hypothalamic tissue sections of the dog was performed.

2. MATERIAL AND METHODS

2.1 Tissue collection and processing

Hypothalamic tissue of one 13-year-old anestrus Beagle bitch was used for this research. The dog was euthanized with dexmedetomidin, 5µg/kg (Dexdomitor® Orion Corporation, Espoo, Finland), due to age related, physical problems, unrelated to endocrine or reproductive function. After bleeding a 4% solution of buffered formalin was perfused through the arteria carotis in order to fixate the brain as fast as possible. The brain was post-fixed in 2% PFA/PBS solution for 24 hours at 4˚C and stored in a fixative until use.

The hypothalamus was dissected from the brain and cryopreserved with ascending solutions of 5%, 10%, and 30% sucrose in PBS before 25 µm coronal sections were cut on a freezing microtome. The tissue sections were conserved in anti-freeze cryoprotectant (30% sucrose solution) and stored at -20˚C until later use.

All animal experiments were conducted according to the welfare protocol of Utrecht University (Utrecht, The Netherlands).

2.2 Immunohistochemistry of GnRH neurons and GPR54

The sections were thawed by putting them in a refrigerator (4°C) overnight. To remove the sucrose, the sections were rinsed three times in 0.1 M PBS (10 min each time). The sections were then mounted on poly-lysine coated slides and dried for at least 48 hours at 26˚C.

Using a Dako Pen, a water repelling circle was drawn around the tissue sections. This circle provided as a barrier to liquids.

To reduce auto fluorescence, the sections were immersed in a solution of sodium borohydride ($NaBH_4$, 0.1%) for 15 minutes (Clancy and Cauller 1998). The sections were then washed once with PBS, followed by antigen retrieval.

Antigen retrieval was accomplished by incubating the sections in 0.01M sodium citrate buffer (pH 6.0) at 80˚C for 20 minutes (Jiao et al. 1999; Kashir et al. 2017). The sections were then rinsed three times with 0.1M PBS (10 min each time) and after this another 3 times with 0.1% PBS-T (45 min each time).

For pre-incubation, the sections were washed with PBS-T + BSA, to reduce any non-specific anti-body binding. The sections were incubated for 1 h at room temperature.

Monoclonal mouse antibody anti-GnRH antibody MAB5456 (Merckmillipore, Billerica, MA) was used for the immunohistochemistry of GnRH and polyclonal rabbit antibody GPR54 antibody NLS1926 (Novus Biologicals, Littleton, CO) was used for the immunohistochemistry of the kisspeptin receptor. Both were diluted 1:200 in PBS-T + BSA and incubated overnight at 4˚C.

The sections were rinsed three times (10 min each time) with PBS-T and incubated with the secondary antibodies. Either goat anti-mouse IgG + IgM 115-035-044 Alexa 488 (Molecular Probes, Eugene, OR) or polyclonal goat anti-rabbit immunoglobulins / HPR P0448 GAR Alexa 568 (Molecular Probes) were applied for staining GnRH and GPR54, respectively.

Both were diluted 1:100 in PBS-T + BSA and incubated in the dark for 2 hours at room temperature.

After rinsing in PBS-T (three times, 10 min each time), a nuclear DAPI (Molecular Probes) stain was performed, consisting of 12 µl of DAPI in 200 µl milliQ diluted in 10 µl of PBS-T. This solution was incubated for approximately 1.5 minutes before quickly rinsing the slides two times with PBS. The slides were mounted in Fluorsave (Calbiochem, Merckmillipore, Billerca, MA) and left to dry in the dark at room temperature.

2.3 Negative control experiments

Negative control experiments were performed by excluding the primary antibody from the protocol and replacing it with normal mouse serum (Vector Labs, Burlingame) (for GnRH) and normal rabbit serum (Vector Labs) (for GPR54). Both were diluted 1:100 in PBS-T + BSA.

2.4 Data analysis

Imaging of fluorescence labelling for GnRH and GPR54 was performed by using an Olympus BX60 (Leica microsystems B.V., The Netherlands) fluorescence – histology microscope equipped with four diode laser lines (455 nm, 488 nm, 561 nm, and 635 nm). For Alexa 488 (green), Alexa 568 (red) and DAPI (blue), the 488, 561, and 455 laser lines were used respectively. A bright-field transmission channel was also used to differentiate between positive and false-positive results. A magnification of 20X and 40X was used and composite images were imported to LAS-AF Lite Leica software (Leica microsystems B.V.) where further analysis of the images was performed.

After obtaining the images, they were later analyzed using the LAS-AF Leica software (Leica microsystems B.V.). Localization of GnRH (green) or GPR54 (red) was considered truly positive when a cell nucleus was associated with the staining. When this was found, the bright-field transmission channel was examined to look for any artefacts that could have caused the fluorescence. When none were found, the results were considered positive. Also, drawings were made to indicate where in the section positive cells were found. Using the drawings made during imaging, the areas in which the positive signals were found could be identified by using a stereotaxic atlas of the Beagle brain (Palazzi 2011). Levels of expression were not quantified, and no statistical comparisons were made.

3. RESULTS

Experiments for detecting GnRH neurons were performed 4 times, while experiments for detecting GPR54 were performed 2 times. In this report, only one experiment for either the detection of GnRH neurons or GPR54 containing neurons was used to give an overview of the location and distribution since other experiments showed inconclusive results. The hypothalamic areas where positive GnRH neurons and GPR54 containing neurons were found, were determined. Only representative sections of the Beagle bitch hypothalamus were taken into account and compared to a section (Plate 59, +29,5 mm) in the stereotaxic atlas of the Beagle brain (Palazzi 2011). Some hypothalamic sections were not cut properly or were torn when they were put on a slide. Therefore, these sections were not representative of the hypothalamus and were not compared to a section in the stereotaxic atlas (Palazzi 2011). This resulted in only one representative section for the detection of GnRH neurons and two for the detection of GPR54 containing neurons.

The arcuate nucleus (ARC) and the preoptic area (POA) were especially examined, as these regions are known for GPR54 expression in GnRH neurons in other species.

3.1 Localization of GnRH neurons

First, the protocol for GnRH detection was performed. During imaging, there appeared to be a high level of auto-fluorescence in the sections which made it impossible to distinguish auto-fluorescence from positive cells (Fig. 1A). The protocol was adjusted to reduce auto-fluorescence by treating the sections with a solution of sodium borohydride ($NaBH_4$, 0.1%) for 15 minutes (Clancy and Cauller 1998). By adding this step to the protocol, a 50% reduction of background fluorescence was accomplished which made it much easier to search for positive fluorescent signals (Fig. 1B).

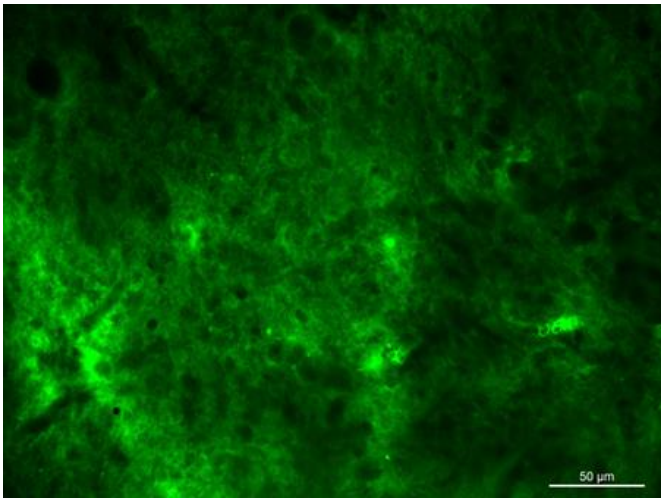


Fig. 1A. Auto-fluorescence seen before treatment with $NABH_4$.

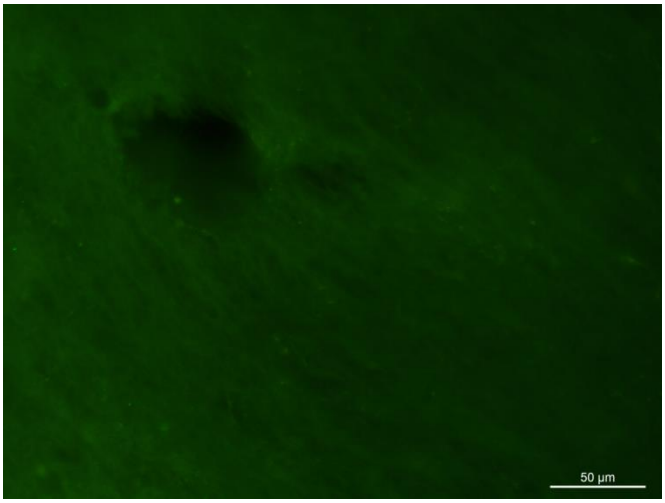


Fig. 1B. Reduction of auto-fluorescence after treatment with $NABH_4$.

GnRH neurons were present in the ARC and the POA (AMN) (Fig. 2). These positive signals showed association with a cell nucleus without the bright field channel showing an artefact which could have caused the fluorescence (Fig. 3). The staining of GnRH neurons was visible as isolated small oval cells. The staining looked solid bright green. On an average, GnRH stained neurons were observed in 6 cells per section in 1 out of 1 section.

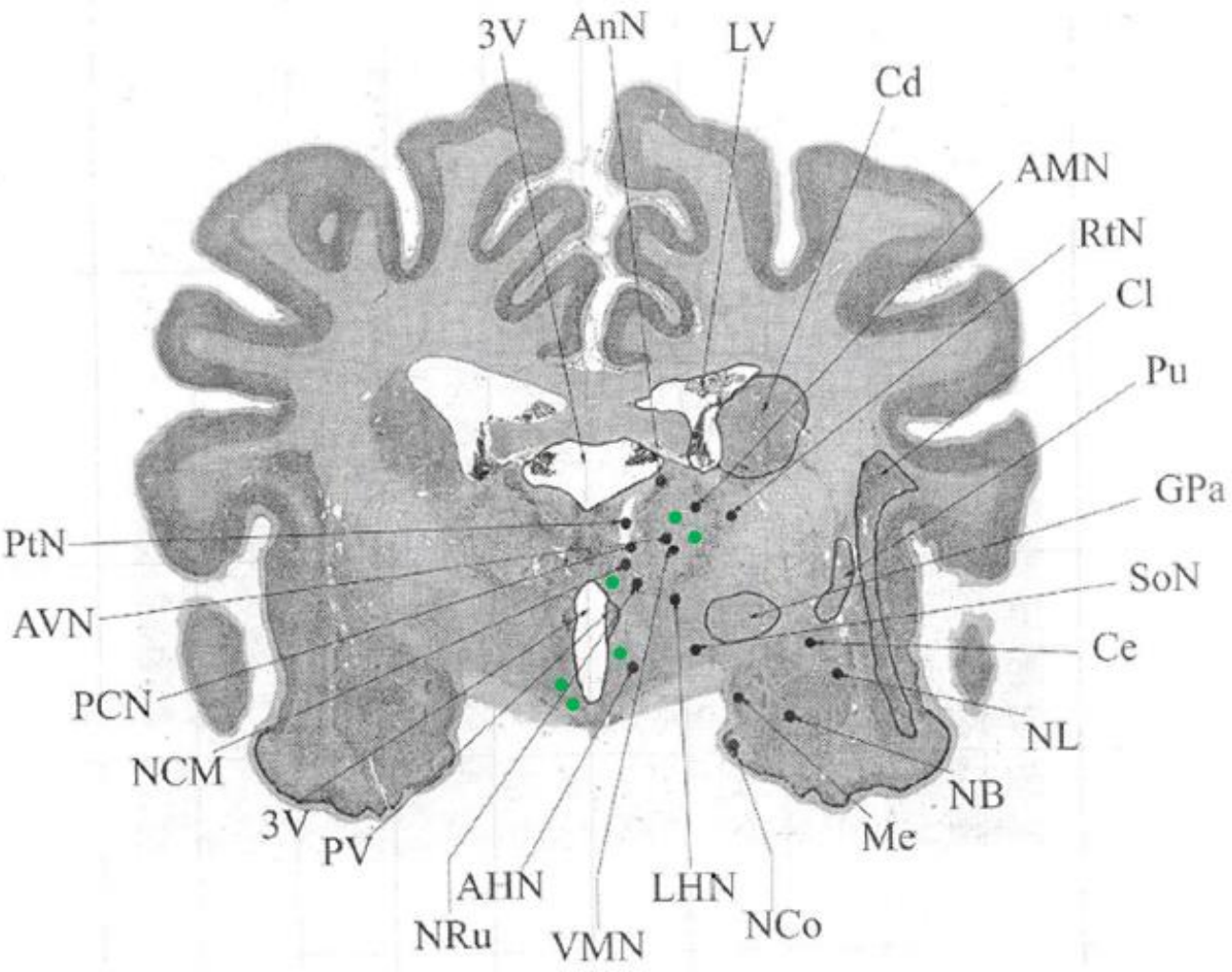


Figure 2. Location of GnRH stained neurons in the hypothalamus of the Beagle bitch. Areas in the hypothalamus where GnRH positive neurons (green dots) were found are shown. Positive neurons were found in the ARC and POA (AMN).

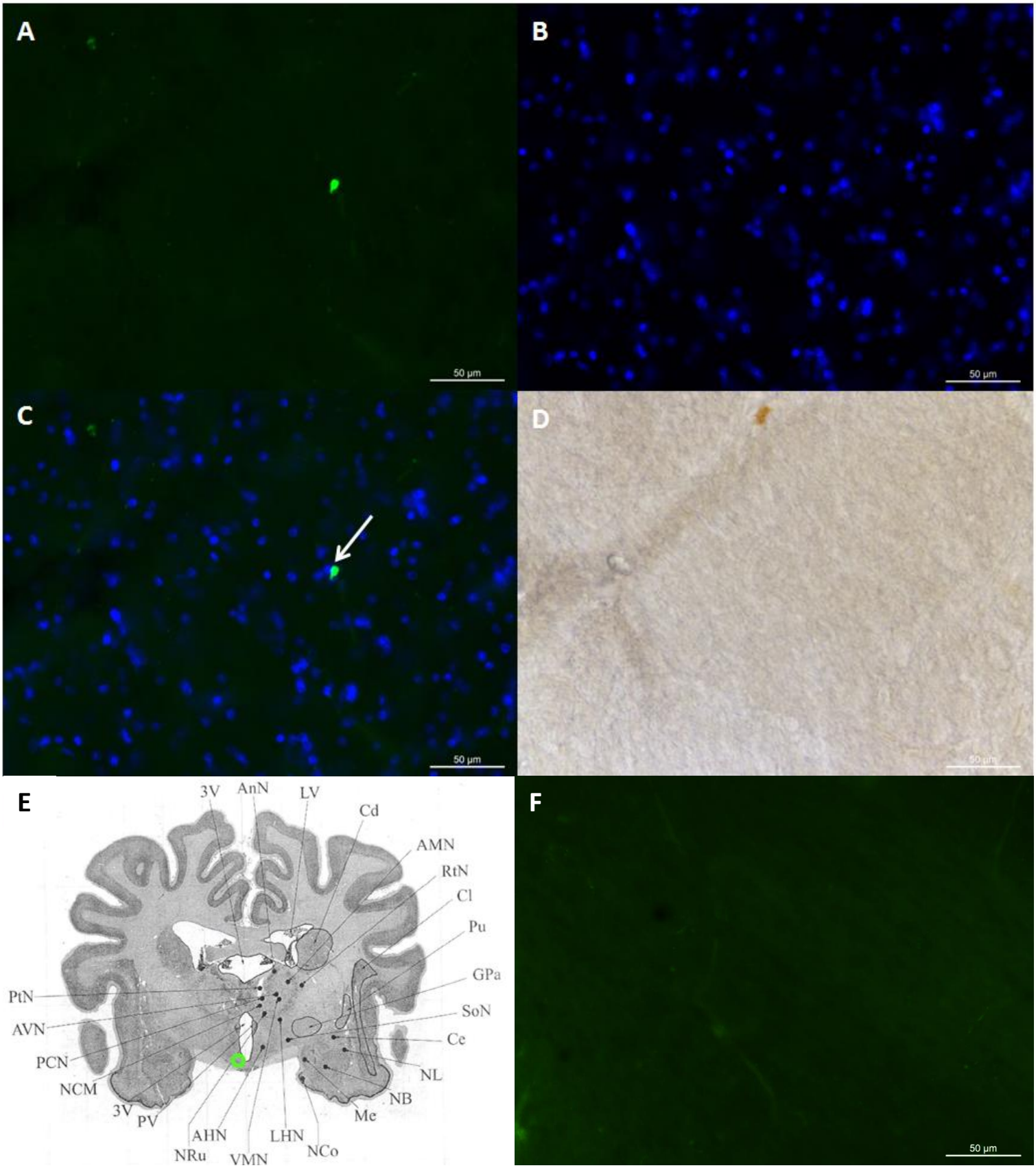


Fig. 3. **Example of a representative GnRH staining.** GnRH stained neurons (green) stained with Alexa 488 (A), cell nuclei (blue) stained with DAPI (B), an overlay (C) of A and B, and the bright field transmission channel (D). The bright field transmission channel shows no artefacts which could have caused the fluorescence, and the GnRH staining seems to associate with a cell nucleus (arrow) and is thus confirmed positive. E shows the location of the GnRH neuron in the ARC. F shows the negative control which shows no fluorescence.

3.2 Localization of GPR54

The same protocol was used for the detection of GPR54 containing neurons. The adjusted protocol for the staining of GnRH neurons resulted in little auto-fluorescence during the staining of GPR54.

GPR54 was also found in the arcuate nucleus (ARC) and the preoptic area (POA) (Fig. 4). Again, the fluorescent structure shows association with a cell nucleus and no artefact was found which could have cause the fluorescence (Fig. 5). GPR54 staining was visible as dots, mostly isolated but sometimes located closely together. The staining looked solid bright red with blurry edges. GPR54 staining was detected in 1-3 cells per section in 2 out of 2 sections.

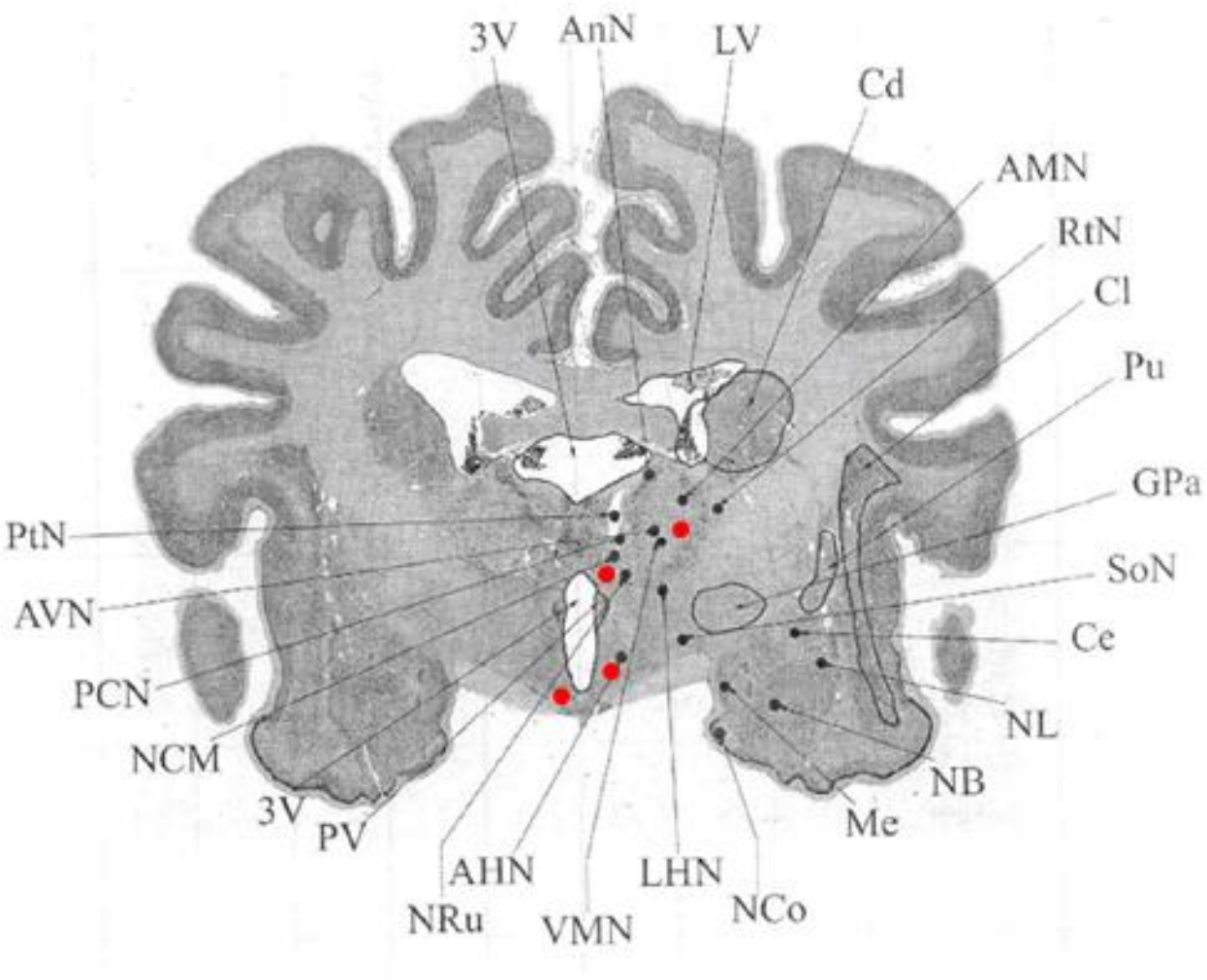


Figure 4. Location of stained GPR54 containing neurons in the hypothalamus of the Beagle bitch. Areas in the hypothalamus where GPR54 containing neurons (red dots) were found are shown. Positive GPR54 staining was found in the ARC and POA (AMN).

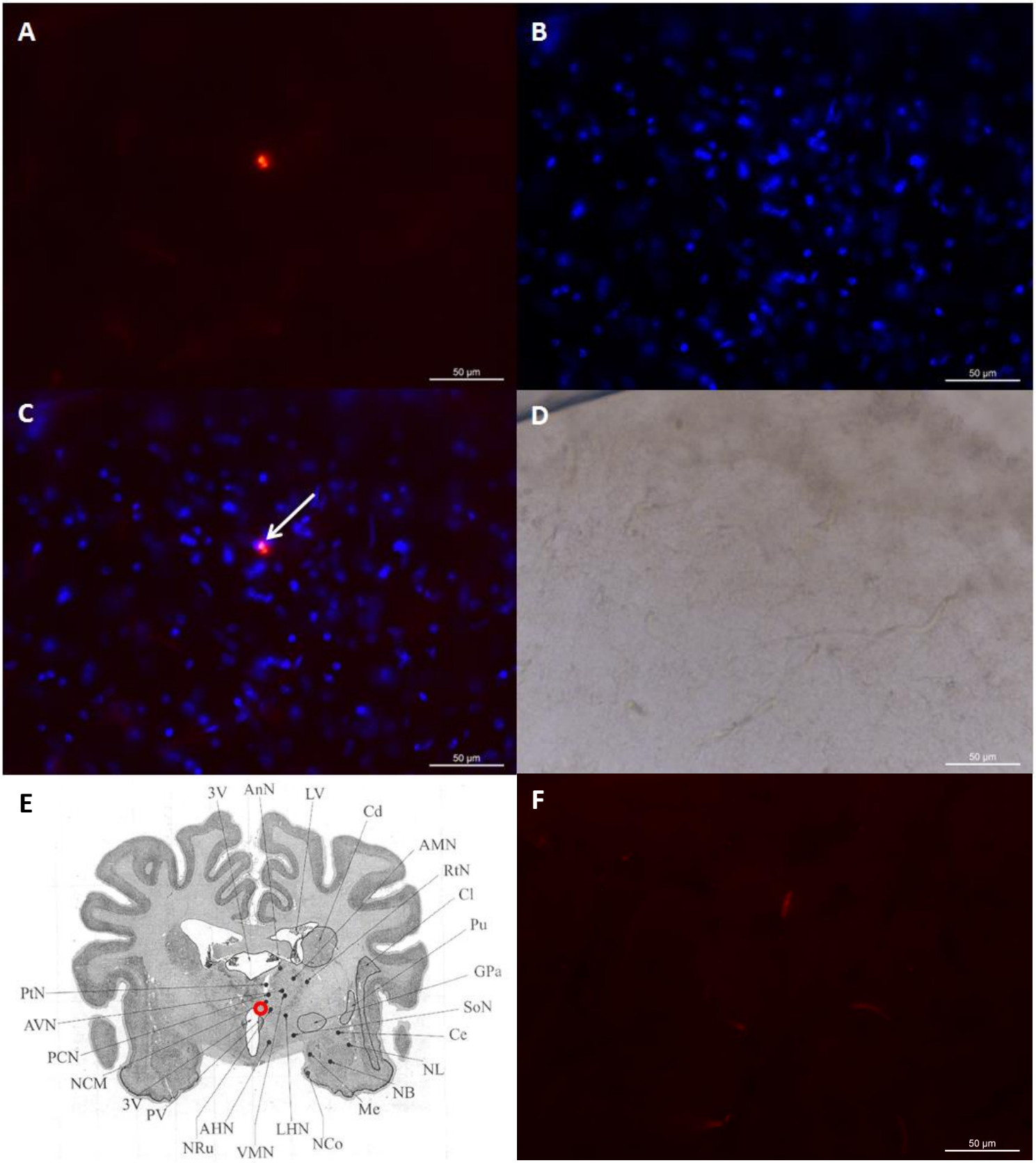


Fig. 5. **Example of a representative GPR54 containing neuron staining.** GPR54 containing neuron (red) stained with Alexa 568 (A), cell nuclei (blue) stained with DAPI (B), an overlay (C) of A and B, and the bright field transmission channel (D). The bright field transmission channel shows no artefacts which could have caused the fluorescence, and the GPR54 containing neuron seems to associate with a cell nucleus (arrow) and is thus confirmed positive. E shows the location of the GPR54 staining in the ARC. F shows the negative control which shows weak fluorescence.

3.3 Co-localization of GPR54 on GnRH neurons

Perfecting the protocols for the optimal staining of GnRH neurons and GPR54 containing neurons has priority in order to perform a co-localization experiment. Because GPR54 is known to co-localize with GnRH neurons in other species (Irwig, et al. 2004; Parhar, et al. 2004; Han, et al. 2005; Messager, et al. 2005; Shibata, et al. 2005; Decourt, et al. 2008), during this research co-localization was determined by combining the localization data of GnRH neurons (Fig. 2) and GPR54 containing neurons (Fig. 4). GPR54 was present on GnRH neurons in the ventromedial area of the hypothalamus, distributed on the sides of the third ventricle (3V). This area corresponds to the arcuate nucleus (ARC). Co-localization was also found slightly more dorsal and lateral, which corresponds to the preoptic area (POA) (Fig. 6). Co-localization could not be properly described, because it was only found theoretically by overlapping the separately detected GnRH neurons and GPR54 containing neurons. Co-localization was theoretically observed in 1-2 cells per section in 2 out of 2 sections.

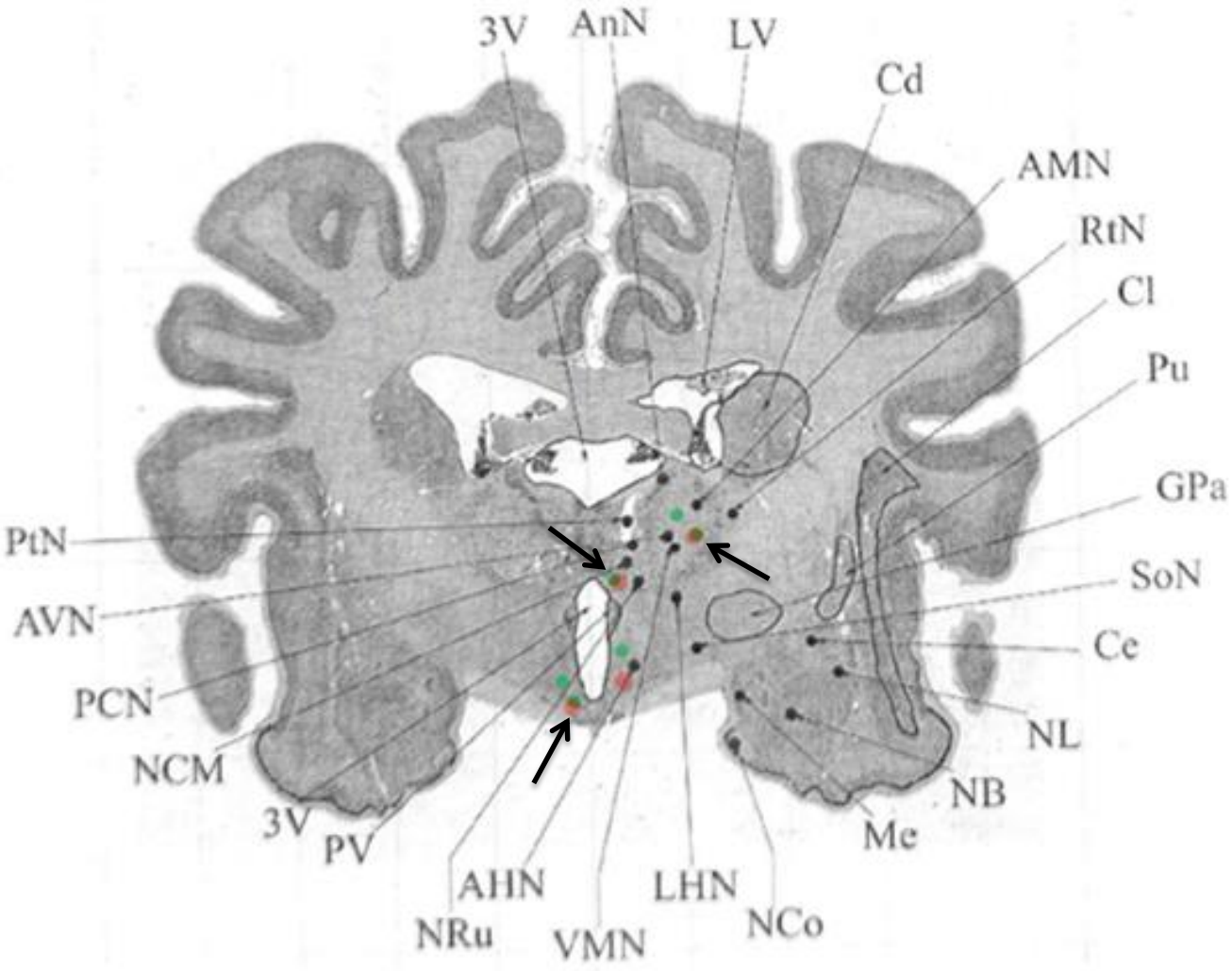


Figure 6. Predicted co-localization of GPR54 containing neurons and GnRH stained neurons based on location analysis of separate GnRH and GPR54 staining in the hypothalamus of the Beagle bitch. Co-localization was found in the ARC and POA (AMN) (arrows).

3.4 False positive results

A large number of false-positive fluorescent structures were found in the negative controls as well as in the experiments. They appeared as positive structures but did not show an association with a cell nucleus, and were therefore considered to be negative. When the bright field channel showed an artefact where a positive signal was observed, this was also considered as false-positive.

During the detection of GnRH neurons, false positive results were found in the ARC and the SoN (supraoptic nucleus) (Fig. 7). Figure 8 shows an example of a false positive result in the ARC. The staining looked solid bright green. False positive structures were observed in 3 structures per section in 1 out of 1 section.

False positive results for GPR54 were found in the ARC, POA, AnN (anterior nucleus), and AMN (anterior medial nucleus) (Fig. 9). Figure 10 shows an example of a false positive result in the caudate nucleus. The staining looked solid bright red. 1-5 structures per section in 2 out of 2 sections showed false positive results.

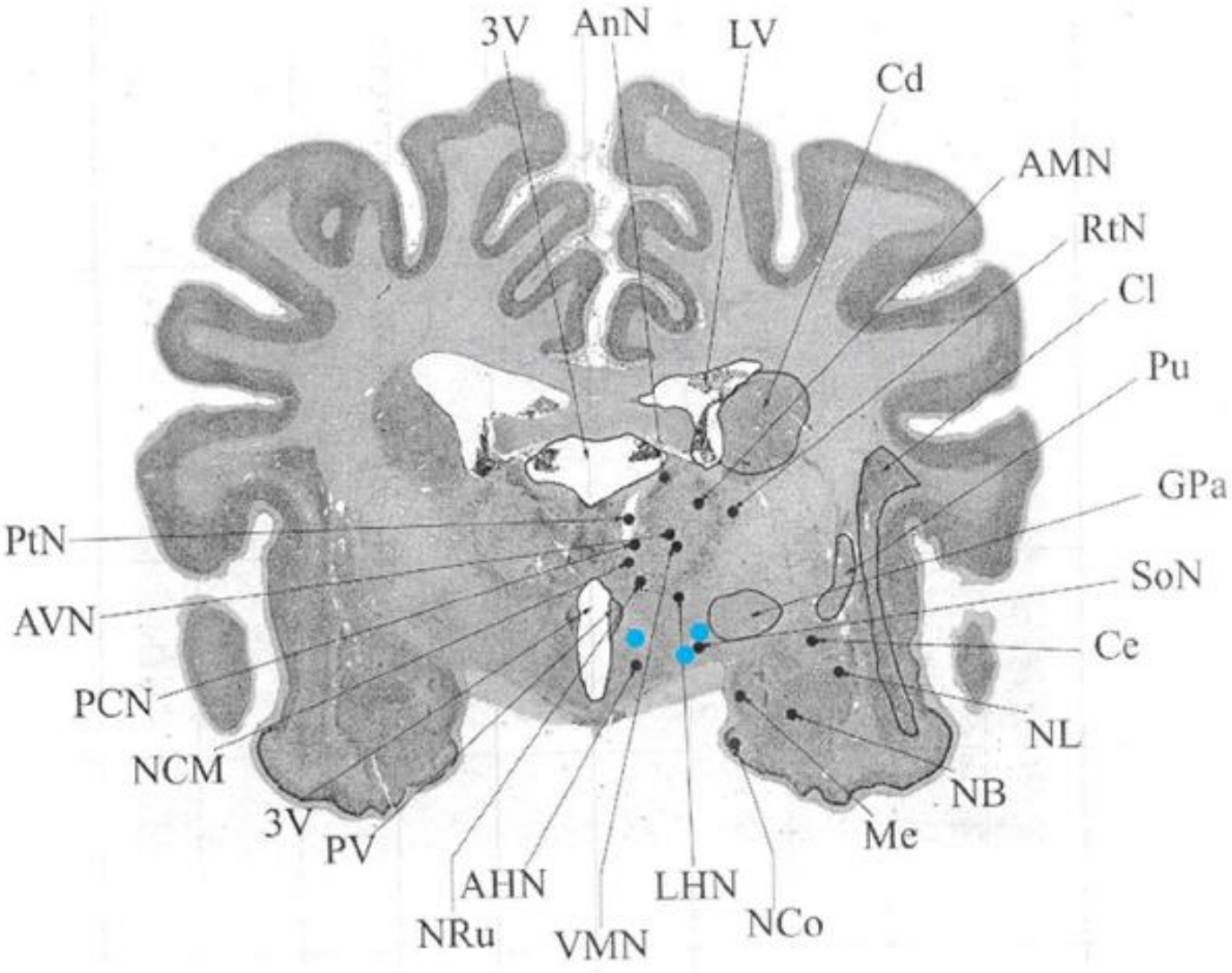
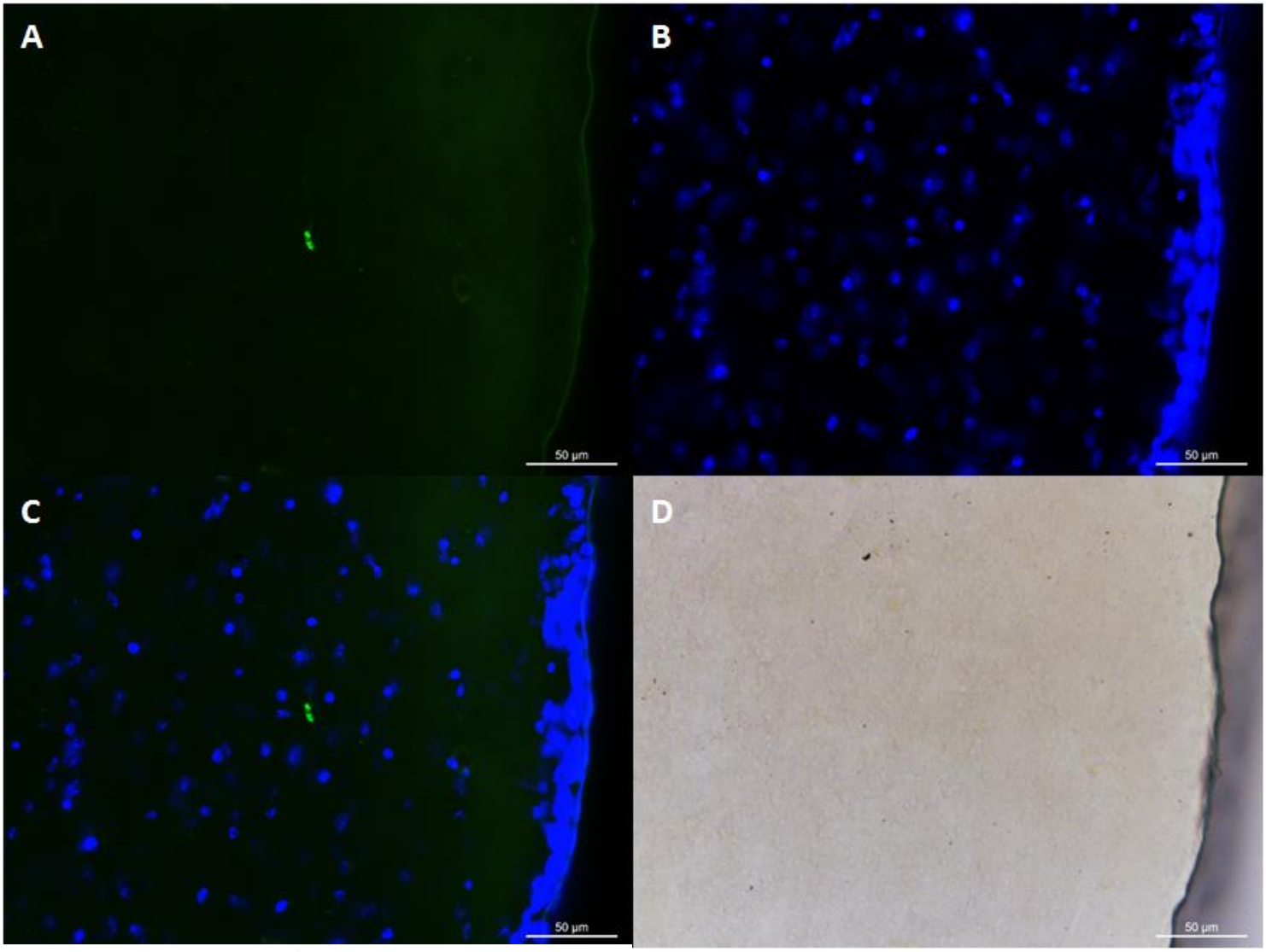


Figure 7. Location of false positive structures after staining GnRH neurons in the hypothalamus of the Beagle bitch. Areas in the hypothalamus where false positive structures (blue dots) were found are shown. False positive structures were found in the ARC and SoN.



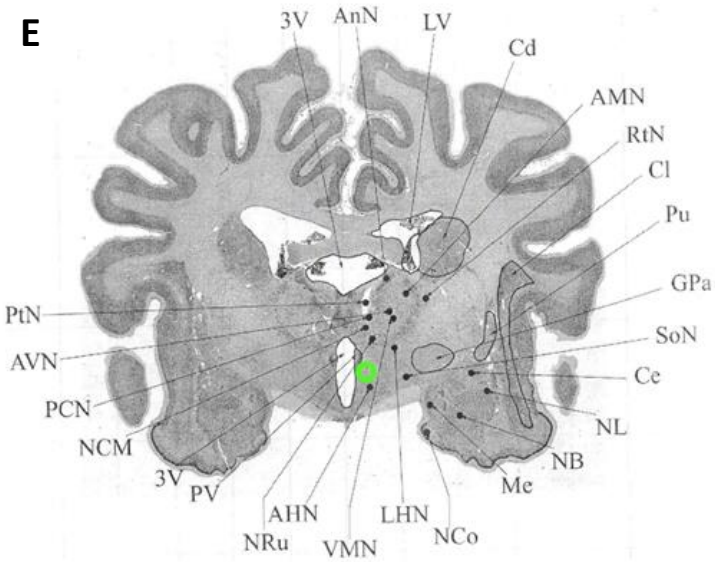


Fig. 8. **Example of a representative false positive structure after staining GnRH neurons in the hypothalamus of the Beagle bitch.** False positive structure (green) stained with Alexa 488 (A), cell nuclei (blue) stained with DAPI (B), an overlay (C) of A and B, and the bright field transmission channel (D). The bright field transmission channel shows no artefacts which could have caused the fluorescence, but the false positive structure does not associate with a cell nucleus and is thus considered false positive. E shows the location of the false positive structure in the ARC.

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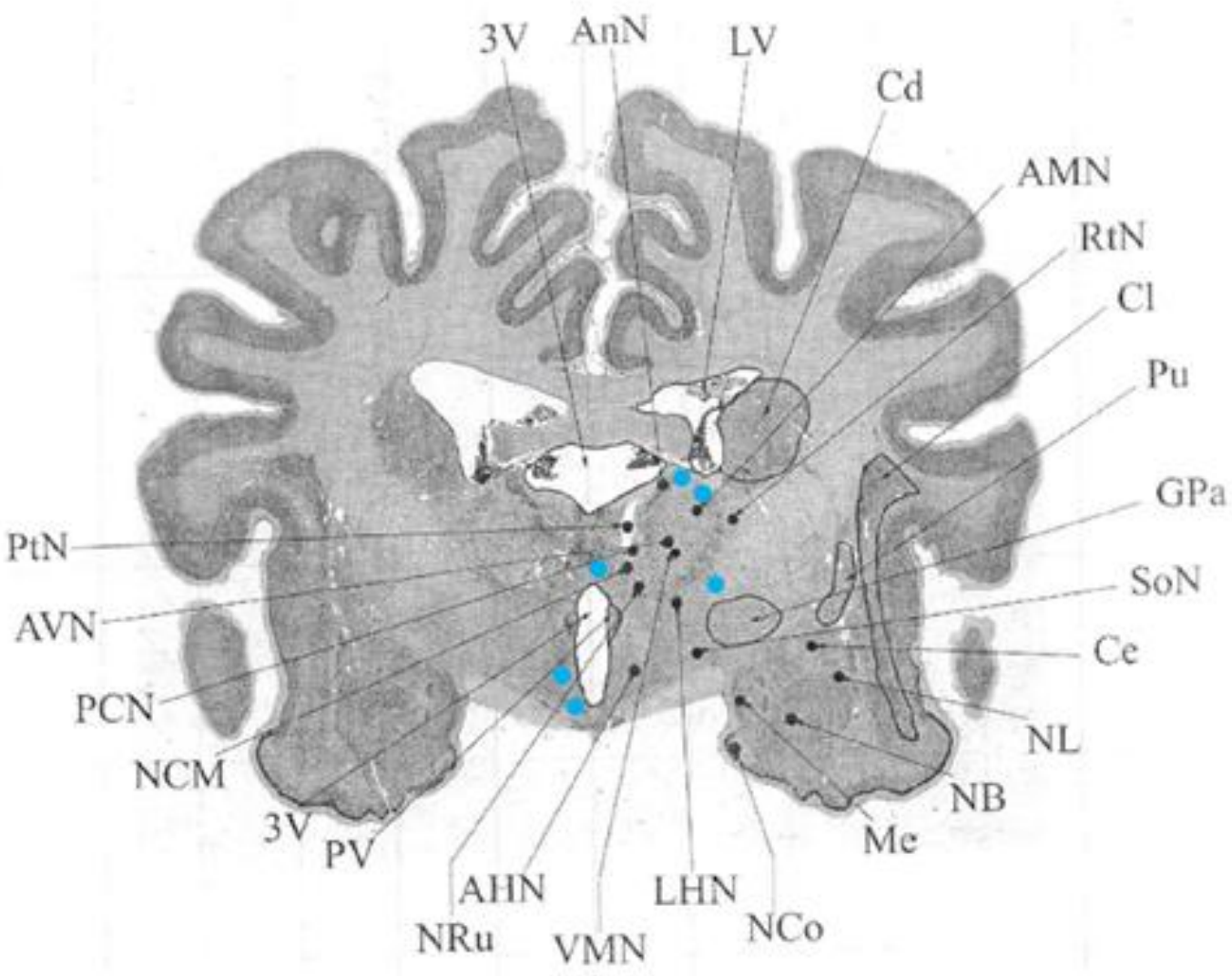

Figure 9. Location of false positive structures after staining GPR54 containing neurons in the hypothalamus of the Beagle bitch. Areas in the hypothalamus where false positive structures (blue dots) were found are shown. False positive structures were found in the GPR54: areas in the hypothalamus were false positive structures (blue dots) were found. False positive structures were found in the ARC, POA (AMN), and AnN.

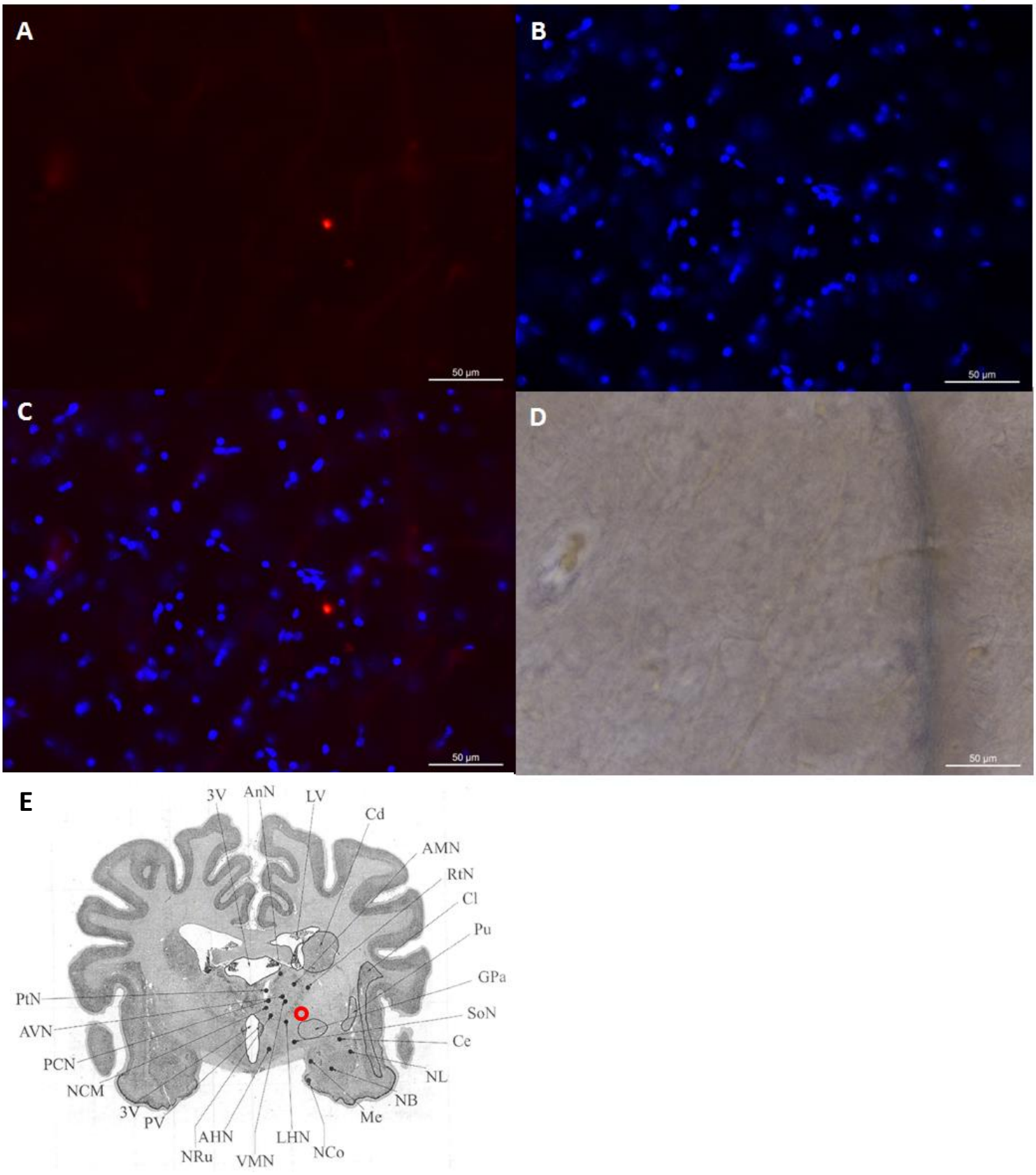

Fig. 10. **Example of a representative false positive structure after staining GPR54 containing neurons in the hypothalamus of the Beagle bitch.** False positive structure (red) stained with Alexa 568 (A), cell nuclei (blue) stained with DAPI (B), an overlay (C) of A and B, and the bright field transmission channel (D). The bright field transmission channel shows no artefacts which could have caused the fluorescence, but the false positive structure does not associate with a cell nucleus and is thus considered false positive. E shows the location of the false positive structure in the caudate nucleus.

3.5 Control experiments

The finding that also the control experiments showed positive results (Fig. 11-12), i.e. association with a cell nucleus and no artefact shown by the bright field channel which could have caused a positive signal, suggests that the experiments may not be conclusive. For the staining of GnRH neurons, 1-3 structures per section in 2 out of 2 sections showed positive structures. For the staining of GPR54 containing neurons, 1-2 structures per section in 2 out of 2 sections showed positive structures. The staining looked bright and solid, either green for GnRH stained neurons or red for GPR54 containing neurons. The control experiments were not representative and were therefore not compared to a section of the hypothalamus in the atlas. Because of this, the exact location of the positive results in Figure 11 and 12 is unknown.

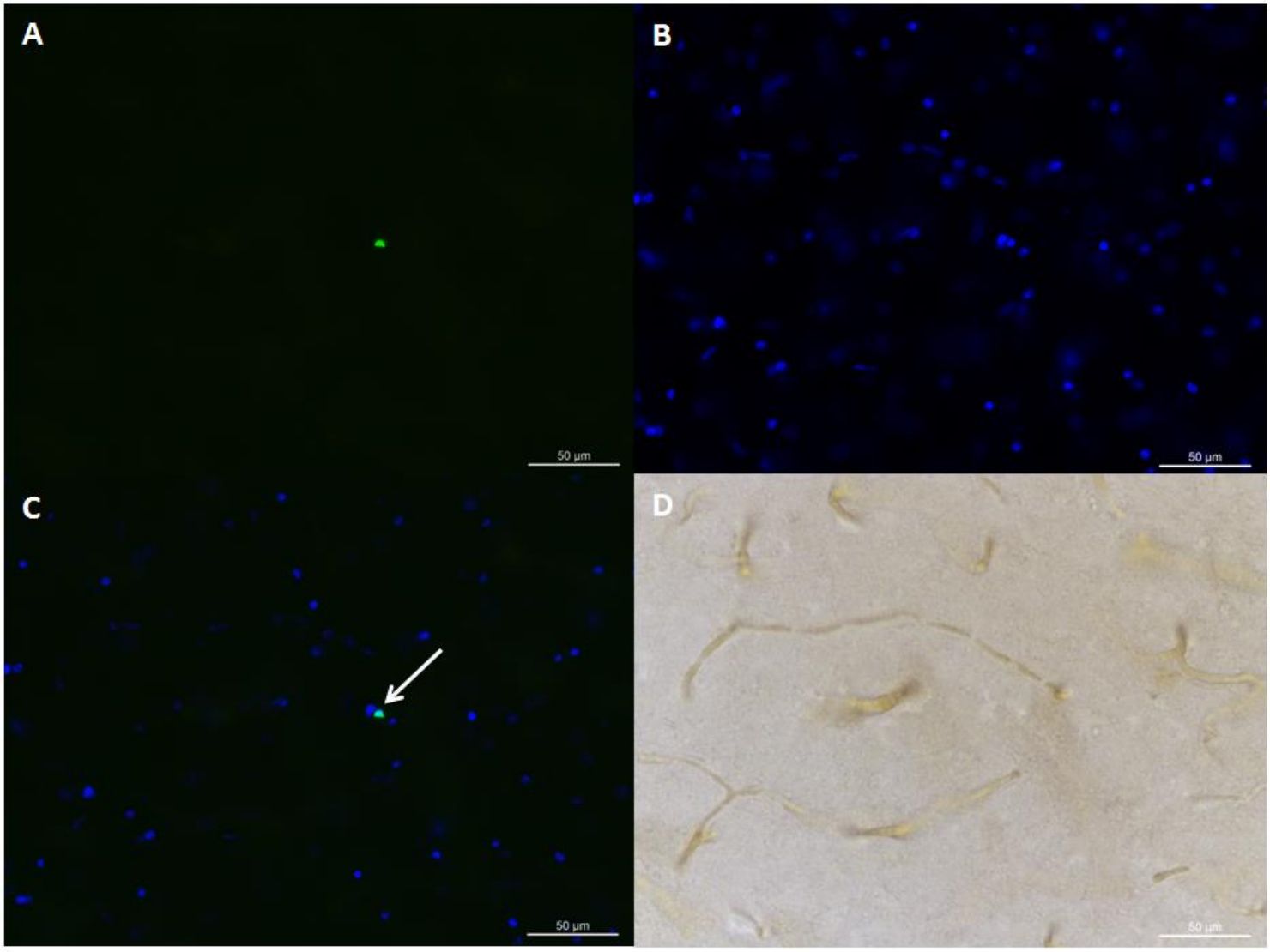

Fig. 11. **Example of a positive structure in the control experiment of staining GnRH neurons.** Positive structure (green) stained with Alexa 488, cell nuclei (blue) stained with DAPI (B), an overlay (C) of A and B, and the bright field transmission channel (D). The bright field transmission channel shows no artefacts which could have caused the fluorescence, and GnRH seems to associate with a cell nucleus and is thus confirmed positive (arrow). The location of the positive structure is unknown.

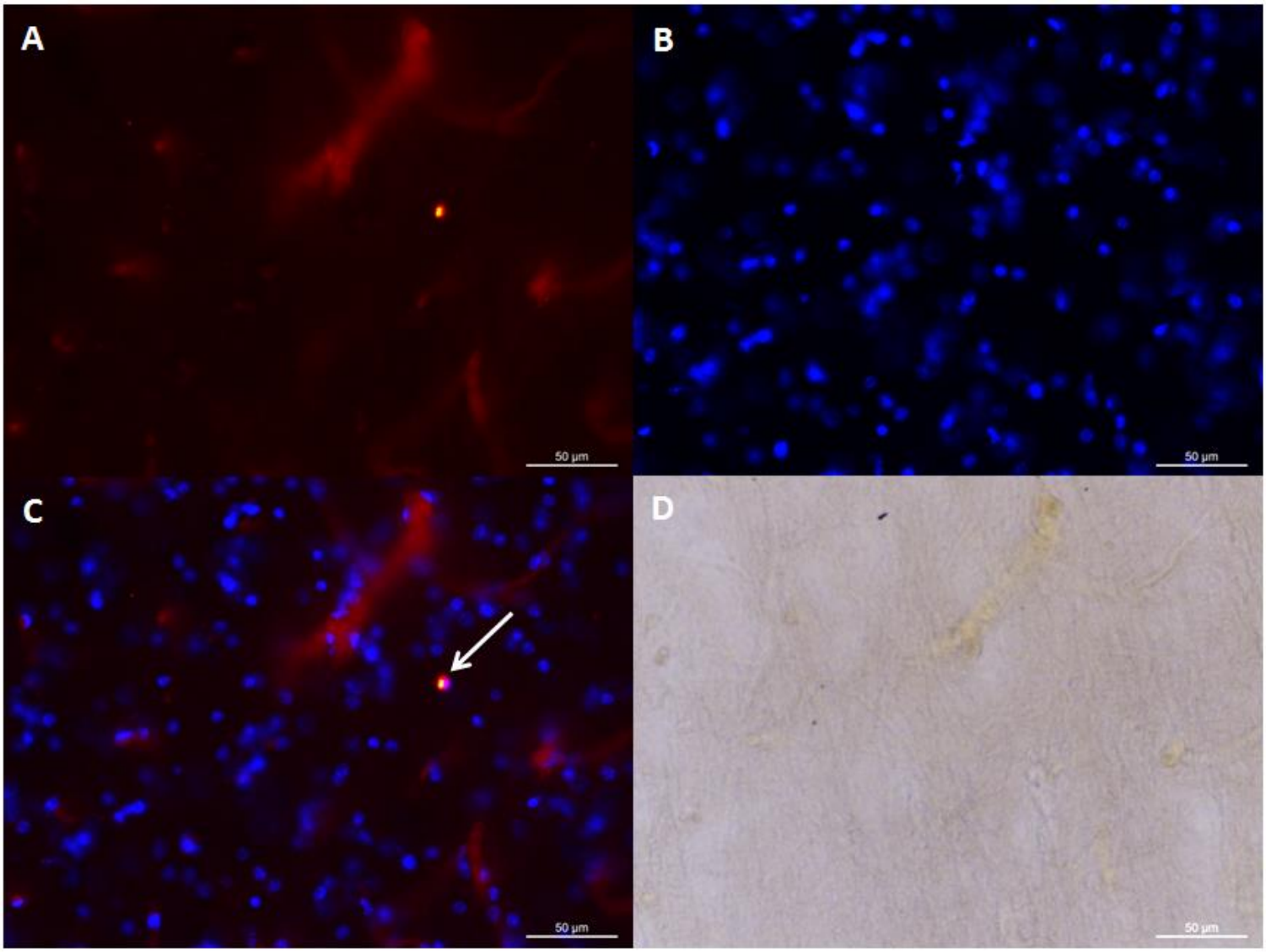

Fig. 12. **Example of a positive structure in the control experiment of staining GPR54 containing neurons.** Positive structure (red) stained with Alexa 568, cell nuclei (blue) stained with DAPI (B), an overlay (C) of A and B, and the bright field transmission channel (D). The bright field transmission channel shows no artefacts which could have caused the fluorescence, and the positive structure seems to associate with a cell nucleus and is thus confirmed positive (arrow). The location of the positive structure is unknown.

4. DISCUSSION

Kisspeptins play a fundamental role in the direct stimulation of GnRH neurons in the hypothalamus by binding to GPR54, which co-localizes with GnRH neurons in several species, including fish (Parhar, et al. 2004), rats (Irwig, et al. 2004), mice (Han, et al. 2005; Messager, et al. 2005), horses (Decourt, et al. 2008), and monkeys (Shibata, et al. 2005). The present report has demonstrated that GnRH neurons and GPR54 containing neurons were present on both sides of the third ventricle in the arcuate nucleus (ARC) region and slightly more dorsal and lateral, which corresponds to the preoptic area (POA) in the hypothalamus of the Beagle bitch. Co-localization of GnRH neurons and GPR54 containing neurons was theoretically seen in the ARC and POA.

The presence of a functional KISS1/GPR54 system in the HGP-axis in dogs could provide new insights in the physiological mechanisms that control reproduction, which is an obvious strategy for improving the fertility of dogs and developing new agents to control reproductive functions. To ensure reproductive success it is essential that the LH surge, which induces ovulation, is properly timed, which could be achieved by acting on the KISS/GPR54 system. Administration of kisspeptin could be a plausible method for timing the LH surge, since it is known that KP administration is able to stimulate GnRH and gonadotropin release in rodents, sheep, cows, primates, dogs and humans (Gottsch et al. 2004; Irwig et al. 2004; Matsui et al. 2004; Thompson et al. 2004; Messager et al. 2005; Shahab et al. 2005; Kauffman et al. 2007; Mikkelsen and Simmoneaux 2008; Albers-Wolthers et al. 2014;). Nevertheless, there appears to be a changing hypothalamic sensitivity to KP-10 during different estrus cycle stages and anestrus (Albers-Wolthers, et al. 2016) which has to be taken into account. Non-surgical contraception and estrus prevention could also be achieved by acting on the KISS1/GPR54 system. Although kisspeptin antagonists appear to be a straightforward way to inhibit GnRH release and thus gonadotropin secretion, Albers-Wolthers, et al. (2016) showed that kisspeptin antagonist p271 did not lower basal LH concentration and could not prevent or decrease the LH response to exogenous KP-10. KP antagonist p271 could therefore not be used in dogs. Kisspeptin receptor GPR54 may also be a target for non-surgical contraception and estrus prevention, as a dysfunction of GPR54 resulted in an insufficient release of LH an FSH from the pituitary and a failure to enter puberty in humans and mice (Funes et al. 2003; De Roux et al. 2003; Seminara et al. 2003). The exact consequences of acting on GPR54 during different estrus cycle stages and anestrus needs to be investigated first, in order to find new agents to control reproductive functions via the KISS1/GPR54 system in dogs.

Not only are kisspeptin neurons in the forebrain recognized as primary afferents of GnRH neurons, as they receive and integrate critical signals for the timing of puberty and the positive and negative feedback of sex steroids, they also play an essential role in the metabolic regulation of the HGP-axis (e.g. leptin) and in the control of reproductive function by photoperiodic cues (Roa, et al. 2008). Although this report did not focus on these aspects, therapeutic inventions could be based on variating in photoperiodic length and changing

food intake.

However, the results shown in this report should be interpreted with caution and could be inaccurate because of the presence of immuno-positive results in the negative controls. Also, the experiments showed false positive results in the ARC and POA, the same regions as where positive results were found.

Although the experiment for the staining of GnRH neurons was performed 4 times and the experiment for the staining of GPR54 containing neurons was performed 2 times, only the last experiment for either the detection of GnRH neurons or GPR54 containing neurons was used to give an overview of the location and distribution. This choice was made, because these last two experiments had the most optimal staining. Moreover, only representative sections of the hypothalamus were taken into account to be able to compare the sections to the stereotaxic atlas of the Beagle brain (Palazzi 2011). This resulted in only 1 representative section for the staining of GnRH neurons and 2 sections for the staining of GPR54 containing neurons. Also, these representative sections are not representing the entire hypothalamus so no exact cell numbers or receptor densities could be determined.

Only one dog was used for the experiments and the bitch used for this research was in anestrous, which means that she did not display estrous cycles. Anestrous is the result of insufficient GnRH release from the hypothalamus to stimulate and maintain gonadotropin release by the pituitary (Senger 2012). KP administration results in an LH response in all estrous cycle phases and anestrous, but the LH response in anestrous bitches is significantly higher (Albers-Wolthers, et al. 2016), which could suggest that the number of GnRH neurons expressing GPR54 is greater during anestrous. Nevertheless, the fact that this bitch was in anestrous could be the reason why GnRH neurons could not be properly stained, simply because GnRH is not abundant in GnRH neurons during anestrous and thus only small numbers of GnRH neurons could be seen.

Although the sections were washed with PBS-T + BSA, control experiments showing immuno-positive results could be caused by nonspecifically binding of the antibodies (monoclonal and polyclonal) used in the protocol (Hewitt, et al. 2014). Specific and non-specific binding could be distinguished by an absorption control (Hewitt, et al. 2014).

While at first auto-fluorescence made it impossible to distinguish auto-fluorescence from positive structures, $NaBH_4$ treatment resulted in a dramatic reduction (50%) of background fluorescence. Auto-fluorescence can be induced by double-bonds in the tissue due to fixation.

Future studies should include more dogs in all different stages of the estrous cycle, and in anestrous. More representative sections of the hypothalamus should be examined in order to get more data. Positive control experiments could be performed for validating the experiments. It is known that GPR54 is abundantly present in the placenta (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001), so using placenta tissue for positive control experiments could be helpful for optimizing the experiment. It is recommended to use $NaBH_4$ treatment for the reduction of background fluorescence.

The finding that GPR54 and kisspeptins are expressed in other areas of the body besides

the brain raises questions about their exact function (Castellano, et al. 2009). GPR54 is also expressed in the heart, muscle, kidney, liver, intestine, thymus, lung, and testis (Kuohung, et al. 2006), and kisspeptins are expressed in the placenta, ovary, testis, pancreas, liver, and small intestines (Muir, et al. 2001; Ohtaki, et al. 2001), yet we have no idea about their physiological relevance in these areas.

In conclusion, this report shows that GPR54 containing neurons and GnRH neurons are located in the ARC and POA in the hypothalamus of the dog. Because the control experiments also showed immuno-positive signals and only few sections were examined, the results shown in this report are debatable. No co-localization experiments were performed, because the problematic protocol for the proper staining of GPR54 and GnRH neurons separately needs to be improved first. Theoretical co-localization was found by merging plotted cells and receptors, but to determine whether co-localization of GPR54 and GnRH neurons is present in the hypothalamus of the dog, further studies are required to define the physiological importance of kisspeptin in the regulation of the HPG-axis in dogs. The effects of sex steroids on KISS1 expression in dogs would be particularly interesting to investigate.

ACKNOWLEDGMENTS

I am most grateful to prof. dr. Theo van Haeften (Biochemistry & Cell biology Utrecht University, the Netherlands) for his guidance and help in the laboratory. Furthermore, I would like to thank dr. Karin Albers-Wolthers and her research group for enabling me to work on this research. I also would like to thank Rob Bleumink and Esther van ‘t Veld from the Centre for Cell Imaging (CCI, Utrecht, the Netherlands) for their technical assistance with the Olympus color microscope. Finally, I would like to thank the department Biochemistry & Cell biology for the opportunity to work there and for having a nice time during my internship.

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