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# Identification of species-specific and general features in the anatomy of the kisspeptin neuron system

PhD thesis

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **ac** | anterior commissure, *commissura anterior* |
| **AgRP** | agouti related protein |
| **AH** | anterior hypothalamic nucleus, *nucleus anterior hypothalami* |
| **ARC** | arcuate nucleus, *nucleus arcuatus* |
| **AVPe, AVPV** | anteroventral periventricular hypothalamic nucleus, *nucleus anterioventralis periventricularis hypothalami* |
| **bv** | blood vessel |
| **CART** | cocaine- and amphetamine-regulated transcript |
| **DMH** | dorsomedial hypothalamic nucleus, *nucleus dorsomedialis hypothalami* |
| **DNA** | deoxyribonucleic acid |
| **Dyn** | dynorphin |
| **E2** | 17ß-estradiol |
| **Enk** | enkephalins |
| **ER-α** | estrogen receptor α |
| **ER-ß** | estrogen receptor ß |
| **ERE** | estrogen response elements |
| **ER** | estrogen receptor |
| **f** | fornix |
| **FSH** | follicle-stimulating hormone |
| **GABA** | γ-aminobutyric acid |
| **Gal** | galanin |
| **GnRH** | gonadotropin-releasing hormone |
| **GPR54** | orphan G protein coupled receptor 54, kisspeptin receptor |
| **HAA** | anterior hypothalamic area, *area hypothalamica anterior* |
| **HLA** | lateral hypothalamic area, *area hypothalamica lateralis* |
| **HPG** | hypothalamic-pituitary-gonadal axis |

| | |
|---|---|
| **INF** | infundibular nucleus,<br>*nucleus infundibularis* |
| **InfS** | infundibular stalk,<br>*infundibulum neurohypophyseos* |
| **IR** | immunoreactive |
| ***KISS1*, *Kiss1*** | kisspeptin gene |
| ***KISS1R*, *Kiss1r*** | kisspeptin receptor gene |
| **KNCART** | kisspeptin/neurokinin B/cocaine- and amphetamine related transcript |
| **KNDy** | kisspeptin/neurokinin B/dynorphin |
| **KNSP** | kisspeptin/neurokinin B/substance P |
| **KP** | kisspeptin |
| **KP-10, -13, -14, -54** | kisspeptin-10, -13, -14, -54 |
| **LH** | luteinizing hormone |
| **MBH** | mediobasal hypothalamus |
| **ME** | median eminence,<br>*eminentia mediana* |
| **mfb** | medial forebrain bundle,<br>*fasciculus medialis telencephali* |
| **MPO** | medial preoptic nucleus,<br>*nucleus preopticus medialis* |
| **mRNA** | messenger ribonucleic acid |
| **mt** | mammillothalamic tract,<br>*tractus mamillothalamicus* |
| **NK1R** | substance P receptor |
| **NK2R** | neurokinin A receptor |
| **NK3R** | neurokinin B receptor |
| **NKB** | neurokinin B |
| **NPY** | neuropeptide Y |
| **oc** | optic chiasm,<br>*chiasma opticum* |
| **ot** | optic tract,<br>*tractus opticus* |
| **OVX** | ovariectomized |
| **Pa** | paraventricular nucleus,<br>*nucleus paraventricularis* |

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| | |
|---|---|
| **PaAP** | paraventricular nucleus, anterior parvocellular subdivision<br>*nucleus paraventricularis, pars parvocellularis anterior* |
| **PaD** | dorsal part of the paraventricular nucleus,<br>*nucleus paraventricularis, pars dorsalis* |
| **PAH** | paraventricular nucleus of the hypothalamus,<br>*nucleus paraventricularis hypothalami* |
| **PaMC** | magnocellular part of the paraventricular nucleus,<br>*nucleus paraventricularis, pars magnocellularis* |
| **PaPC** | parvocellular part of the paraventricular nucleus,<br>*nucleus paraventricularis, pars parvocellularis* |
| **PEH** | periventricular complex of the hypothalamus |
| **POA** | preoptic area,<br>*area preoptica* |
| **POMC** | proopiomelanocortin |
| **preproKP** | prepro-kisspeptin |
| **RFRP1, -2, -3** | RF amide-related peptide 1, -2, -3 |
| **RP3V** | rostral periventricular area of the third ventricle |
| **RT-PCR** | real-time polymerase chain reaction |
| **SCH** | suprachiasmatic nucleus,<br>*nucleus suprachiasmaticus* |
| **SMN** | medial septal nucleus,<br>*nucleus septalis medialis* |
| **SON** | supraoptic nucleus,<br>*nucleus supraopticus* |
| **SP** | substance P |
| **SST** | somatostatin |
| ***TAC3*** | neurokinin B |
| ***TACR3, tacr3*** | neurokinin B receptor |
| **TCA** | area of the tuber cinereum |
| **TH** | tyrosine hydroxylase |
| **v3** | third cerebral ventricle,<br>*ventriculus tertius cerebri* |
| **VMH** | ventromedial hypothalamic nucleus,<br>*nucleus ventromedialis hypothalami* |

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# 1. INTRODUCTION

## 1. 1. Neuroendocrine regulation of mammalian reproduction

Mammalian reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. The menstrual and estrous cycles depend upon complex inter-communication between the three levels of the HPG axis. A key element of this axis is the hypothalamic gonadotropin-releasing hormone (GnRH) neuronal system which serves as the final output component of a neuronal network that integrates multiple environmental and internal factors to regulate reproductive hormone secretion. GnRH neurons release GnRH in an episodic manner into the pituitary portal circulation to generate distinct pulses of the two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gonadotropes. The gonadotropins regulate gametogenesis and steroidogenesis in the gonads of both males and females. The levels of the HPG axis are bidirectionally linked to each other. In addition to regulating reproductive functions in the peripheral tissues, the gonadal sex steroid hormones (estradiol and progesterone from the ovary, testosterone from the testicle) feed back to the hypothalamus to regulate GnRH secretion, and to the pituitary to modulate the responsiveness of gonadotropes to GnRH (**Fig. 1**). Each level of the HPG axis represents an important site in the regulation of reproductive status [1-4].

The topography of GnRH neurons, the mechanisms of the GnRH pulse and surge generation, factors influencing pulsatile hormone secretion (especially the roles of estrogens) and neurons that play a crucial role in afferent regulation of GnRH cells and in mediating estrogen action on GnRH cells will be described in the upcoming chapters.

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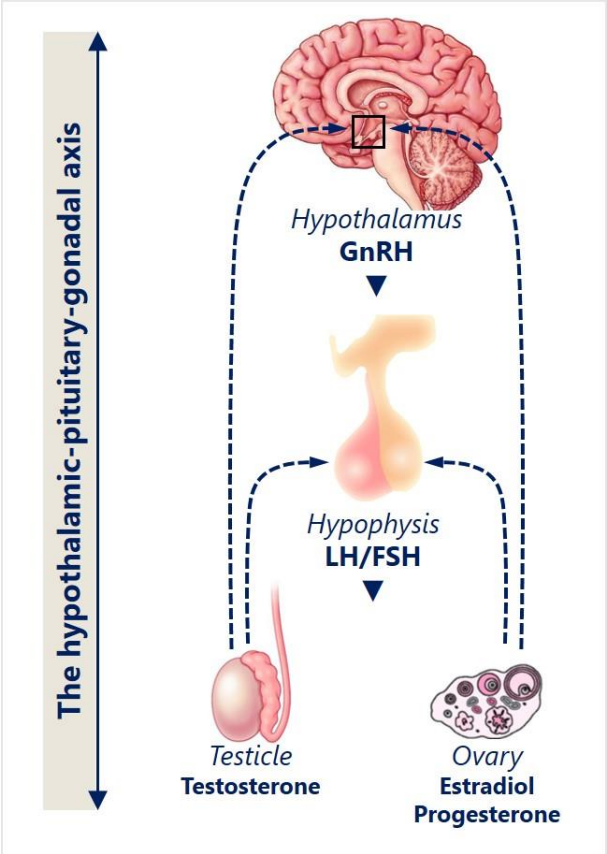


**Figure 1. The communication between the levels of the hypothalamic-pituitary-gonadal axis.** Gonadotropin-releasing hormone (GnRH) is secreted form the hypothalamus by GnRH neurons into the pituitary portal circulation. GnRH promotes the production and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotropic cells in the anterior pituitary. The secreted gonadotropins (LH and FSH) are responsible for gametogenesis and sex steroid synthesis of the gonads. Gonadal steroids feed back to the hypothalamus to regulate GnRH secretion, and to the anterior pituitary to modulate the responsiveness of gonadotropes to GnRH.

## 1. 2. GnRH neurons

### *1. 2. 1. The development, topography and projections of GnRH neurons*

GnRH neurons are born in the medial part of the nasal placode and enter the brain along a migratory route formed by the vomeronasal/terminal nerve during vertebrate embryogenesis [5-8]. GnRH neurons follow two different migratory streams. The ventral migratory pathway is directed towards to presumptive hypothalamic regions, whereas the dorsal migratory pathway leads developing GnRH cells towards pallial and subpallial telencephalic regions. The number of GnRH neurons in mice is higher during embryogenesis (1000-1200) and declines at adulthood [9, 10]. About 10 000 GnRH neurons were counted in the developing brain in humans [8]. Given that the number of

GnRH neurons in the adult brain is similar, the majority of migrating GnRH neurons may survive through adulthood [11].

The undisrupted migration of GnRH neurons is critical for the onset of puberty and subsequent sexual maturation. Kallmann syndrome is a form of congenital hypogonadotropic hypogonadism, a rare reproductive disorder that is due to the dysregulation of GnRH neuron migration and is characterized by low plasma levels of LH, FSH and circulating sex steroids, leading to the failure of pubertal development [12, 13].

While species differences exist in the topographic distribution of postmigratory GnRH neurons, these cells are not concentrated in defined hypothalamic nuclei in any species [14]. For example, GnRH cell bodies are located in the septal-preoptic regions in laboratory rodents [15, 16], whereas they show wider distribution in the septal-preoptic regions, the ventrolateral anterior hypothalamus, the lateral hypothalamus and in the arcuate nucleus (ARC; called infundibular nucleus in humans, INF) of the mediobasal hypothalamus (MBH) in ewes [17]. In primates including humans, GnRH neurons are widely scattered from the septal-preoptic region to the retromammillary area, with high cell numbers in the infundibular nucleus within MBH [18, 19].

Comparative analysis of the GnRH neuronal system in a series of mammals revealed prominent species differences in the path of GnRH axon projections [20]. However, in all species the majority of hypophysiotropic GnRH fibers enter the median eminence (ME) (also called postinfundibular eminence in the human) which is the functional link between the hypothalamus and the anterior pituitary gland. The ME is composed of extensive arrays of blood vessels and nerve endings. Small capillary loops extend into the internal and external zones of the ME. The axons of the hypohysiotropic neurons, including GnRH cells, target the external layer where neurons secreting hypothalamic releasing and release-inhibiting factors release their neurohormone products into the pituitary portal vessels [21].

### *1. 2. 2. „GnRH pulse generator”*

The „GnRH pulse generator” is a neural ensemble generating episodic GnRH release which is ultimately responsible for pulsatile LH secretion from the pituitary [22]. In rodents, GnRH fibers projecting to the ME function simultaneously as dendrites and

axons, giving rise to the recently introduced "dendron" terminology [23]. The pulse generator may regulate GnRH neuron's perikarya, dendrons and/or terminals to control episodic GnRH/LH secretion [24].

The frequency and amplitude of GnRH pulses direct the secretion pattern of the gonadotropins. Accordingly, the profile of pulsatile GnRH release maps closely to that of pulsatile LH secretion. In rodents, GnRH/LH pulses appear with a relatively constant frequency of approximately one pulse per hour in the follicular phase of the ovarian cycle and then slows in the luteal phase (one pulse every 3-4 hours) as pulse amplitude increases [1, 22]. The changing patterns of pulsatile GnRH release across the ovarian cycle differentially regulate pituitary gonadotropin biosynthesis and secretion. Pulsatility is a prerequisite for reproduction, and indeed, constant GnRH receptor activation suppresses, instead of activating the pituitary-gonadal axis [24].

At least a subpopulation of GnRH neurons must be synchronized to generate episodic GnRH release. Two models of GnRH cell synchronization exist in the literature.

The initial concept of an intrinsic pulse generator (**Fig. 2a**) came from studies of the immortalized GnRH-secreting GT1 cells and fetal GnRH cells which were able to generate pulsatile GnRH release without an extrinsic signal with an interpulse interval of 20-40 minutes. These observations suggested that GnRH neurons alone can synchronize their secretion and thus the GnRH system *per se* is the pulse generator [25, 26]. The main difficulty in translating results from these cell lines to adult GnRH neurons *in vivo* was the lack of knowledge how individual GnRH cells may intercommunicate with each other under physiological conditions. Because GnRH neurons are widely scattered within the hypothalamus, gap junctions and humoral communication reported in the *in vitro* preparations are unlikely to exist *in vivo* [22]. The extrinsic pulse generation is based on the concept that an entirely separate group of neurons direct the generation of GnRH pulses (**Fig. 2b**). There is growing evidence that GnRH neurons have no substantial direct interactions with each other. Therefore, it is possible that the synchronized activity of GnRH cells during *in vivo* pulses is mostly regulated by afferent neuronal signals [23, 27-29]. According to Herbison [24], it seems most likely that GnRH pulse generation emerges from the combination of the intrinsic interconnectedness of GnRH cells and the external pulse generator function. Now it is known that multiple afferent systems converge on GnRH neurons producing a dynamic and finely tuned reproductive control

system. GnRH itself is also involved in regulating the activity of GnRH neurons through autocrine signaling [2, 22].

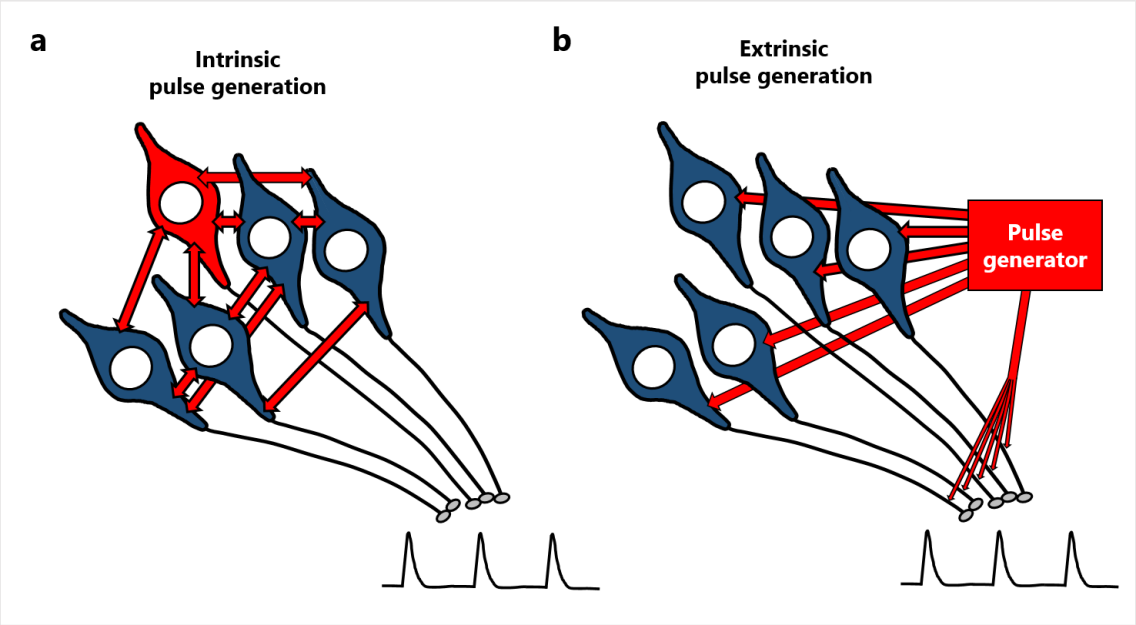


**Figure 2. Two potential models of gonadotropin-releasing hormone (GnRH) neuron synchronization.** Intrinsic model (**a**): GnRH neurons alone are inter-connected with each other and, therefore, they are capable of generating synchronized pulsatile secretory patterns by themselves. The episodic activity of the neurons might be driven by a special subset of GnRH neurons (red). Extrinsic model (**b**): a separate group of neurons (the actual pulse generator neurons) might synchronize the activity of GnRH neurons via providing afferent inputs to the latter ([24]; modified).

The extrinsic pulse generator might be located within the hypothalamic arcuate nucleus [30-32], as suggested by studies in several species [33-38].

### *1. 2. 3. GnRH surge*

In the middle of the cycle the pattern of GnRH secretion changes from a strictly episodic to a nonepisodic one. This relatively sudden and prolonged elevation (>24h) of GnRH release into the portal vasculature that is caused by increased GnRH pulse frequency generates a preovulatory LH surge in female mammals [39-43]. Exceptions although, may exist in this respect: in rats minor alteration of GnRH pulse frequency can be measured during LH surge but an obvious increase in GnRH pulse amplitude occurs [44].

### *1. 2. 4. Effects of sex steroids on the „GnRH pulse generator”*

Gonadal sex steroids, especially 17ß-estradiol (E2), can feed back negatively or positively to the hypothalamus to regulate GnRH secretion according to the actual phase of the reproductive cycle. Two types of nuclear receptors – ER-α and ER-ß – acting as ligand-dependent transcription factors are responsible for the mediation of these feedback effects [45-47]. Upon estrogen binding, the receptors dimerize and bind to DNA sequences known as estrogen response elements (EREs). This changes the gene expression of estrogen-dependent genes. This mechanism is responsible for the classical genomic effects of estrogens which may be involved in the regulation of GnRH biosynthesis and release [2, 48]. Non-genomic estrogen signaling – mediated by plasma membrane-associated ERs – can lead to fast activation of intracellular secondary messengers and signaling pathway elements. This fast action of estrogens can modulate the electrical activity of GnRH neurons [49, 50].

Regulation through feedback mechanisms of sex steroids varies among the sexes. Due to the organizational and activational effects of gonadal steroids sex differences in brain structure and function – thus in the GnRH network – emerge during development. The organizational effect of a perinatal testosterone exposure from the male gonads in the mice accounts for the inability of adult males to mount estrogen-induced LH surges [51, 52]. In the rat, the masculinization is due to estrogens produced by aromatization of testosterone [53, 54]. As a result of this neonatal imprinting regulating neuronal connections in the male hypothalamus (organizational effects), testosterone and estrogen continuously inhibit the function of GnRH neurons in adults.

In females estrogen level fluctuates across the ovarian cycle. Low-dose estrogen in the follicular and luteal phase exhibit inhibitory actions on „GnRH pulse generator” and thus on GnRH secretion (negative feedback). The mid-cycle GnRH/LH surge is induced by the positive feedback effects of rising serum estrogen levels and plays a crucial role in triggering ovulation. While positive feedback involves both hypophysial [55] and hypothalamic [56] components, the relative importance of each and the preoptic/hypothalamic sites where feedback actions take place, may differ considerably among species [29].

In women, the menstrual periods stop permanently during menopause (at an average of 50 years of age). The prominent decrease of ovarian hormone secretion overlaps with

the menopausal transition. The lack of estrogens leads to physiological alterations in estrogen target organs, including the central nervous system, among others [57].

### *1. 2. 5. Interneurons influencing GnRH neuronal functions*

One major discovery in reproductive neurobiology has been the observation by Shivers and co-workers [58] which showed that GnRH neurons are not able to concentrate estradiol. This result was confirmed with immunocytochemical methods in several species proving the absence of ER-α in GnRH neurons [59-64]. Therefore, the feedback effects of estrogen on GnRH neurons do not occur through a classic ER-mediated process, but instead, must be mediated by estrogen sensitive (inter)neurons. Later studies provided evidence that a second estrogen receptor isoform – ER-ß – is expressed by GnRH neurons [65-69]. This observation reopened the questions whether estrogens may directly influence the function of GnRH cells. Currently, it is generally accepted that neurons expressing ER-α are essential for estrogen negative and positive feedback mechanism and ER-ß expressing cells are not key components of feedback signaling [70]. In accordance with this concept, ER-α receptor knockout animals are infertile [71].

Common features of estrogen sensitive interneurons that mediate estrogenic signals to GnRH cells include (1) the expression of ER-α receptor, (2) neuronal connection with GnRH neurons and (3) communication through specific postsynaptic receptors to classical neurotransmitters (e.g. GABA, glutamate), neuromodulators (e.g. histamine) or neuropeptides (e.g. NPY) [2, 72]. Among these estrogen-sensitive interneurons, those synthesizing the neuropeptide kisspeptin (KP) play a central role in the afferent regulation of GnRH neurons.

## 1. 3. Kisspeptin

The preprokisspeptin protein is derived from the KISS1 gene. Its proteolytic cleavage results in 54-, 14-, 13- and 10-amino acid long peptides (KP-54; KP-14, KP-13 and KP-10). The decapeptide form represents the minimal length which can fully stimulate the kisspeptin receptor [73-75]. The kisspeptin peptide products of KISS1 are commonly referred to as “kisspeptins”. These peptides possess a conserved RF-amide C terminus core sequence [76]. Kisspeptin was initially identified as a tumor metastasis suppressor [77]. It has been several years after that its essential role in the regulation of reproduction

and puberty was also discovered [78, 79]. In 1999, an orphan G protein coupled receptor (GPR54) was isolated [80] and in 2001, two independent groups revealed that kisspeptin is the natural ligand of this receptor. Therefore, the receptor was renamed *KISS1R* [73, 74, 81]. Mutations in *KISS1* or *KISS1R* cause hypogonadotropic hypogonadism in humans and laboratory animals [82-85], whereas activating mutations can lead to precocious puberty [86, 87]. These clinical phenotypes clearly demonstrate the crucial role of KISS1/KISS1R system in reproduction.

#### *1. 3. 1. Main functions of kisspeptin*

Evidence accumulated over the recent years indicates that neurons in the ARC/INF expressing kisspeptin play a crucial role in the episodic GnRH/LH secretion. KP, indeed, stimulates LH release [88-91] via direct activation of GnRH neurons, which express KP receptor mRNA (*Kiss1r*) [90, 92, 93]. GnRH neurons show c-Fos expression [92, 94] and depolarization [93, 95, 96] in response to KP. KP influences the function of many other cell types but it is suggested that the only effect fundamental for reproduction is exerted directly on GnRH neurons. Accordingly, specific deletion of *Kiss1r* from GnRH cells causes infertility and this phenotype can be restored via selective reinsertion of the *Kiss1r* into GnRH neurons of mice bearing a global deletion of *Kiss1r* [97], serving as proof of the necessity of KP signaling at GnRH cells.

#### *1. 3. 2. Distribution of kisspeptin neurons in the hypothalamus*

KP expressing neurons in mammals have been localized to the preoptic area (POA)/rostral hypothalamus and to the arcuate nucleus of the mediobasal hypothalamus [98]. The MBH contains the largest population of KP neurons in rodents [99], sheep [100], goat [101] and primates [102-104].

## 1. 4. The roles and neurochemical characteristics of kisspeptin neurons

#### *1. 4. 1. KP neurons in negative feedback*

MBH KP neurons are commonly referred to as „KNDy" neurons because of their neurokinin B (NKB) and dynorphin (Dyn) co-contents [105]. Irrespective of species, these neurons have been implicated in negative sex steroid feedback [106] and the generation of pulsatile GnRH/LH secretion [107]. KNDy neurons are reciprocally

connected and communicate with each other autosynaptically, forming a critical component of the GnRH/LH pulse generator (KNDy hypothesis; [108]). Accordingly, NKB activates and Dyn inhibits KNDy neurons via NK3 receptor and κ-opioid receptor, respectively, while KP serves as the output signal driving GnRH secretion. Indeed, NKB stimulates the firing frequency of KNDy neurons in mice [109, 110] and goat [101]. Likewise, Dyn also appears to play an important role in the regulation of GnRH/LH pulses and in the KNDy neuron activity, respectively, in both rodents and ruminants [29]. Moreover, administration of the opioid receptor antagonist naltrexone increases LH pulsatility in healthy male volunteers [111], suggesting a similar role of Dyn (or other opioid peptides) in pulse generation in human. Interestingly, however, Dyn presented in virtually all KP cells of sheep [100] and mice [108] can be visualized very rarely with immunohistochemistry in human KP cells [112-114]. Similarly, galanin (Gal) colocalized with KP in mice, but not in human [113]. On the other hand, substance P (SP) was detectable in human KP and NKB neurons [115], a phenomenon not reported earlier in any other species [116], and these cells also expressed cocaine- and amphetamine-regulated transcript (CART) in nonhuman primates [117] and humans [118] but not in rodents and ruminants (**Fig. 3**).

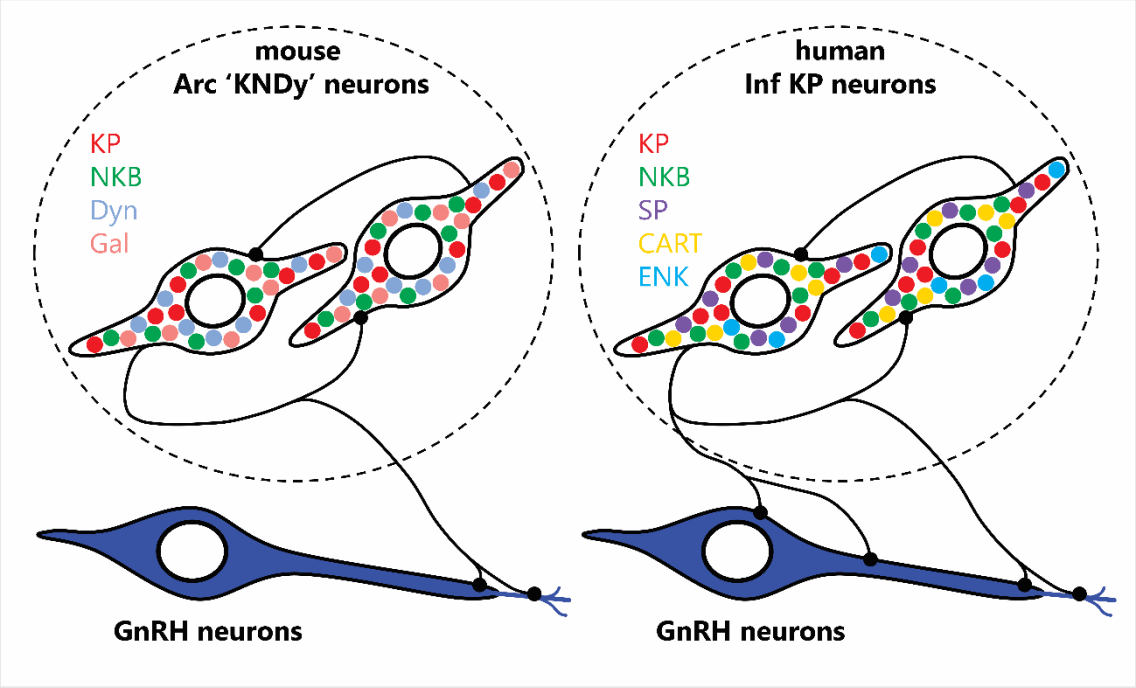


**Figure 3. Comparative neurochemistry of kisspeptin neurons in the arcuate nucleus/infundibular nucleus of mice *versus* humans** [119]. The neurochemistry of KP neurons

differs considerably in these species. KP neurons of the mouse ARC contain neurokinin B (NKB), dynorphin (Dyn) and galanin (Gal), while human INF KP neurons contain NKB but show no Gal and only rarely Dyn expression. In contrast to mouse KNDy neurons, human KP cells contain substance P (SP), cocaine- and amphetamine-regulated transcript (CART) and sometimes, enkephalin (Enk) as well. Presence of SP and CART and absence of Dyn and Gal represent species-specific neurochemical characteristics of KP neurons in the MBH.

The species-specific neurochemistry of KP neurons questions the validity of the popular „GnRH/LH pulse generator" models based on the interactions of the above-mentioned three neuropeptides (KP, NKB, and Dyn) and their receptors for all mammalian species. Accordingly, the comparison of KP neurons of poorly investigated non-rodent mammals (e.g. carnivorous domestic animals) with the KP neurons of laboratory rodents and humans may help to determine which peptides are obligatory or optional in the function of pulse generator (KNDy) neurons.

### *1. 4. 3. KP neurons in positive feedback*

In laboratory rodents, surgical lesions between the preoptic area and the mediobasal hypothalamus block ovulation [30], indicating the critical importance of the preoptic/rostral hypothalamic area in positive feedback. Much of this feedback is mediated to GnRH neurons by kisspeptin neurons located in the rostral periventricular region of the third ventricle (RP3V). Accordingly, KP neuron-specific deletion of ER-α disrupts positive feedback in mice [56]. The rodent RP3V is sexually dimorphic, with 10 times as many KP neurons in adult females compared with males. This sex difference is due to the organizational effect of a perinatal testosterone exposure from the male gonads and accounts for the inability of adult males to mount estrogen-induced LH surges [52, 120, 121]. KP neurons in the rodent RP3V also contain other neurotransmitters/ neuromodulators; they express galanin [122, 123], met-enkephalin (Enk) [122], and markers for dopamine synthesis/release [124], GABA [125] and glutamate [125].

In contrast, the MBH has long been considered to be the major positive feedback site for estrogens in primates [1]. While controversial [126, 127], surgical isolation of this region in some studies did not interfere with the spontaneous ovulation and estrogen-induced LH surges of rhesus monkeys [1, 128, 129], providing basis for the prevailing view that the MBH contains all neuronal elements required for positive feedback. In sheep and primates, KP neurons of the MBH may also mediate positive feedback to GnRH

neurons. *Kiss1* expression in the MBH increases prior to the LH surge in sheep and primates [130, 131] and KP neurons in the caudal and mid-ARC of the sheep express c-Fos in response to estrogen [131]. However, these observations do not exclude the important contribution of preoptic/rostral hypothalamic KP neurons to positive estrogen feedback. Accordingly, preoptic KP neurons show increased *Kiss1* and c-Fos expression during the LH surge in both sheep [131, 132] and monkeys [133, 134].

Previous immunohistochemical studies of human *post mortem* tissues have thoroughly characterized the topographic distribution, network connectivity, neurochemistry, sexual dimorphism and aging-dependent morphological plasticity of KP neurons in the MBH [135]. In contrast, to date only a single pilot study addressed whether KP neurons also occur in the human rostral hypothalamus [104]; if such neurons exist in the human as in monkeys [133, 134], they could be homologous anatomically and possibly, also functionally to KP neurons of the rodent RP3V which are implicated in positive estrogen feedback. Recent availability of newly developed highly specific preproKP antibodies [114] and hypothalamic tissue samples from a large number of human subjects now allowed us to more thoroughly characterize KP neurons in the human rostral hypothalamus and thus answer the questions whether this neuron population exhibits sex differences and whether it is regulated by estrogens.

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# 2. OBJECTIVES

In contrast to rodent KP neurons which express NKB, Dyn and Gal, the KP neurons of the human infundibular nucleus do not synthetize Gal and Dyn, but co-express NKB, CART and SP. These species differences question the validity of the popular „GnRH/LH pulse generator" models based on the interactions of the three peptides (KP, NKB, and Dyn) and their receptors for all mammalian species. Studying the neurochemistry of KP neurons in the MBH of other species may provide critical information about the obligatory and facultative neuropeptide players in the mechanism acting upstream from GnRH cells which generate pulsatile GnRH/LH secretion.

In addition, a second critical question relates to the mechanism of positive feedback in primates, including humans. The prevailing view over the past decades has been the lack of importance of the rostral hypothalamic/preoptic region, which left the MBH as the only site that might play a role in positive feedback. Putative existence and estrogen dependence of a rostral hypothalamic KP cell population in the human brain, which would be homologous with KP neurons of the rodent RP3V, would put a challenge to this prevailing view.

The aims of the present study were to investigate further the species-specific and the general characteristics of the two kisspeptin neuronal systems.

## 2. 1. Characterization of carnivore KP neurons within the infundibular nucleus

According to our hypothesis, comparison of KP neurons of poorly investigated non-rodent mammals (e.g. carnivorous domestic animals) with the much better characterized KP neurons of laboratory rodents and the human could help us determine which neuropeptides are obligatory *versus* optional in the pulse generator function of (KNDy) neurons.

Therefore, the first aim of our studies was to characterize KP neurons of the feline and canine hypothalamic infundibular nuclei. In addition to describe the neuropeptide neurochemistry of these cells, we planned to map the distribution of KP and GnRH neurons in the canine and feline hypothalamus and to investigate the connection between

KP and GnRH neurons, via examining KP fiber appositions on the somatic and dendritic compartment of the GnRH cells.

## 2. 2. Identification and characterization of KP neurons in the human rostral hypothalamus

The second aim of our study was to identify a KP cell group that could homologous to the rodent RP3V KP neuron population. We investigated

1) the topography,
2) sex- and age dependence,
3) the neuropeptide/neurotransmitter phenotype and
4) neuronal interactions

of this presumptive KP cell population in the human rostral hypothalamus.

Identification and positive estrogenic regulation of KP neurons in the human rostral hypothalamus could challenge the long-held view that positive estrogen feedback may be restricted to the mediobasal part of the hypothalamus in humans.

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# 3. RESULTS

## 3. 1. Anatomy and neurochemistry of kisspeptin neurons in the infundibular nucleus of ovariectomized dogs and cats

### *3. 1. 1. Topography of canine and feline kisspeptin neurons and GnRH cells*

*3. 1. 1. 1. Distribution of kisspeptin-immunoreactive neurons in the infundibular nucleus*

Immunohistochemical studies of tissue sections from different rostro-caudal levels of the hypothalamus (**Fig. 4a-i**) visualized numerous heavily labeled KP-immunoreactive (IR) perikarya in the canine and feline MBH (**Fig. 4e-i**). The majority of these neurons were concentrated in the caudal part of the INF in both dogs (**Fig. 4g'**) and cats (**Fig. 4h'**). In OVX cats, fewer perikarya also occurred in the rostral INF and occasionally, in the periventricular nucleus. In both species, KP-IR fibers formed dense plexuses in the septal-preoptic region, the periventricular nucleus, the INF, the ventromedial and dorsomedial hypothalamic nuclei and the median eminence (ME).

*3. 1. 1. 2. Differential distribution of canine and feline GnRH neurons*

While KP cells displayed similar basic topography in the canine and feline mediobasal hypothalami with relatively minor species differences, the distribution of GnRH neurons considerably differed between the two species (**Fig. 4a-h**). Accordingly, the distribution of canine GnRH neurons covered wide areas from the preoptic region to the mediobasal hypothalamus (**Fig. 4b-h**), including the supraoptic nucleus, the medial preoptic area, the ventrolateral preoptic nucleus, the lateroanterior hypothalamic nucleus (**Fig. 4d'**), the area of the tuber cinereum-lateral hypothalamic area continuum, the medial tuberal nucleus and the INF. In contrast, GnRH cells of the cat exhibited a much more restricted distribution in the septal-preoptic area (**Fig. 4a, b**), including the medial septal nucleus (**Fig. 4a'**) and the periventricular complex of the hypothalamus; only few scattered neurons were observed in the INF. The bulk of GnRH-IR fibers targeted the ME in both species.

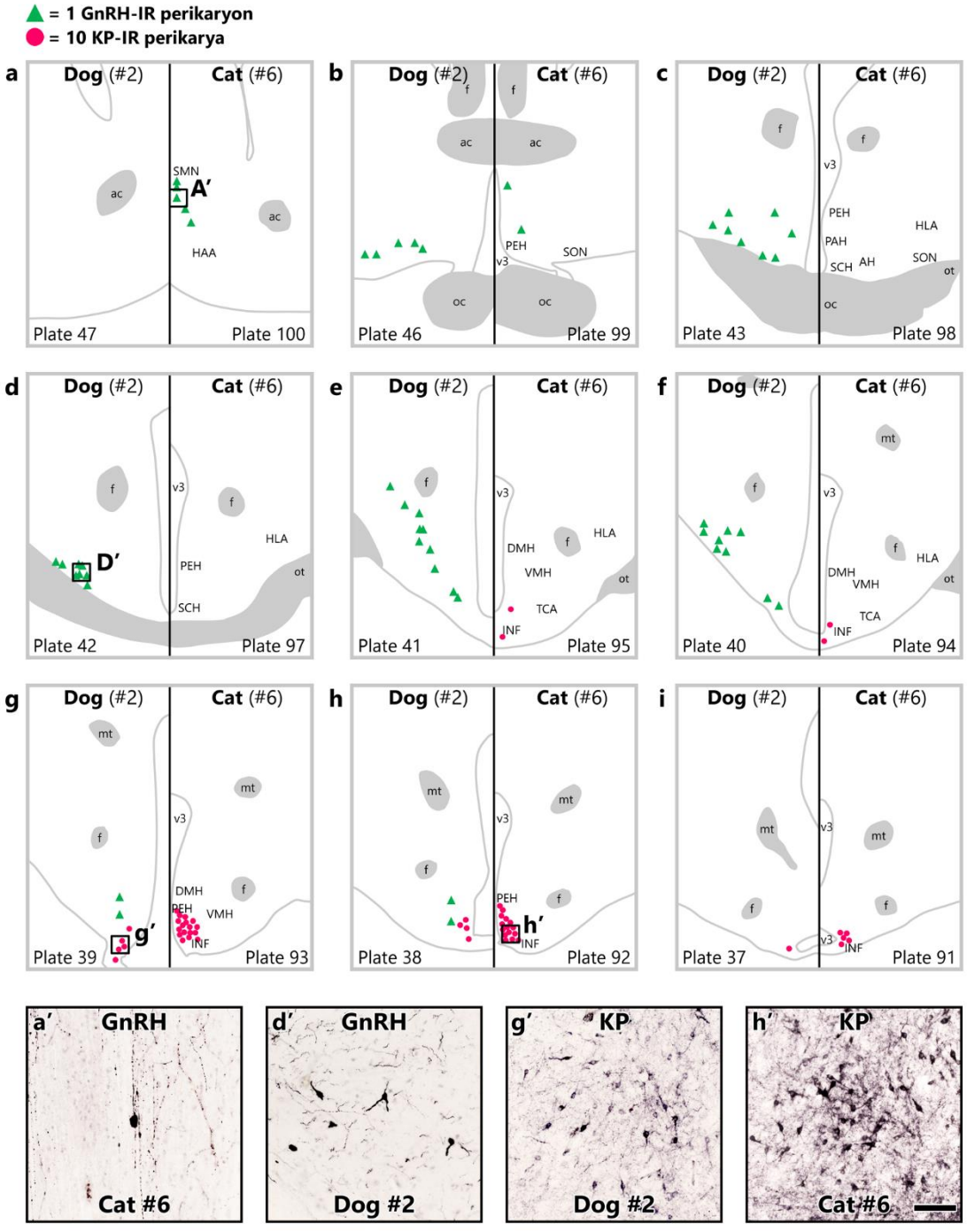


**Figure 4. Topography of gonadotropin-releasing hormone and kisspeptin neurons in ovariectomized dogs and cats.** Gonadotropin-releasing hormone (GnRH) neurons (green triangles) are scattered throughout the hypothalamus in ovariectomized (OVX) dogs and restricted to the septal-preoptic region in OVX cats. While kisspeptin-immunoreactive (KP-IR) neurons (red dots) are concentrated in the caudal infundibular nucleus in OVX cats as well as dogs, they also occur somewhat more rostrally in the feline mediobasal hypothalamus. Serial coronal schemas (**a-i**) were obtained from BrainMaps: An Interactive Multiresolution Brain Atlas (BrainMaps.org) [136]. Each green triangle corresponds to a single GnRH neuron and each red

dot represents 10 KP-IR somata from two representative animals (D#2 and C#6). Representative photomicrographs at the bottom display immunolabeled GnRH and KP neurons from OVX cats (**a', h'**) and dogs (**d', g'**), respectively. Abbreviations: **ac**, anterior commissure; **AH**, anterior hypothalamic nucleus; **DMH**, dorsomedial hypothalamic nucleus; **f**, fornix; **HAA**, anterior hypothalamic area; **HLA**, lateral hypothalamic area; **INF**, infundibular nucleus; **mt**, mammillothalamic tract; **oc**, optic chiasm; **ot**, optic tract; **PAH**, paraventricular nucleus of the hypothalamus; **PEH**, periventricular complex of the hypothalamus; **SCH**, suprachiasmatic nucleus; **SMN**, medial septal nucleus; **SON**, supraoptic nucleus; **TCA**, area of the tuber cinereum; **v3**, third cerebral ventricle; **VMH**, ventromedial hypothalamic nucleus. Scale bar: 100 μm.

### *3. 1. 2. Neuropeptide phenotype of canine and feline kisspeptin neurons*

#### *3. 1. 2. 1. Partial overlap of neurokinin B and dynorphin neurons with kisspeptin neurons*

Triple-immunofluorescent studies provided evidence for the presence of both NKB and Dyn in canine and feline KP neurons (**Fig. 5a, b**). The quantitative analysis of 833 immunoreactive cell bodies from two OVX dogs and 931 immunoreactive somata from four OVX cats identified Dyn signal in ~60% of KP neurons in both species (55.7±5.2% in dogs, **Fig. 5a,** upper column; 64.4±7.3% in cats, **Fig. 5b,** upper column), in 36.9±3.8% of NKB neurons in dogs (**Fig. 5a,** bottom column) and in 56.6±9.6% of NKB neurons in cats (**Fig. 2b,** bottom column). 66.8±2.4% of KP perikarya contained NKB (**Fig. 5b,** upper column) and 84.0±3.0% of NKB neurons contained KP in cats (**Fig. 5b,** bottom column), whereas virtually all (98.8±0.8%) KP neurons expressed NKB (**Fig. 5a,** upper column) and 49.3±5.3% of NKB neurons expressed KP (**Fig. 5a,** bottom column) in dogs. Triple-labeled (KNDy) somata contributed to 25.9±1.4% and 22.3±2.5% of the labeled neurons in dogs and cats, respectively (white slices of pie charts in **Figs. 5a** and **b**). Interestingly, the most frequently encountered phenotypes were NKB-only neurons in dogs (38.9±0.7%; **Fig. 5a,** green slice of pie chart), and Dyn-only neurons in cats (38.2±1.8%; **Fig. 5b,** blue slice of pie chart).

#### *3. 1. 2. 2. Substance P synthesis in canine and feline kisspeptin and neurokinin B neurons*

The putative expression of SP in KP and NKB neurons was addressed in additional triple-immunofluorescent experiments. Only two out of the four OVX dogs contained SP signal in KP and NKB cells and SP was present in less than 2% of these cells. In contrast, quantitative analysis of 189 immunolabeled perikarya from a gonadally-intact male dog revealed SP signal in 25.6% of KP neurons (**Fig. 5c,** upper column) and 24.3% of NKB

neurons (**Fig. 5c,** bottom column); 11.1% of the labeled neurons were triple-labeled (white slice of the pie chart in **Fig. 5c**). As opposed to OVX dogs, OVX cats contained SP signal in 20.0±3.4% of KP perikarya (**Fig. 5d,** upper column**)** and 28.8±3.7 of NKB perikarya (**Fig. 5d,** bottom column); 8.9±2.2% of the 695 immunolabeled neurons were KP/NKB/SP-positive (white slice of pie chart in **Fig. 5d**).

*3. 1. 2. 3. Cocaine- and amphetamine regulated transcript in kisspeptin and neurokinin B neurons of cats but not dogs*

Analysis of sections triple-labeled for KP, NKB and CART provided evidence that subpopulation of feline KP and NKB neurons also synthesize CART. The quantitative analysis of 838 cell bodies from four OVX cats provided evidence for the presence of CART in 22.9±2.0% of KP-IR (**Fig. 5f,** upper column) and 7.4±2.9% of NKB-IR (**Fig. 5f,** bottom column) neurons; triple-labeled somata contributed to 2.4±0.9% of the IR perikarya (white slice of pie chart in **Fig. 5f**). CART was not detectable in canine KP or NKB neurons (**Fig. 5e).**

*3. 1. 2. 4. Absence of galanin in kisspeptin and neurokinin B cells*

Galanin, which was previously detected in KP neurons of mice [122, 123] but not humans [113], was not present in KP and NKB cells of OVX dogs or cats (**Figs. 5g, h**).

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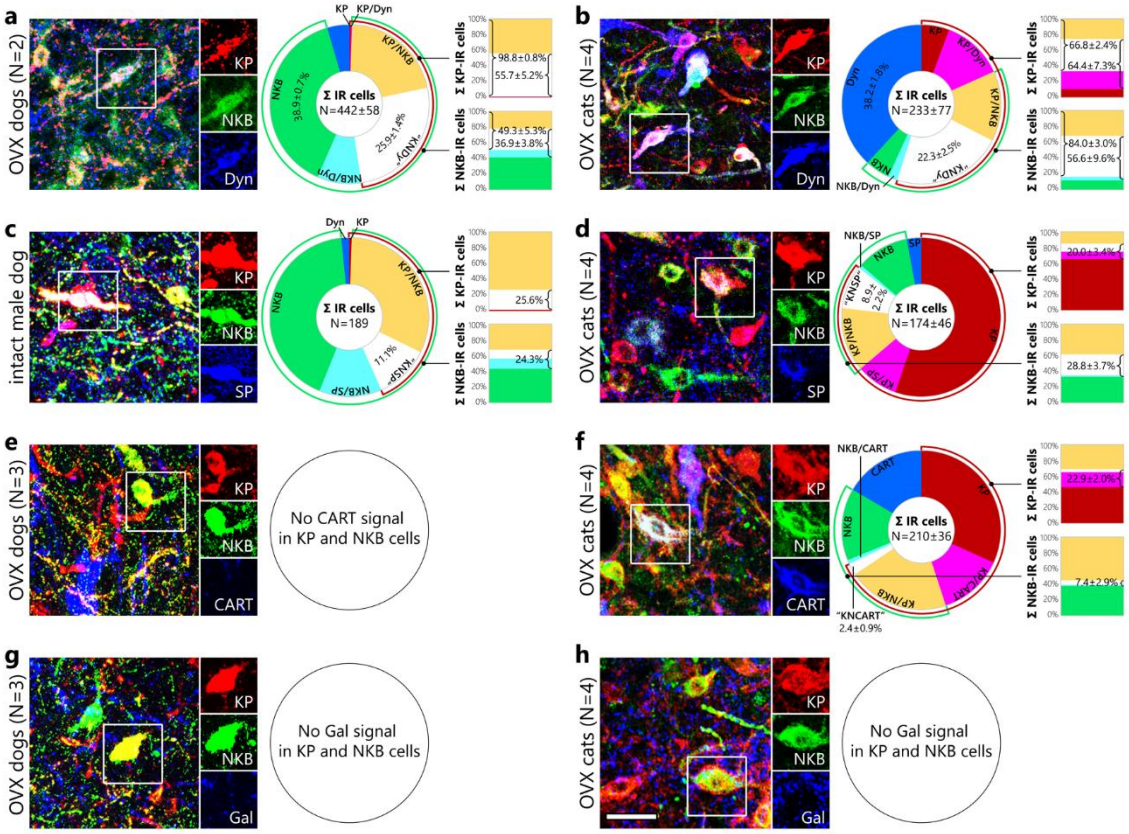


**Figure 5. Neurochemistry of canine and feline kisspeptin and neurokinin B cells.** Representative photomicrographs illustrate the neuropeptide phenotype of canine and feline kisspeptin (KP; red) and neurokinin B (NKB; green) neurons. Cells framed in the three-color figures are shown in separate color channels on the right side of each panel. KP and NKB immunoreactivities overlap considerably but incompletely in the infundibular nucleus of dogs (**a, c, e, g**) and ovariectomized (OVX) cats (**b, d, f, h**). Pie charts illustrate the percent distribution of distinct neuronal phenotypes from triple-immunofluorescence experiments addressing dynorphin (Dyn; **a**), substance P (SP; **b**) and cocaine- and amphetamine-regulated transcript (CART; **c**) co-expression in KP and NKB neurons. Bar graphs show percentages of neuropeptide colocalization in KP neurons (upper columns) and NKB neurons (bottom columns). Dynorphin signal (blue) occurs in large subsets of canine (**a**) and feline (**b**) KP and NKB cells; ~25% of the immunolabeled infundibular neurons are triple-labeled („KNDy") cells in both dogs and cats. SP (blue) is detectable in 26% of KP and 24% of NKB neurons in an intact male dog (**c**) as well as in 20% of KP and 29% of NKB cells in OVX cats (**d**). CART (blue) is also present in 23% of KP and 7% of NKB neurons in OVX cats (**f**), but no CART signal is detectable in canine KP and NKB cells (**e**). Galanin (Gal) is not expressed in KP and NKB neurons of either species (**g, h**). Abbreviations: "KNCART", kisspeptin/neurokinin B/ cocaine- and amphetamine regulated transcript-positive cells; "KNSP", kisspeptin/neurokinin B/substance P-positive cells. Scale bar: 50 µm.

### *3. 1. 3. Kisspeptin innervation of GnRH cells*

*3.1.3.1. Kisspeptin inputs to the somatodendritic compartment of GnRH neurons*

In OVX cats, KP neurons sent dense projections to the septal-preoptic region where GnRH neurons were located. Virtually all (94.4±5.6%) GnRH neurons received at least one KP-IR input; appositions were observed on both the somatic (6.9±0.2 inputs/soma, **Fig. 6c'**) and the dendritic (7.5±1.4 contacts/100 μm dendrite, **Fig. 6c"**) compartments. The feline hypothalamus showed very poor axonal labeling for INF KP neuron markers NKB or Dyn, making it impossible to assess whether and to what extent, KP afferents to GnRH neurons arise from the INF. In OVX dogs 77.6±17.6% of GnRH neurons (75.8±18.3% in the preoptic region and 83.3±16.7% in the INF) were innervated by at least one KP fiber and GnRH neurons in the INF also received more KP-IR contacts than those located in more rostral hypothalamic regions [6.3±1.0 (**Fig. 6a'**) *vs*. 2.0±0.7 inputs/soma and 7.9±5.5 (**Fig. 6a"**) *vs*. 0.9±0.6 inputs/100 μm dendrite]. 57.6±14.1% of inputs (93.3±1.5% in the INF) targeting the somatodendritic compartment were also immunopositive for NKB, indicating that more than half of these KP afferents arise from the INF (**Fig. 6a**).

*3.1.3.2. Axo-axonal communication between kisspeptin and GnRH neurons in the median eminence*

The distribution of hypophysiotropic GnRH fibers in the ME overlapped with a dense KP-IR fiber plexus in both dogs and cats (**Fig. 6b, d**). Contacts suggesting axo-axonal interactions were often detected between the juxtaposed GnRH and KP profiles. In dogs, many KP fibers contained NKB signal as well, indicating their mediobasal hypothalamic origin (**Fig. 6b**).

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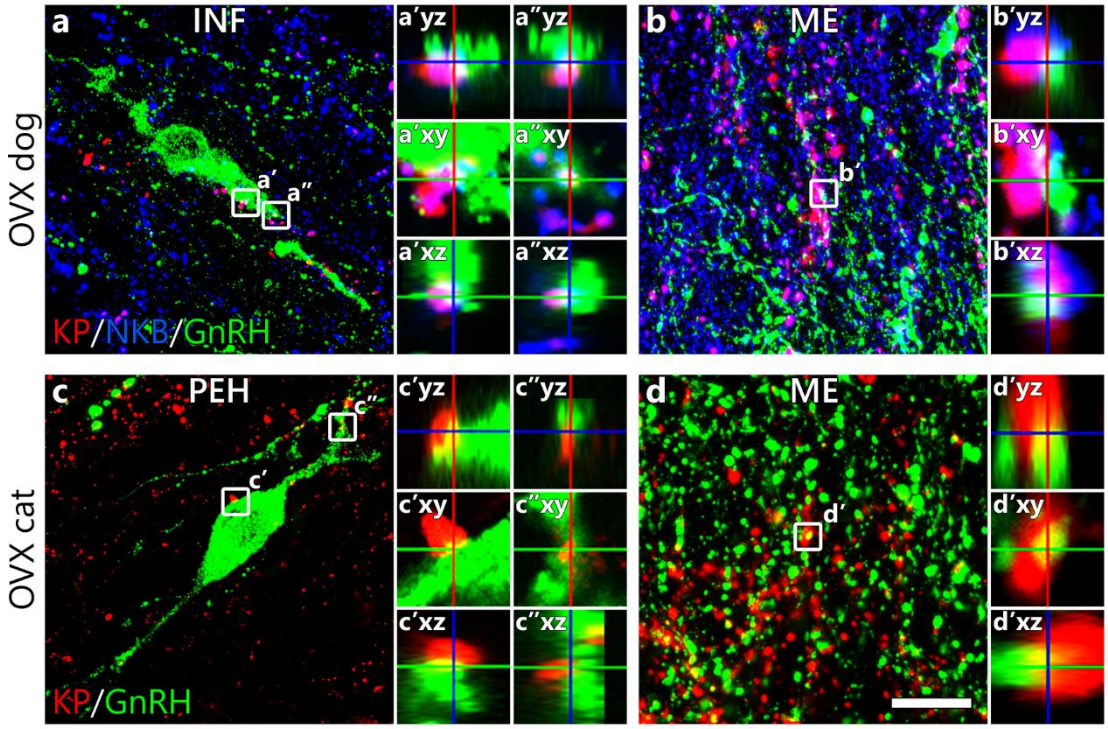


**Figure 6. Kisspeptin innervation of canine and feline gonadotropin-releasing hormone neurons.** Results of triple- and dual-immunofluorescent studies on ovariectomized dogs (**a, b**) and cats (**c, d**), respectively, reveal that kisspeptin (KP) fibers (red) establish axo-somatic (**a'**, **c'**), axo-dendritic (**a''**, **c''**) and axo-axonal (**b'**, **d'**) contacts with gonadotropin-releasing hormone-immunoreactive (green) neurons. Successful detection of neurokinin B (NKB) in fibers of the canine hypothalamus allows the identification of KP fibers that arise from KP/NKB dual-phenotype neurons of the infundibular nucleus. High-power images of framed regions show absence of gaps between the juxtaposed profiles in orthogonal side-views of contacts. Abbreviations: INF, infundibular nucleus; ME, median eminence; PEH, periventricular complex of the hypothalamus; Scale bar: 20 μm in **a**, **c** (4 μm in insets), 16 μm in **b**, **d** (3 μm in insets).

## 3. 2. Characterization of kisspeptin neurons in the human rostral hypothalamus

### *3. 2. 1. Mapping of KP immunoreactivity in the human rostral hypothalamic region*

Immunohistochemical studies using the GQ2 KP-54 antibodies visualized numerous IR cell bodies in rostral hypothalamic sections. The topography of these perikarya is illustrated schematically in **Fig. 7,** and a representative photomicrograph is shown in **Fig. 7a1.** Most rostrally and corresponding to atlas plates 20-21 of Mai et al. (Mai et al., 1997) (**Fig. 7a** and **b**, respectively), KP neurons occurred in the anterior parvocellular paraventricular nucleus (PaAP). These levels contained the highest number of labeled cells per section (mean±SEM) in each group (young women: 36.2±6.2; aged women: 12.2±2.8; young men: 33.8±6.8; aged men: 45.8±7.8). More caudally at the level of atlas plate 22 (**Fig. 7c**), labeled neurons were observed in the dorsal (PaD) and magnocellular (PaMC) parts of the paraventricular nucleus. Level 23 contained labeled neurons in both the parvocellular (PaPC) and magnocellular (PaMC) subdivisions of the paraventricular nucleus (**Fig. 7d**).

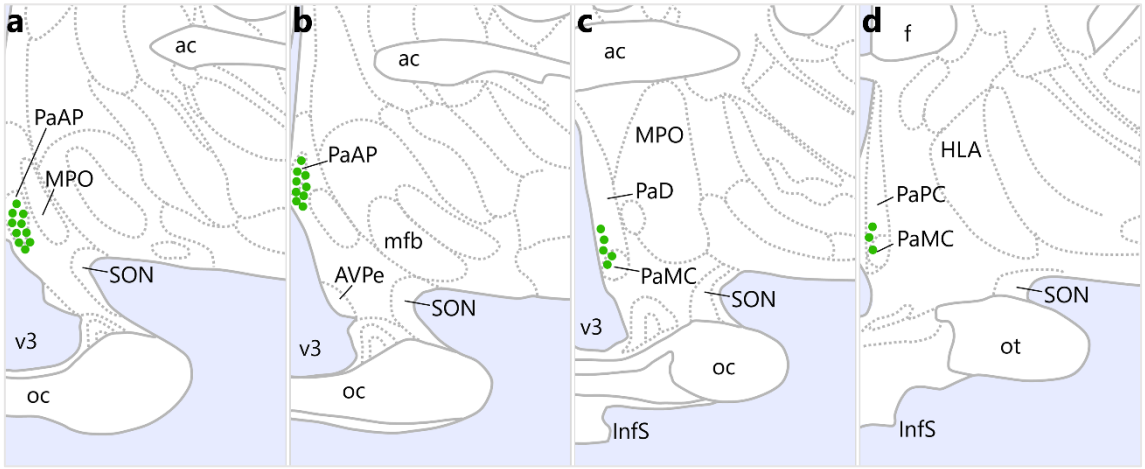


**Figure 7. Topographic distribution of kisspeptin-immunoreactive perikarya in the human rostral hypothalamus.** The green dots in the schematic diagrams illustrate the topographic distribution of kisspeptin-immunoreactive cell bodies at different rostrocaudal levels of the rostral hypothalamus. Panels **a-d** correspond to atlas plates 20-23, respectively, of Mai et al. (Mai et al., 1997). Labeled neurons occur in highest numbers in the anterior parvocellular (**PaAP**), dorsal (**PaD**), magnocellular (**PaMC**) and parvocellular (**PaPC**) subnuclei of the hypothalamic paraventricular nucleus. **ac**, anterior commissure; **AVPe**, anteroventral periventricular hypothalamic nucleus; **f**, fornix; **InfS**, infundibular stalk; **HLA**, lateral hypothalamic area; **mfb**, medial forebrain bundle; **MPO**, medial preoptic nucleus; **ot**, optic tract; **oc**, optic chiasm; **SON**, supraoptic nucleus; **v3**, third cerebral ventricle.

### *3. 2. 2. Demonstration of KP labeling specificity*

The sheep KP-54 (GQ2) and the mouse preproKP (BG#01) antibodies visualized similarly distributed KP neurons in the rostral hypothalamus (**Fig. 8a1, a2**), making it unlikely that KP-54 immunolabeling is due to nonspecific antibody binding to unwanted RF-amide peptides. In addition, dual-immunofluorescence studies combining the GQ2 with the rabbit AAS26420C (against preproKP) antibodies mostly revealed an overlapping signal in cell bodies and fibers, providing compelling evidence for the detection of authentic KP signals in the human rostral hypothalamus **(Fig. 8b1-3)**.

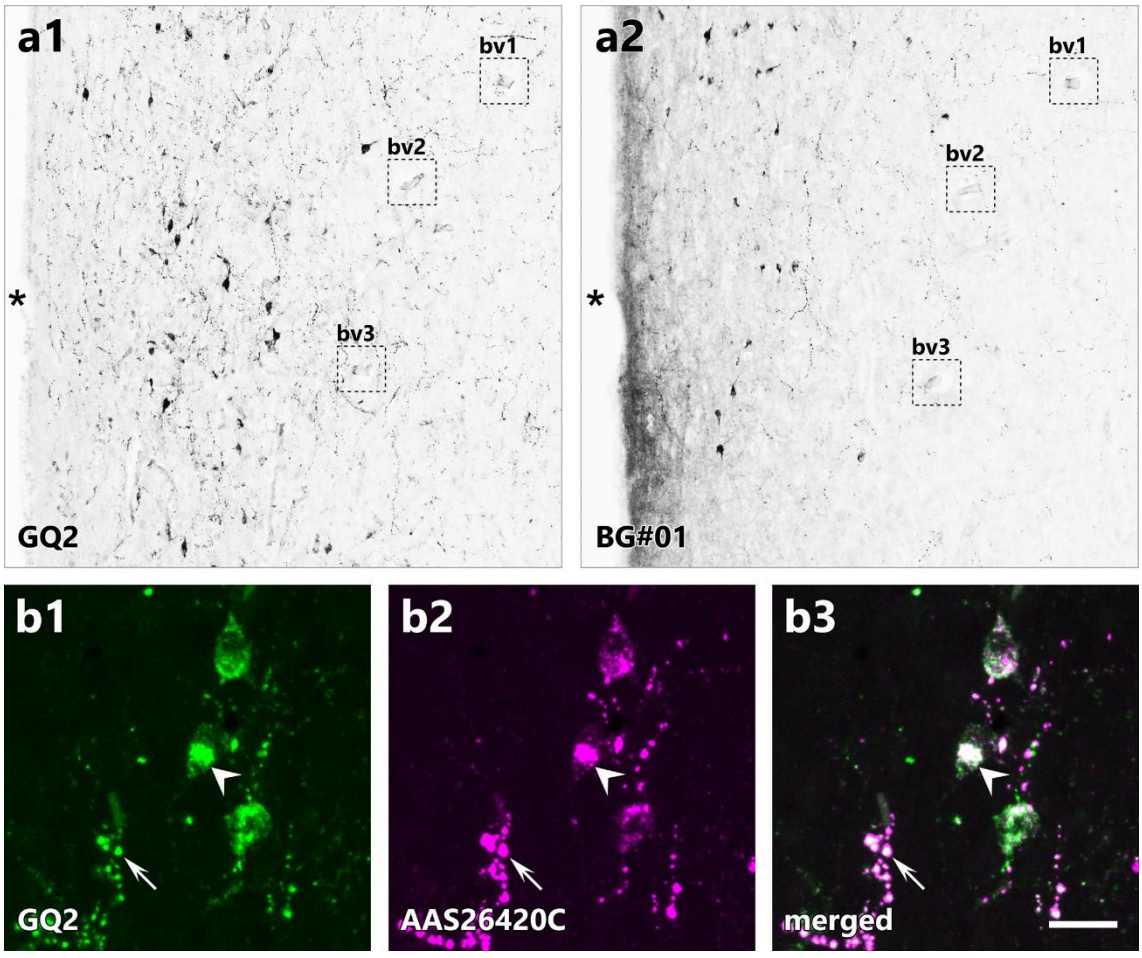


**Figure 8. Demonstration of a kisspeptin (KP) cell group in the human rostral hypothalamus. a:** Somewhat dispersed KP-immunoreactive neurons occur in the paraventricular nucleus (Pa) of the rostral hypothalamus. Neurons with this topography are labeled in adjacent sections with the GQ2 antiserum against the full-length KP-54 (aa 68-121 of NP_002247.3) (**a1**) as well as with the polyclonal BG#01 control antibodies against preproKP (**a2**). Note that BG#01 has been raised in a mouse against aa 70-93 of NP_002247.3. This sequence does not contain the conserved C-terminal RF-amide motif of KP-54 to rule out possible cross-reactions with unwanted members of the RF-amide peptide family. The dashed frames in **a1** and **a2** contain the same blood vessels

(bv 1-3) serving as reference points. The asterisks designate the 3rd cerebral ventricle. **b:** Dual-immunofluorescence experiments with the combined use of the reference sheep GQ2 KP-54 antiserum (green) and a rabbit polyclonal control antiserum (AAS26420C; magenta) targeting aa 21-80 of preproKP (NP_002247.3) detect mostly double-labeled perikarya (arrowheads) and fibers (arrows) in the Pa. These control experiments prove authentic KP synthesis in the rostral hypothalamus of the human brain. The Photomicrographs are representative images from immunolabeled samples of young women. Scale bar: 100 μm in (**a)** and 20 μm in (**b**).

### *3. 2. 3. Absence of known KP neuron markers within rostral hypothalamic KP cells*

A series of dual-immunofluorescence experiments with well-characterized primary antibodies found no evidence for Enk, TH, Gal or SST synthesis in KP neurons in the rostral hypothalamus (**Fig. 9)**. Furthermore, KP neurons of the rostral hypothalamus were also devoid of NKB, SP and CART, three known markers of KP neurons in the human MBH (**Fig. 10a-c)**.

### *3. 2. 4. Detection of afferent input from KP neurons of the mediobasal hypothalamus*

Triple-immunofluorescence studies established that KP/SP/NKB triple-labeled axons formed occasional contacts with KP neurons of the rostral hypothalamus **(Fig. 11)**. Reciprocity of this innervation could not be addressed in the absence of distinctive markers for fibers originating from the rostral hypothalamic KP cell population.

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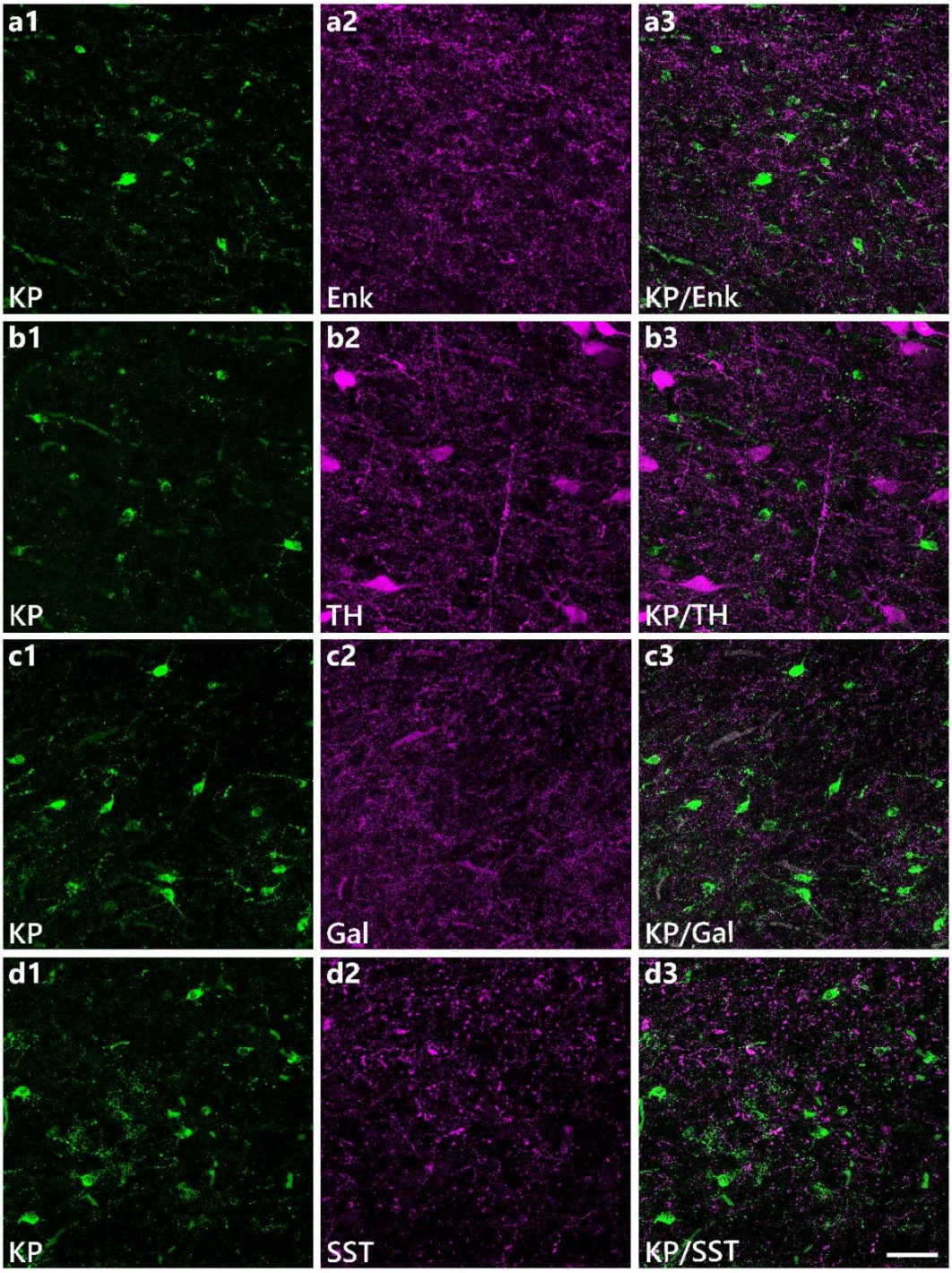


**Figure 9. Immunofluorescence experiments to address the synthesis of rodent kisspeptin (KP) neuron markers and somatostatin (SST) in the rostral hypothalamic kisspeptin cell population. a-c**: Dual-label immunofluorescence experiments aimed to determine whether enkephalins (Enk; **a**), the dopaminergic marker enzyme tyrosine hydroxylase (TH; **b**) or galanin (Gal; **c**) occur in human rostral hypothalamic KP neurons. These three well-established markers of murine KP neurons of the rostral periventricular region of the third ventricle are undetectable in the homologous human cell group. **d**: Human KP neurons are similarly devoid of SST immunolabeling, although SST is abundant in perikarya and neuronal fibers surrounding KP neurons. Scale bar: 50 μm.

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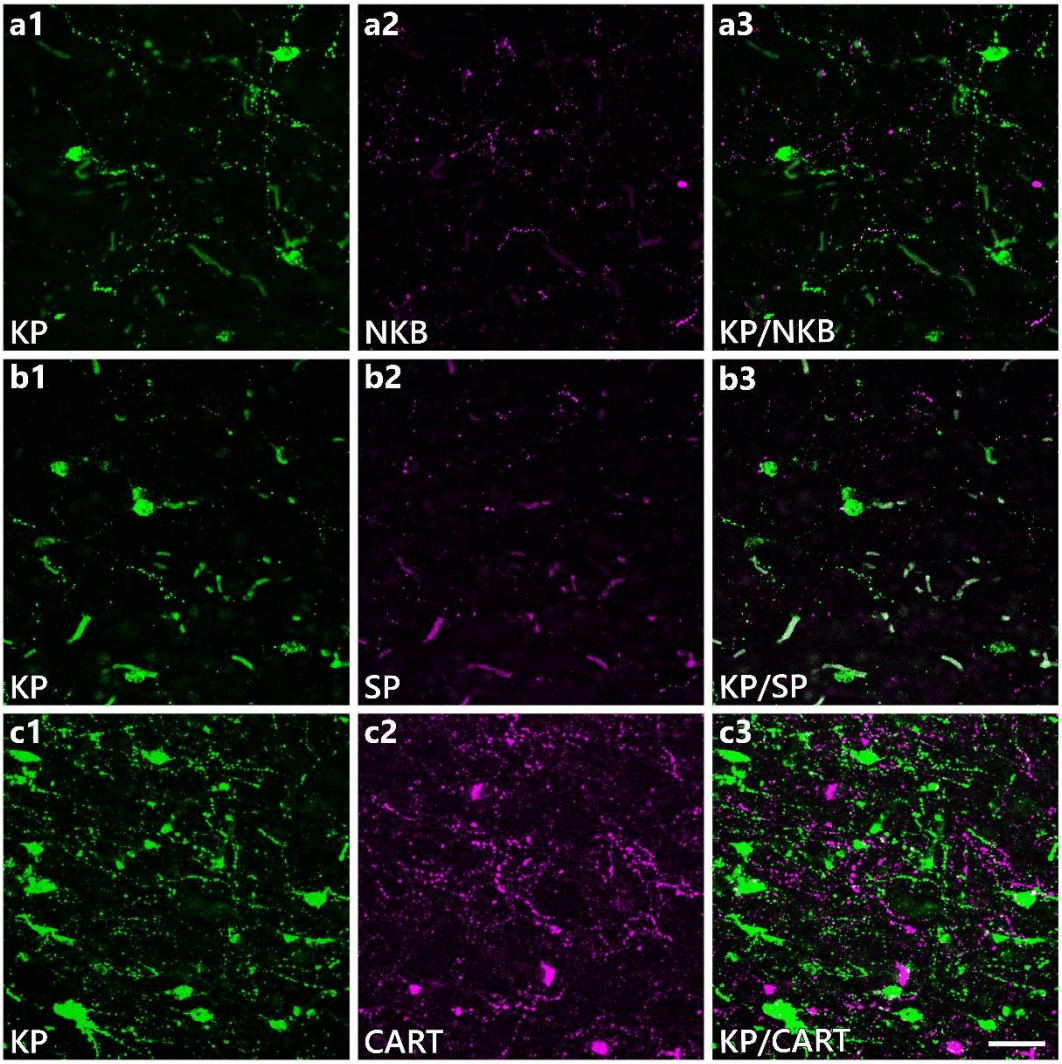


**Figure 10. Colocalization studies of marker neuropeptides identified previously in mediobasal hypothalamic kisspeptin (KP) neurons. a-c**: Further immunofluorescence experiments addressed whether rostral hypothalamic KP neurons contain the neuropeptide markers (shown in magenta) of the human mediobasal hypothalamic KP neuron population. Neurokinin B (NKB; **a**), substance P (SP; **b**), and cocaine- and amphetamine-regulated transcript (CART; **c)** are all absent from KP neurons (green) of the rostral hypothalamus. Scale bar: 50 μm in (**a-c**).

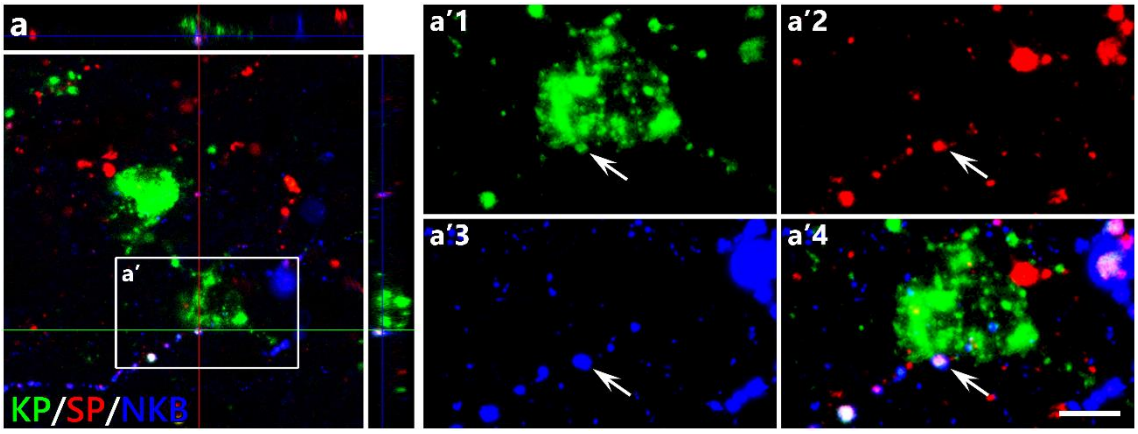


**Figure 11. Detection of afferent inputs from KP cells of the infundibular nucleus. a**: Fibers co-containing KP (green) with NKB (blue) and/or SP (red) in the rostral hypothalamus likely originate in the mediobasal hypothalamus. The images from confocal Z-stacks illustrate an axosomatic apposition between a KP/NKB/SP-triple-labeled axon (arrow in **a'1-4**) and a KP perikaryon. Note the absence of a gap between the juxtaposed profiles in the orthogonal side views (z axis) of the neuronal contact (**a**). The existence of these contacts reveals that KP neurons of the mediobasal hypothalamus communicate with the rostral hypothalamic KP cell population. Scale bar: 12 μm in (**a**) and 6 μm in (**a'1-4**).

### *3. 2. 5. Significant reduction of KP cell numbers after menopause but no sex difference between young males and females*

While an earlier pilot study revealed rostral hypothalamic KP neurons only in histological samples of young women [104], here we were able to detect KP-IR neurons in both males and females and their arbitrarily defined "young" and "aged" subgroups. The highest number of labeled perikarya found in a single section was recorded for each individual and used for statistical comparisons (average cell number/section ± SEM: young female – 36.2±6.15; aged female – 12.17±2.82; young male – 33.83±6.78; aged male – 45.83±7.82). Two-way ANOVA identified a significant effect of sex ($F_{(1,19)}$=6.30, $p$<0.05) but not age ($F_{(1,19)}$=0.97, $p$=0.34) on KP cell numbers, and a significant interaction between sex and age ($F_{(1,19)}$=8.55, $p$<0.01). A comparison of the four groups with a Newman-Keuls *post hoc* test showed that aged (postmenopausal) women had significantly lower cell numbers than the other groups ($p$<0.05), including young women likely in their fertile years ($p$<0.05, **Fig. 12**). The 66.4% postmenopausal reduction was likely attributed to the loss of estrogens. The cell numbers in young males and females did not differ ($p$=0.77), indicating a species difference from rodents, which display much higher cell numbers in females.

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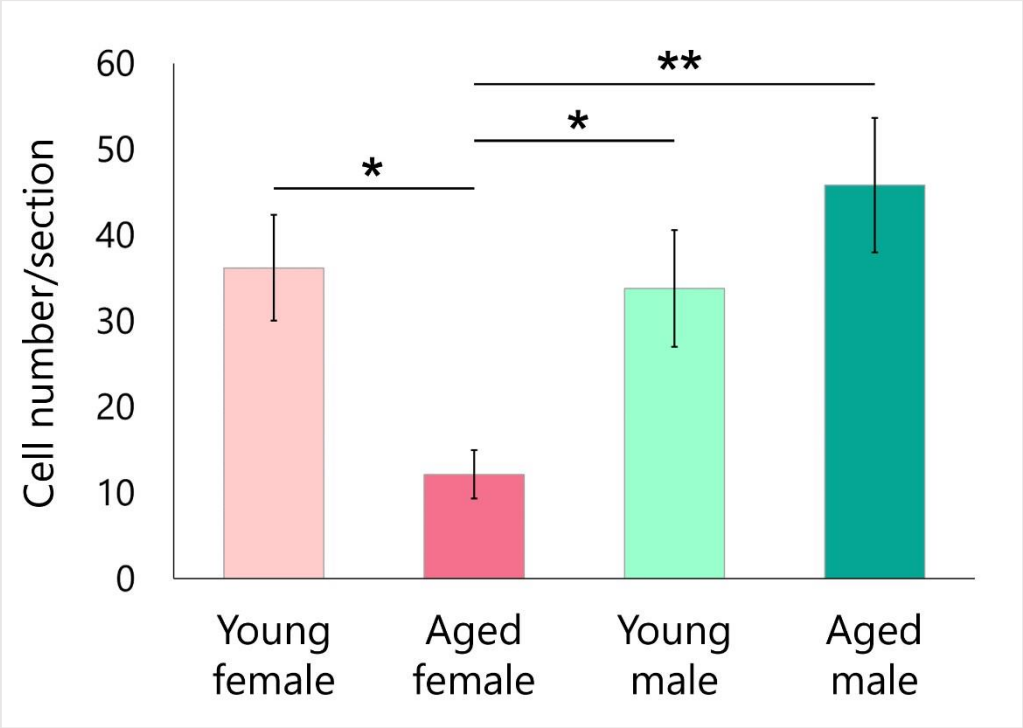


**Figure 12. Assessment of effects of age and sex on kisspeptin (KP) cell numbers.** Each human individual was characterized semi-quantitatively with the maximal number of KP perikarya found in a single section to address putative age and sex effects. The group of aged (postmenopausal) women (>55 years) shows a 66.4 % reduction in labeled cell numbers in comparison with young females (≤45 years), and it differed significantly from the arbitrarily defined young (<50 years) and aged (≥50 years) male groups as well. The reduced number of KP neurons after menopause is likely caused by the loss of estrogens. The cell numbers in the other three groups did not differ. The similar cell numbers in young men and young women suggest a lack of robust sexual dimorphism and a species difference from rodents, which have more neurons in females than in males. *p<0.05; **p<0.01 (2-way ANOVA followed by Newman-Keuls test).

# 4. DISCUSSION

## 4. 1. The topography and neurochemistry of infundibular kisspeptin neurons and their connectivity to GnRH cells

The topography of GnRH and KP neurons and the peptide neurochemistry of the putative pulse generator KP neurons within the mediobasal hypothalamus differ substantially between the most widely studied laboratory rodent species and humans. Although the GnRH/LH pulse generator function seems to be highly conserved across mammals, species differences, especially in the neurochemistry of MBH KP neurons, indicate that the anatomical basis and molecular mechanisms of episodic GnRH secretion can vary between species. Here we carried out the neuroanatomical characterization of KP and GnRH neurons in OVX dogs and cats. Information about the pulse generator network of these two poorly studied carnivore species may provide important insight into evolutionarily conserved *vs.* variable mechanisms of episodic GnRH/LH secretion.

### *4. 1. 1. Distribution of GnRH and kisspeptin neurons in dogs and cats*

GnRH neurons are not concentrated within distinct hypothalamic nuclei and their distribution varies considerably between species [20]. For example, while GnRH cell bodies are located exclusively within the septal-preoptic region in laboratory rodents [16], in humans they are scattered quite widely from the septo-preoptic region to the retromammillary area [137, 138]. Similarly to human, OVX dogs used in our study contained GnRH-IR perikarya throughout the hypothalamus, including the INF. The functional difference between the rostral and the caudal GnRH cell groups of this species requires clarification. In sheep, both the preoptic and the mediobasal hypothalamic GnRH neuron populations express Fos during the LH surge [139], whereas Fos only occurs in GnRH neurons of the ARC at times of enhanced pulsatile GnRH/LH secretion [140].

In contrast with dogs, OVX cats contained the majority of GnRH neurons in the septal-preoptic region. This observation is in line with the previously reported distribution of GnRH-immunopositive perikarya in cats [141-143], except that in one study adult male [143] cats also contained GnRH cell bodies in the INF.

A variety of mammals contain two major KP cell populations in the preoptic region and in the ARC/INF of the mediobasal hypothalamus, respectively [98]. An additional

small group of KP neurons has been identified recently in the medial amygdala of rodents [144]. In rats and mice, preoptic KP neurons play important roles in positive estrogen feedback and the mid-cycle GnRH/LH surge [145]. *Kiss1* expression in these cells is regulated positively by estrogens [120]. The largest KP cell group in all species studied so far has been localized to the ARC/INF of the mediobasal hypothalamus [98]; these neurons have been implicated in negative sex steroid feedback [106] and their *Kiss1* expression is inhibited by gonadal sex steroid hormones [146]. There is accumulating evidence that KP neurons of the ARC/INF play a critical role in the generation of GnRH/LH pulses [107].

Our mapping experiments localized the majority of KP neurons to the INF of OVX dogs and cats. In addition, the feline, but not the canine hypothalamus contained scattered IR neurons in the periventricular nucleus, in line with results of a recent immunohistochemical study by Amelkina et al. [147]. These authors studied intact female cats and identified two additional KP cell populations in the anterior periventricular nucleus and the amygdaloid complex, respectively [147]. From these, the first population may be homologous with KP neurons of the rodent anteroventral periventricular nucleus which are activated during positive feedback [145]. On the other hand, the role of KP neurons in the cat amygdala is less clear and it was proposed that these KP neurons do not correspond to KP cells described in the medial amygdala in rodents [147].

KP neurons of the anterior periventricular nucleus and the amygdaloid complex remained undetected in our present study. This is most likely explained by the use of OVX animals, in view that the expression of *Kiss1* is positively regulated by estrogens both in the preoptic region [120] and the amygdala [144]. In the context of ovulation, it is important to note that the cat is a reflex ovulator species in which the preovulatory GnRH/LH surge is induced by the receipt of genital somatosensory stimuli during mating. In such species, activation of brainstem noradrenergic neurons is thought to act in the mediobasal hypothalamus to promote GnRH release from nerve terminals in the median eminence [148]. While induced ovulating species generally do not show steroid-induced preovulatory LH surges, the cat seems to be an exception in this respect because group-housed females are capable of ovulating spontaneously [149]. It remains to be clarified how the hypothalamic KP and GnRH neuronal systems of the cat are integrated into the neuronal networks regulating spontaneous *vs.* reflex ovulation.

### *4. 1. 2. Connections between kisspeptin cells and GnRH neurons in dogs and cats*

KP input to GnRH neurons is critical for reproduction in mice [97] and humans [79, 82], and unlikely to be less important in other mammals like dogs and cats. KP fibers in different species innervate the somatodendritic compartment of GnRH neurons [52, 103, 104, 150, 151]. In mice, these inputs tend to arise from the preoptic region because most of them are devoid of the arcuate nucleus marker NKB [152]. The excitatory responses of GnRH neurons to KP [93, 95, 96] are likely mediated by somatodendritic Kiss1r during positive estrogen feedback. Humans whose GnRH neurons are rather scattered within the hypothalamus [137] differ from mice in that at least 10-30% of their KP afferents contain the INF marker NKB [153, 154]. In our present study on OVX dogs and cats, the cell bodies and dendrites of GnRH neurons received abundant innervation from KP axons. In dogs, successful detection of NKB signal in over 90% of KP-IR afferents also allowed us to conclude that a higher number of these KP inputs originate in the INF. In contrast, lack of sufficient axonal signal for NKB and Dyn within KP axons of the cat did not allow us to firmly establish the importance of KP inputs arising from the INF in this species.

It is known that KP-IR neurons of the mediobasal hypothalamus also establish axo-axonal contacts with hypophysiotropic GnRH fiber varicosities in the ME of various species [103, 155-157]. KP likely binds to Kiss1r on these GnRH fibers when inducing GnRH release from mediobasal hypothalamic explants (which contain only a few GnRH cell bodies in rodents) in an action potential-independent manner [84]. Systemic KP injection is also thought to stimulate LH secretion via acting on these processes [89, 94, 158, 159]. Finally, axo-axonal contacts may also play a paramount role in the communication between the putative pulse generator KNDy neurons and the GnRH neurosecretory system. Temporal correlation between the pulsatile KP output and GnRH secretory pulses in the ME of monkeys provide strong support for this notion [160]. Results of our present study established that, similarly to other species, dogs and cats also contain axo-axonal appositions between GnRH-IR and KP-IR fibers in the ME. In addition to these *bona fide* axo-axonal appositions, it remains possible that KP axons also innervate more proximal segments of GnRH fibers before they spray into terminal axon branches. In mice, GnRH fibers bear mixed dendritic and axonal properties (called “dendrons”) and at the border of the median eminence they receive a particularly high density of synaptic inputs. This connectivity may represent an important anatomical

pathway in the regulation of episodic GnRH/LH secretion [29]. The existence of such GnRH dendrons and their synaptic regulation in other species, including cats and dogs, will require clarification.

### *4. 1. 3. Species differences in the neurochemistry of kisspeptin cells*

The most important message of our present study is the unique neurochemistry of canine and feline mediobasal hypothalamic KP neurons, with both similarities to and differences from the KP system of other species.

Similarities to rodents [108] and the sheep [100] include the co-expression of the three KNDy neuropeptides in the same cells. This evolutionarily conserved phenomenon suggests that all three neuropeptides play obligate roles in the generation of GnRH/LH pulses. However, it is worth to note that KP neurons in the INF of *postmortem* human brains only shows partial overlap between the KNDy neuropeptides which also varies considerably with sex and age. For example, only ~36% of NKB neurons express KP immunoreactivity in young human males [112, 154]. In addition, Dyn immunoreactivity can only be detected very rarely in human KP and NKB neurons and their processes [112-114], in contrast with much higher degrees of co-expression in sheep [100] and mice [108]. An interesting similarity between dogs and cats and the human is the relatively high abundance of NKB neurons without KP signal, whereas most KP neurons in all three species contain NKB signal. Our immunofluorescence results on OVX dogs and cats indicate that about ~25% of labeled neurons in the canine and feline INF are triple-labeled (KNDy) neurons, supporting the notion that the overlap of the three KNDy peptides might be functionally important, although far from being complete. It will be important to more thoroughly investigate the relationship between the reproductive status and the co-synthesis/co-secretion of each KNDy peptide in different species.

In addition to identifying the above species similarities, we also observed clear differences between OVX cats and dogs and the more extensively studied rodent and primate species. As opposed to mice [161] and similarly to humans [115, 118], OVX cats showed a considerable co-expression of CART and SP in KP and NKB neurons. Interestingly, the two carnivores differed from each other in this context; canine KP and NKB neurons did not co-contain CART signal, whereas feline KP and NKB neurons did. Similarly, we did not observe SP signal in KP and NKB neurons in OVX dogs. Despite

this negative finding, SP co-synthesis likely plays some role in the physiology of these neurons in this species because a gonadally intact male dog included in our study showed SP signal in many KP and NKB neurons. This finding raises the possibility that sex and/or gonadal status exerts critical effects on the SP synthesis of KP neurons. Finally, galanin, which has only been observed in murine KP neurons [122, 152], was absent from KP cells of both dogs and cats which is similar to our earlier observation on human KP neurons [113]. Neurochemical differences and common features of different species suggest that some neuropeptides play facultative roles in the generation or fine-tuning of GnRH/LH pulses, whereas the role of others is evolutionarily more conserved. One should keep in mind that colocalization results from different species were obtained using different antibodies and different immunohistochemical techniques. Therefore, comparison of the exact colocalization percentages has innate pitfalls.

## 4. 2. Characterization of kisspeptin neurons in the human rostral hypothalamus

### *4. 2. 1. Preoptic/rostral hypothalamic kisspeptin neurons are detectable in a variety of mammalian species, including primates*

With somewhat variable topography, preoptic/rostral hypothalamic KP neurons are detectable in various mammalian species, including rodents [88, 146, 162, 163], sheep [100, 164], pigs [165] and nonhuman primates [133, 134, 166]. In rats and mice, this cell population is located in the RP3V and includes two subsets within the anteroventral periventricular and the periventricular preoptic nuclei, respectively [145, 167]. In naked mole-rats, additional KP neurons have also been reported more caudally in the anterior periventricular nucleus [168], and in pigs, the rostral-most KP neurons have been described in the periventricular nucleus [165]. The rostral KP cell group has been localized to the preoptic area in both sheep [164, 169] and nonhuman primates [133, 134, 166]. Somewhat unexpectedly, a careful distribution analysis of the homologous KP neurons in the present study found the largest overlap with four distinct subdivisions of the paraventricular nucleus in humans.

The functional significance and putative contribution of preoptic/rostral hypothalamic KP neurons to positive sex steroid feedback may considerably vary from species to species.

### *4. 2. 2. Positive feedback and the LH surge in primates may involve both rostral and caudal hypothalamic sites*

In spontaneous ovulators, rising concentrations of circulating estradiol in the late follicular phase of the estrous cycle provide a key trigger for the mid-cycle GnRH/LH surge. KP neurons in the rodent RP3V play a critical role in positive estrogen feedback to GnRH neurons [145]. These cells innervate GnRH neurons [52, 123, 150] and express c-Fos during the LH surge [120, 167]. Further, KP neuron-specific deletion of estrogen receptor-α disrupts positive feedback, as expected [56]. The rodent RP3V is sexually dimorphic, with many more KP neurons in females than in males [52, 120, 121]. This perinatally established sex difference is in accordance with the inability of adult male rodents to mount an LH surge in response to estrogens [170].

The site/s and mechanisms of positive estrogen feedback may differ substantially among species. Relatively few and scattered KP neurons can be detected in the ovine preoptic region [100, 164]. In this species, both the preoptic area and the MBH have been implicated in positive feedback. Frontal hypothalamic deafferentation in female sheep did not prevent estrous cycles but reduced the estrogen-induced LH surges, indicating that the input from the rostral hypothalamus to the MBH is required for a normal LH surge but not for ovulation [171]. Accordingly, the percentage of KP neurons expressing *c-Fos* is significantly higher during the LH surge in both the ARC and the preoptic area [132], and both sites also exhibit enhanced *Kiss1* expression during the estrogen-induced LH surge [131].

Although KP neurons occur both in the preoptic area/rostral hypothalamus and the ARC/INF of primates [104, 133, 134, 166], the primary sites of positive estrogen feedback is considered to be the MBH [1]. Surgical isolation of the MBH in rhesus monkeys did not interfere with spontaneous ovulation and the estrogen-induced LH surge [128, 129], providing a basis for the prevailing view that the MBH contains all neuronal elements necessary for positive feedback. While these data also argue against the reproductive significance of the preoptic/anterior hypothalamic region, other

investigators found that bilateral destruction of the ventral preoptic area/anterior hypothalamic area actually did block the spontaneous ovulation in rhesus monkeys and also compromised the ability of the hypothalamic-hypophysial axis to release LH in response to estrogens [126]. Along the same line, monkeys with bilateral anterior hypothalamic disconnection ceased to have cyclic gonadotropin release and ovulation after surgery and estrogen-induced release of LH [127]. A follow-up examination of lesioned animals 4-7 months after surgery showed spontaneous resumption of menstrual cyclicity and gonadotropin responsiveness to estrogen. Although these observations make it likely that cycles can be maintained by an anatomically isolated MBH-pituitary unit, they also indicate that the preoptic area/anterior hypothalamus plays important modulatory roles in normal menstrual cyclicity [127]. In further support of a putative preoptic/rostral hypothalamic involvement in positive feedback, preoptic KP neurons of the rhesus monkey show increased *Kiss1* mRNA expression in response to a surge-inducing estradiol regimen [166]. Moreover, in Japanese monkeys, estradiol only activates c-Fos expression in the preoptic, but not in the ARC KP cell population [133]. The above data from nonhuman primates make it likely that the KP neurons of the human rostral hypothalamus that we characterize in this study may also be recruited during the positive estrogen feedback.

### *4. 2. 3. Immunohistochemistry using different preproKP antibodies reveals authentic KP neurons in the human rostral hypothalamus*

A pilot study from our laboratory [104] which used the GQ2 sheep antiserum against the full-length human KP-54 sequence revealed a group of KP neurons in the rostral hypothalamus of human females, in addition to the bulk of KP-IR neurons in the MBH. This antiserum reacts with human KP-54, KP-14 and KP-10 and shows virtually no *in vitro* cross-reactivity (<0.01%) with other related human RF amide peptides, including prolactin releasing peptide, neuropeptide FF, neuropeptide AF and RF amide-related peptides (RFRP1, RFRP2, RFRP3) [89]. To exclude the possibility that we mistakenly detect unwanted RF-amide peptides in the rostral hypothalamus, here we used additional polyclonal antibodies against different preproKP peptide segments which did not contain the conserved RF-amide motif. Mapping studies including these strong positive controls

have established that authentic KP neurons occur in the PaAP, PaD, PaMC and PaPC subnuclei of the hypothalamic paraventricular nucleus, as defined by Mai et al. [172].

### *4. 2. 4. KP neurons in the human rostral hypothalamus are devoid of known KP neuron markers*

In rodents, the RP3V KP cell population contains a series of additional neurotransmitters/neuromodulators, including Gal [122, 123], Enk [122] and the dopaminergic marker TH [124]. Expression of GABAergic and glutamatergic markers [125] indicates that these cells also use classic amino acid neurotransmitters to communicate with GnRH neurons and/or other cell types.

Here, based on the rodent RP3V studies [122-124], we made an attempt to detect Enk, Gal and TH in KP neurons of the human rostral hypothalamus. Additional experiments also addressed the possible co-expression of neuropeptides that have been detected previously in human INF KP neurons, including NKB [104], SP [115] and CART [118]. None of the above immunofluorescent double-labeling experiments was able to reveal other neurotransmitters in human KP neurons. These observations suggest considerable species differences and also indicate that KP neurons in the INF and those in the human rostral hypothalamus differ in their neurochemistry and, likely, in their functions. As mentioned above, NKB which is an accepted topographic marker of ARC KP neurons, can only be found rarely within KP fibers that establish neuronal contacts with the GnRH neurons of mice [123], indicating that the major KP input to the somatodendritic cellular compartment of preoptic GnRH neurons arises from the RP3V KP neurons in this species. In contrast, at least 10-25% of the KP-IR afferent contacts observed previously on human GnRH cells also contain NKB [112]. This suggests that, unlike in rodents, the major KP cell group of the human MBH directly innervates the somatodendritic compartment of GnRH neurons, in addition to forming numerous contacts with the hypophysiotropic GnRH processes within the infundibular region [157]. The identification of a unique topographic marker would greatly facilitate the identification of the projections fields and target cells of KP neurons in the human rostral hypothalamus.

### *4. 2. 5. KISS1 expression in the rostral hypothalamus is regulated positively by estrogens*

In the absence of information about *post mortem* hormone levels in our study, the estrogenic regulation of *KISS1* expression by rostral hypothalamic KP neurons was assessed from the comparison of young (below the expected age of menopause) to aged (postmenopausal) samples. We found that the rostral hypothalamus had significantly reduced KP cell numbers in aged subjects. The postmenopausal involution of the KP system likely results from the loss of estrogens in view that estrogens positively regulate *KISS1* expression in the preoptic area/rostral hypothalamus of rodent [162, 169], ovine [173], porcine [165], and nonhuman primate [133, 134, 166] species.

### *4. 2. 6. Absence of sexual dimorphism in KP cell numbers of young individuals represents a species difference from rodents*

KP neurons in the rodent RP3V (which includes the anteroventral periventricular nucleus and the rostral periventricular nucleus) play a critical role in LH surge triggered by positive estrogen feedback [145]. Male rodents are unable to mount spontaneous or estrogen-induced LH surges in adulthood [170], which can be correlated with their much lower KP cell number at this site [52, 120, 121].

In the present study, we sought to find sex differences between the staining patterns of male and female human subjects being in their fertile years. Maximal numbers of KP neurons/section were compared between young males and females, assuming that sex steroids upregulate *KISS1* expression similarly in both sexes and that, therefore, any differences we might see partly reflect organizational effects of sex steroids during early development. Our *post hoc* analysis did not reveal significant sex difference in KP cell numbers in the rostral hypothalamus of young individuals. In the functional context, the key neuronal elements of positive feedback may not necessarily differ between sexes so much in primates compared to rodents. Likewise, a single estrogen injection into gonadectomized monkeys can induce an LH surge [170] and c-Fos activation in preoptic KP neurons [133] of both sexes. Similarly, estrogen [174, 175] or estrogen plus progesterone [176] administration to castrate human males is capable of inducing positive feedback on LH levels.

### *4. 2. 7. Connections between the two main KP cell populations*

In this study, we showed that KP/NKB/SP-triple-IR axon varicosities form contacts on rostral hypothalamic KP neurons. Together with presence of SP and NKB in large subsets of KP neurons in the MBH [104, 115] but not in the rostral hypothalamus (present study), this observation provides evidence that the two KP cell populations communicate with each other. It would be difficult to study the quantitative aspects of this communication, because not all MBH KP neurons co-contain NKB [154] or SP [115] and even lower percentages of their fibers may co-contain the marker neuropeptides [154]. We made an effort here to find some selective neuropeptide marker for KP neurons of the rostral hypothalamus. As we were not able to identify such a marker, it is currently impossible to determine if these neurons also provide neuronal inputs to KP neurons of the MBH.

The interaction between the two KP cell populations likely exists in rodents as well. At least, the KNDy neurons of the MBH also send projections to the RP3V region in rats [177] and mice [177, 178]. Moreover, the KP connection between the ARC and the RP3V regions are reciprocal [177].

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# 5. CONCLUSIONS

The aim of the two studies underlying this thesis was to deepen our understanding about the mechanisms whereby the two KP systems regulate mammalian fertility. (1) In our first study, we characterized the neuroanatomy of the KP system in two carnivores, the dogs and the cats.

We described the distribution of GnRH and KP neurons in the hypothalamus of OVX dogs and cats and identified conspicuous anatomical similarities to and differences from laboratory rodents and primates. We showed that the majority of GnRH neurons receive input from KP neurons. The vast majority of these inputs, at least in OVX dogs, originated from KP neurons of the INF. Finally, we determined the unique neurochemistry of KP and NKB neurons in the INF of OVX dogs and cats. Results of immunofluorescent colocalization studies indicated that the three KNDy peptides show partial co-expression, with ~25% of neurons expressing all three substances. Anatomical and neurochemical similarities to and differences from homologous and more extensively investigated rodent, domestic and primate KP cells will contribute to our understanding of obligate and facultative players in the molecular mechanisms underlying episodic GnRH/LH secretion in mammals.

(2) In the second study, we addressed the existence and studied the neurochemistry of KP neurons in the human rostral hypothalamus.

We reported that human KP neurons in the human rostral hypothalamus differ from the homologous KP neurons of the rodent RP3V; they did not contain the same cotransmitters and did not exhibit an obvious sexual dimorphism, unlike their rodent counterparts. On the other hand, their reduced number after menopause suggested that estrogen positively regulates *KISS1* expression in these neurons in humans, similarly to rodents. These new anatomical data from the human hypothalamus challenged the long-held view that positive estrogen feedback may be restricted to the mediobasal part of the hypothalamus in primates and raised the intriguing possibility that the contribution of the rostral hypothalamus to the regulation of positive estrogen feedback and reproduction might be currently underestimated thus pointed to the need of further anatomical, molecular and functional studies of rostral hypothalamic KP neurons.

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# 6. SUMMARY

The two KP systems of the mediobasal hypothalamus MBH and rostral hypothalamus, play crucial roles in negative and positive estrogen feedback mechanisms, respectively.

Neurons co-synthesizing KP, NKB, and Dyn („KNDy" neurons) in the MBH form a crucial component of the GnRH/LH „pulse generator" and mediate negative estrogen feedback. One of our studies aimed to characterize the neuroanatomy of the KP system of two carnivore species, the dog and the cat. Studies of MBH sections from OVX animals, established that the KP and NKB cell populations overlapped substantially. Dyn was detected in large subsets of canine KP (56%) and NKB (37%) cells and feline KP (64%) and NKB (57%) cells; triple-labeled (KNDy) somata formed ~25% of all immunolabeled neurons. SP was present in subsets of KP and NKB neurons in OVX cats but not in OVX dogs, although KP and NKB neurons of a gonadally intact male dog also synthesized SP. Only in cats, CART was also colocalized with KP (23%) and NKB (7%). In contrast with reports from mice, KP neurons did not express Gal in either carnivore. Finally, KP neurons innervated virtually all GnRH neurons in both species. Although the role of MBH KP neurons in pulse generation is conserved in mammals, results of our studies indicate that peptidergic signaling mechanisms used by these cells exhibit surprising species differences.

We identified the KP neurons in the paraventricular nucleus of the human rostral hypothalamus. These cells seem to be homologous with the rodent KP neurons in the RP3V which account for the regulation of positive estrogen feedback. Cell numbers here decreased after menopause, indicating that estrogens positively regulate KP gene expression in the human rostral hypothalamus, as reported in other species. Young adult women and men had similar cell numbers, as opposed to rodents having more KP neurons in the RP3V of females. Human KP neurons differed neurochemically from the homologous rodent cells in that they were devoid of Enk, Gal and TH. Further, they did not contain known KP neuron markers of the human infundibular nucleus, NKB, SP and CART but received afferent input from these KP neurons. Detection of KP neurons in the human rostral hypothalamus challenges the long-held view that positive feedback in the human is restricted to the MBH.

# 7. REFERENCES


1. Knobil E. (1980) The neuroendocrine control of the menstrual cycle. Recent Prog Horm Res, 36: 53-88.
2. Herbison AE. (1998) Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocr Rev, 19: 302-330.
3. Acevedo-Rodriguez A, Kauffman AS, Cherrington BD, Borges CS, Roepke TA, Laconi M. (2018) Emerging insights into hypothalamic-pituitary-gonadal axis regulation and interaction with stress signalling. J Neuroendocrinol, 30: e12590.
4. Herbison AE. (2020) A simple model of estrous cycle negative and positive feedback regulation of GnRH secretion. Front Neuroendocrinol, 57: 100837.
5. Schwanzel-Fukuda M, Pfaff DW. (1989) Origin of luteinizing hormone-releasing hormone neurons. Nature, 338: 161-164.
6. Wray S, Grant P, Gainer H. (1989) Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. Proc Natl Acad Sci U S A, 86: 8132-8136.
7. Quanbeck C, Sherwood NM, Millar RP, Terasawa E. (1997) Two populations of luteinizing hormone-releasing hormone neurons in the forebrain of the rhesus macaque during embryonic development. J Comp Neurol, 380: 293-309.
8. Casoni F, Malone SA, Belle M, Luzzati F, Collier F, Allet C, Hrabovszky E, Rasika S, Prevot V, Chedotal A, Giacobini P. (2016) Development of the neurons controlling fertility in humans: new insights from 3D imaging and transparent fetal brains. Development, 143: 3969-3981.
9. Messina A, Ferraris N, Wray S, Cagnoni G, Donohue DE, Casoni F, Kramer PR, Derijck AA, Adolfs Y, Fasolo A, Pasterkamp RJ, Giacobini P. (2011) Dysregulation of Semaphorin7A/beta1-integrin signaling leads to defective GnRH-1 cell migration, abnormal gonadal development and altered fertility. Hum Mol Genet, 20: 4759-4774.
10. Parkash J, Cimino I, Ferraris N, Casoni F, Wray S, Cappy H, Prevot V, Giacobini P. (2012) Suppression of beta1-integrin in gonadotropin-releasing hormone cells disrupts migration and axonal extension resulting in severe reproductive alterations. J Neurosci, 32: 16992-17002.

11. Rance NE, Young WS, 3rd, McMullen NT. (1994) Topography of neurons expressing luteinizing hormone-releasing hormone gene transcripts in the human hypothalamus and basal forebrain. J Comp Neurol, 339: 573-586.
12. Schwanzel-Fukuda M, Bick D, Pfaff DW. (1989) Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. Brain Res Mol Brain Res, 6: 311-326.
13. Kim SH. (2015) Congenital Hypogonadotropic Hypogonadism and Kallmann Syndrome: Past, Present, and Future. Endocrinol Metab (Seoul), 30: 456-466.
14. Skrapits K, Hrabovszky E. The anatomy of the GnRH neuron network in the human, in The GnRH neuron and its control. Herbison AE, Plant TM, Editors. Wiley Online Library, 2018: 149-175.
15. Setalo G, Vigh S, Schally AV, Arimura A, Flerko B. (1976) Immunohistological study of the origin of LH-RH-containing nerve fibers of the rat hypothalamus. Brain Res, 103: 597-602.
16. Merchenthaler I, Lengvari I, Horvath J, Setalo G. (1980) Immunohistochemical study of the LHRH-synthesizing neuron system of aged female rats. Cell Tissue Res, 209: 499-503.
17. Lehman MN, Robinson JE, Karsch FJ, Silverman AJ. (1986) Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) pathways in the sheep brain during anestrus and the mid-luteal phase of the estrous cycle. J Comp Neurol, 244: 19-35.
18. Barry J. (1976) Characterization and topography of LH-RH neurons in the human brain. Neurosci Lett, 3: 287-291.
19. Silverman AJ, Antunes JL, Abrams GM, Nilaver G, Thau R, Robinson JA, Ferin M, Krey LC. (1982) The luteinizing hormone-releasing hormone pathways in rhesus (Macaca mulatta) and pigtailed (Macaca nemestrina) monkeys: new observations on thick, unembedded sections. J Comp Neurol, 211: 309-317.
20. King JC, Anthony EL. (1984) LHRH neurons and their projections in humans and other mammals: species comparisons. Peptides, 5 Suppl 1: 195-207.
21. Low MJ. Neuroendocrinology, in Williams textbook of endocrinology. Melmed S, Polonsky K, Larsen PR, Editors. Elsevier, 2016: 110-175.

22. Herbison AE. (2018) The Gonadotropin-Releasing Hormone Pulse Generator. Endocrinology, 159: 3723-3736.
23. Herde MK, Iremonger KJ, Constantin S, Herbison AE. (2013) GnRH neurons elaborate a long-range projection with shared axonal and dendritic functions. J Neurosci, 33: 12689-12697.
24. Herbison AE. Physiology of the adult gonadotropin-releasing hormone neuronal network, in Knobil and Neill's physiology of reproduction. Plant TM, Zeleznik AJ, Editors. Academic Press, London, 2015: 399-443.
25. Martinez de la Escalera G, Choi AL, Weiner RI. (1992) Generation and synchronization of gonadotropin-releasing hormone (GnRH) pulses: intrinsic properties of the GT1-1 GnRH neuronal cell line. Proc Natl Acad Sci U S A, 89: 1852-1855.
26. Terasawa E, Schanhofer WK, Keen KL, Luchansky L. (1999) Intracellular Ca(2+) oscillations in luteinizing hormone-releasing hormone neurons derived from the embryonic olfactory placode of the rhesus monkey. J Neurosci, 19: 5898-5909.
27. Campbell RE, Ducret E, Porteous R, Liu X, Herde MK, Wellerhaus K, Sonntag S, Willecke K, Herbison AE. (2011) Gap junctions between neuronal inputs but not gonadotropin-releasing hormone neurons control estrous cycles in the mouse. Endocrinology, 152: 2290-2301.
28. Constantin S, Jasoni C, Romano N, Lee K, Herbison AE. (2012) Understanding calcium homeostasis in postnatal gonadotropin-releasing hormone neurons using cell-specific Pericam transgenics. Cell Calcium, 51: 267-276.
29. Moore AM, Coolen LM, Porter DT, Goodman RL, Lehman MN. (2018) KNDy Cells Revisited. Endocrinology, 159: 3219-3234.
30. Halasz B, Gorski RA. (1967) Gonadotrophic hormone secretion in female rats after partial or total interruption of neural afferents to the medial basal hypothalamus. Endocrinology, 80: 608-622.
31. Blake CA, Norman RL, Sawyer CH. (1974) Localization of inhibitory actions of estrogen and nicotine on release of luteinizing hormone in rats. Neuroendocrinology, 16: 22-35.

32. Soper BD, Weick RF. (1980) Hypothalamic and extrahypothalamic mediation of pulsatile discharges of luteinizing hormone in the ovariectomized rat. Endocrinology, 106: 348-355.
33. Thiery JC, Pelletier J. (1981) Multiunit activity in the anterior median eminence and adjacent areas of the hypothalamus of the ewe in relation to LH secretion. Neuroendocrinology, 32: 217-224.
34. Kawakami M, Uemura T, Hayashi R. (1982) Electrophysiological correlates of pulsatile gonadotropin release in rats. Neuroendocrinology, 35: 63-67.
35. Knight PG, Wilson SC, Gladwell RT, Cunningham FJ. (1984) Hypothalamic contents of LHRH and catecholamines during the ovulatory cycle of the hen (Gallus domesticus). J Reprod Fertil, 71: 289-295.
36. Mori Y, Nishihara M, Tanaka T, Shimizu T, Yamaguchi M, Takeuchi Y, Hoshino K. (1991) Chronic recording of electrophysiological manifestation of the hypothalamic gonadotropin-releasing hormone pulse generator activity in the goat. Neuroendocrinology, 53: 392-395.
37. Kimura F, Nishihara M, Hiruma H, Funabashi T. (1991) Naloxone increases the frequency of the electrical activity of luteinizing hormone-releasing hormone pulse generator in long-term ovariectomized rats. Neuroendocrinology, 53: 97-102.
38. O'Byrne KT, Knobil E. (1993) Electrophysiological approaches to gonadotrophin releasing hormone pulse generator activity in the rhesus monkey. Hum Reprod, 8 Suppl 2: 37-40.
39. Sarkar DK, Chiappa SA, Fink G, Sherwood NM. (1976) Gonadotropin-releasing hormone surge in pro-oestrous rats. Nature, 264: 461-463.
40. Clarke IJ, Cummins JT. (1982) The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinology, 111: 1737-1739.
41. Caraty A, Locatelli A, Martin GB. (1989) Biphasic response in the secretion of gonadotrophin-releasing hormone in ovariectomized ewes injected with oestradiol. J Endocrinol, 123: 375-382.
42. Xia L, Van Vugt D, Alston EJ, Luckhaus J, Ferin M. (1992) A surge of gonadotropin-releasing hormone accompanies the estradiol-induced gonadotropin surge in the rhesus monkey. Endocrinology, 131: 2812-2820.

43. Moenter SM, Brand RC, Karsch FJ. (1992) Dynamics of gonadotropin-releasing hormone (GnRH) secretion during the GnRH surge: insights into the mechanism of GnRH surge induction. Endocrinology, 130: 2978-2984.
44. Sisk CL, Richardson HN, Chappell PE, Levine JE. (2001) In vivo gonadotropin-releasing hormone secretion in female rats during peripubertal development and on proestrus. Endocrinology, 142: 2929-2936.
45. Green S, Walter P, Greene G, Krust A, Goffin C, Jensen E, Scrace G, Waterfield M, Chambon P. (1986) Cloning of the human oestrogen receptor cDNA. J Steroid Biochem, 24: 77-83.
46. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A, 93: 5925-5930.
47. Gruber HE, Yamaguchi D, Ingram J, Leslie K, Huang W, Miller TA, Hanley EN, Jr. (2002) Expression and localization of estrogen receptor-beta in annulus cells of the human intervertebral disc and the mitogenic effect of 17-beta-estradiol in vitro. BMC Musculoskelet Disord, 3: 4.
48. Radovick S, Levine JE, Wolfe A. (2012) Estrogenic regulation of the GnRH neuron. Front Endocrinol (Lausanne), 3: 52.
49. Genazzani AR, Bernardi F, Pluchino N, Begliuomini S, Lenzi E, Casarosa E, Luisi M. (2005) Endocrinology of menopausal transition and its brain implications. CNS Spectr, 10: 449-457.
50. Chimento A, Sirianni R, Casaburi I, Pezzi V. (2014) Role of estrogen receptors and g protein-coupled estrogen receptor in regulation of hypothalamus-pituitary-testis axis and spermatogenesis. Front Endocrinol (Lausanne), 5: 1.
51. Barraclough CA, Gorski RA. (1961) Evidence that the hypothalamus is responsible for androgen-induced sterility in the female rat. Endocrinology, 68: 68-79.
52. Clarkson J, Herbison AE. (2006) Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology, 147: 5817-5825.
53. Doughty C, Booth JE, McDonald PG, Parrott RF. (1975) Inhibition, by the anti-oestrogen MER-25, of defeminization induced by the synthetic oestrogen RU 2858. J Endocrinol, 67: 459-460.

54. Vreeburg JT, van der Vaart PD, van der Schoot P. (1977) Prevention of central defeminization but not masculinization in male rats by inhibition neonatally of oestrogen biosynthesis. J Endocrinol, 74: 375-382.
55. Ottowitz WE, Dougherty DD, Fischman AJ, Hall JE. (2008) [18F]2-fluoro-2-deoxy-D-glucose positron emission tomography demonstration of estrogen negative and positive feedback on luteinizing hormone secretion in women. J Clin Endocrinol Metab, 93: 3208-3214.
56. Dubois SL, Acosta-Martinez M, DeJoseph MR, Wolfe A, Radovick S, Boehm U, Urban JH, Levine JE. (2015) Positive, but not negative feedback actions of estradiol in adult female mice require estrogen receptor alpha in kisspeptin neurons. Endocrinology, 156: 1111-1120.
57. Morrison JH, Brinton RD, Schmidt PJ, Gore AC. (2006) Estrogen, menopause, and the aging brain: how basic neuroscience can inform hormone therapy in women. J Neurosci, 26: 10332-10348.
58. Shivers BD, Harlan RE, Morrell JI, Pfaff DW. (1983) Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature, 304: 345-347.
59. Herbison AE, Theodosis DT. (1992) Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. Neuroscience, 50: 283-298.
60. Watson RE, Jr., Langub MC, Jr., Landis JW. (1992) Further Evidence that Most Luteinizing Hormone-Releasing Hormone Neurons are not Directly Estrogen-Responsive: Simultaneous Localization of Luteinizing Hormone-Releasing Hormone and Estrogen Receptor Immunoreactivity in the Guinea-Pig Brain. J Neuroendocrinol, 4: 311-317.
61. Herbison AE, Robinson JE, Skinner DC. (1993) Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone. Neuroendocrinology, 57: 751-759.
62. Lehman MN, Karsch FJ. (1993) Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and beta-endorphin-immunoreactive neurons contain estrogen

receptors? A double-label immunocytochemical study in the Suffolk ewe. Endocrinology, 133: 887-895.

63. Herbison AE, Horvath TL, Naftolin F, Leranth C. (1995) Distribution of estrogen receptor-immunoreactive cells in monkey hypothalamus: relationship to neurones containing luteinizing hormone-releasing hormone and tyrosine hydroxylase. Neuroendocrinology, 61: 1-10.
64. Sullivan KA, Witkin JW, Ferin M, Silverman AJ. (1995) Gonadotropin-releasing hormone neurons in the rhesus macaque are not immunoreactive for the estrogen receptor. Brain Res, 685: 198-200.
65. Hrabovszky E, Shughrue PJ, Merchenthaler I, Hajszan T, Carpenter CD, Liposits Z, Petersen SL. (2000) Detection of estrogen receptor-beta messenger ribonucleic acid and 125I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology, 141: 3506-3509.
66. Hrabovszky E, Steinhauser A, Barabas K, Shughrue PJ, Petersen SL, Merchenthaler I, Liposits Z. (2001) Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology, 142: 3261-3264.
67. Kallo I, Butler JA, Barkovics-Kallo M, Goubillon ML, Coen CW. (2001) Oestrogen receptor beta-immunoreactivity in gonadotropin releasing hormone-expressing neurones: regulation by oestrogen. J Neuroendocrinol, 13: 741-748.
68. Skinner DC, Dufourny L. (2005) Oestrogen receptor beta-immunoreactive neurones in the ovine hypothalamus: distribution and colocalisation with gonadotropin-releasing hormone. J Neuroendocrinol, 17: 29-39.
69. Hrabovszky E, Kallo I, Szlavik N, Keller E, Merchenthaler I, Liposits Z. (2007) Gonadotropin-releasing hormone neurons express estrogen receptor-beta. J Clin Endocrinol Metab, 92: 2827-2830.
70. Cheong RY, Porteous R, Chambon P, Abraham I, Herbison AE. (2014) Effects of neuron-specific estrogen receptor (ER) alpha and ERbeta deletion on the acute estrogen negative feedback mechanism in adult female mice. Endocrinology, 155: 1418-1427.
71. Hamilton KJ, Hewitt SC, Arao Y, Korach KS. (2017) Estrogen Hormone Biology. Curr Top Dev Biol, 125: 109-146.

72. Petersen SL, Ottem EN, Carpenter CD. (2003) Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biol Reprod, 69: 1771-1778.
73. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G, Parmentier M. (2001) The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem, 276: 34631-34636.
74. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M. (2001) Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature, 411: 613-617.
75. Tena-Sempere M. (2006) KiSS-1 and reproduction: focus on its role in the metabolic regulation of fertility. Neuroendocrinology, 83: 275-281.
76. de Roux N. (2006) GnRH receptor and GPR54 inactivation in isolated gonadotropic deficiency. Best Pract Res Clin Endocrinol Metab, 20: 515-528.
77. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, Welch DR. (1996) KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst, 88: 1731-1737.
78. Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL. (2003) The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun, 312: 1357-1363.
79. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH. (2003) The GPR54 gene as a regulator of puberty. N Engl J Med, 349: 1614-1627.
80. Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-Nguyen AT, George SR, O'Dowd BF. (1999) Discovery of a receptor related to the galanin receptors. FEBS Lett, 446: 103-107.

81. Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekeres PG, Sarau HM, Chambers JK, Murdock P, Steplewski K, Shabon U, Miller JE, Middleton SE, Darker JG, Larminie CG, Wilson S, Bergsma DJ, Emson P, Faull R, Philpott KL, Harrison DC. (2001) AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem, 276: 28969-28975.
82. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A, 100: 10972-10976.
83. Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, Le WW, Hoffman GE, Seminara SB. (2007) Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. Endocrinology, 148: 4927-4936.
84. d'Anglemont de Tassigny X, Fagg LA, Carlton MB, Colledge WH. (2008) Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology, 149: 3926-3932.
85. Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, Gurbuz F, Temiz F, Millar RP, Yuksel B. (2012) Inactivating KISS1 mutation and hypogonadotropic hypogonadism. N Engl J Med, 366: 629-635.
86. Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC. (2008) A GPR54-activating mutation in a patient with central precocious puberty. N Engl J Med, 358: 709-715.
87. Silveira LG, Noel SD, Silveira-Neto AP, Abreu AP, Brito VN, Santos MG, Bianco SD, Kuohung W, Xu S, Gryngarten M, Escobar ME, Arnhold IJ, Mendonca BB, Kaiser UB, Latronico AC. (2010) Mutations of the KISS1 gene in disorders of puberty. J Clin Endocrinol Metab, 95: 2276-2280.
88. Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. (2004) A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology, 145: 4073-4077.
89. Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR. (2005) Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab, 90: 6609-6615.

90. Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA. (2005) Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc Natl Acad Sci U S A, 102: 1761-1766.
91. Plant TM, Ramaswamy S, Dipietro MJ. (2006) Repetitive activation of hypothalamic G protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (Macaca mulatta) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology, 147: 1007-1013.
92. Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA. (2004) Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology, 80: 264-272.
93. Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE. (2005) Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci, 25: 11349-11356.
94. Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T. (2004) Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochem Biophys Res Commun, 320: 383-388.
95. Dumalska I, Wu M, Morozova E, Liu R, van den Pol A, Alreja M. (2008) Excitatory effects of the puberty-initiating peptide kisspeptin and group I metabotropic glutamate receptor agonists differentiate two distinct subpopulations of gonadotropin-releasing hormone neurons. J Neurosci, 28: 8003-8013.
96. Pielecka-Fortuna J, Chu Z, Moenter SM. (2008) Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. Endocrinology, 149: 1979-1986.
97. Kirilov M, Clarkson J, Liu X, Roa J, Campos P, Porteous R, Schutz G, Herbison AE. (2013) Dependence of fertility on kisspeptin-Gpr54 signaling at the GnRH neuron. Nat Commun, 4: 2492.
98. Lehman MN, Merkley CM, Coolen LM, Goodman RL. (2010) Anatomy of the kisspeptin neural network in mammals. Brain Res, 1364: 90-102.

99. Navarro VM, Gottsch ML, Wu M, Garcia-Galiano D, Hobbs SJ, Bosch MA, Pinilla L, Clifton DK, Dearth A, Ronnekleiv OK, Braun RE, Palmiter RD, Tena-Sempere M, Alreja M, Steiner RA. (2011) Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. Endocrinology, 152: 4265-4275.
100. Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ. (2007) Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology, 148: 5752-5760.
101. Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H. (2010) Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. J Neurosci, 30: 3124-3132.
102. Rometo AM, Krajewski SJ, Voytko ML, Rance NE. (2007) Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab, 92: 2744-2750.
103. Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM. (2008) Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (Macaca mulatta) as revealed by double immunofluorescence and confocal microscopy. Endocrinology, 149: 4387-4395.
104. Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Kallo I. (2010) The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. Eur J Neurosci, 31: 1984-1998.
105. Lehman MN, Coolen LM, Goodman RL. (2010) Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. Endocrinology, 151: 3479-3489.
106. Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, Lai J, Ciofi P, McMullen NT, Rance NE. (2012) Arcuate kisspeptin/neurokinin B/dynorphin (KNDy)

neurons mediate the estrogen suppression of gonadotropin secretion and body weight. Endocrinology, 153: 2800-2812.

107. Clarkson J, Han SY, Piet R, McLennan T, Kane GM, Ng J, Porteous RW, Kim JS, Colledge WH, Iremonger KJ, Herbison AE. (2017) Definition of the hypothalamic GnRH pulse generator in mice. Proc Natl Acad Sci U S A, 114: E10216-E10223.
108. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. (2009) Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci, 29: 11859-11866.
109. Ruka KA, Burger LL, Moenter SM. (2013) Regulation of arcuate neurons coexpressing kisspeptin, neurokinin B, and dynorphin by modulators of neurokinin 3 and kappa-opioid receptors in adult male mice. Endocrinology, 154: 2761-2771.
110. de Croft S, Boehm U, Herbison AE. (2013) Neurokinin B activates arcuate kisspeptin neurons through multiple tachykinin receptors in the male mouse. Endocrinology, 154: 2750-2760.
111. Narayanaswamy S, Prague JK, Jayasena CN, Papadopoulou DA, Mizamtsidi M, Shah AJ, Bassett P, Comninos AN, Abbara A, Bloom SR, Veldhuis JD, Dhillo WS. (2016) Investigating the KNDy Hypothesis in Humans by Coadministration of Kisspeptin, Neurokinin B, and Naltrexone in Men. J Clin Endocrinol Metab, 101: 3429-3436.
112. Hrabovszky E, Sipos MT, Molnar CS, Ciofi P, Borsay BA, Gergely P, Herczeg L, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z. (2012) Low Degree of Overlap Between Kisspeptin, Neurokinin B, and Dynorphin Immunoreactivities in the Infundibular Nucleus of Young Male Human Subjects Challenges the KNDy Neuron Concept. Endocrinology, 153: 4978-4989.
113. Skrapits K, Borsay BA, Herczeg L, Ciofi P, Liposits Z, Hrabovszky E. (2015) Neuropeptide co-expression in hypothalamic kisspeptin neurons of laboratory animals and the human. Front Neurosci, 9: 29.
114. Hrabovszky E, Takacs S, Gocz B, Skrapits K. (2019) New Perspectives for Anatomical and Molecular Studies of Kisspeptin Neurons in the Aging Human Brain. Neuroendocrinology, 109: 230-241.

115. Hrabovszky E, Borsay BA, Racz K, Herczeg L, Ciofi P, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z. (2013) Substance P immunoreactivity exhibits frequent colocalization with kisspeptin and neurokinin B in the human infundibular region. PLoS One, 8: e72369.
116. Kalil B, Ramaswamy S, Plant TM. (2016) The Distribution of Substance P and Kisspeptin in the Mediobasal Hypothalamus of the Male Rhesus Monkey and a Comparison of Intravenous Administration of These Peptides to Release GnRH as Reflected by LH Secretion. Neuroendocrinology, 103: 711-723.
117. True C, Takahashi D, Kirigiti M, Lindsley SR, Moctezuma C, Arik A, Smith MS, Kievit P, Grove KL. (2017) Arcuate nucleus neuropeptide coexpression and connections to gonadotrophin-releasing hormone neurones in the female rhesus macaque. J Neuroendocrinol, 29.
118. Skrapits K, Borsay BA, Herczeg L, Ciofi P, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Hrabovszky E. (2014) Colocalization of cocaine- and amphetamine-regulated transcript with kisspeptin and neurokinin B in the human infundibular region. PLoS One, 9: e103977.
119. Hrabovszky E, Takacs S, Rumpler E, Skrapits K. The human hypothalamic kisspeptin system. Functional neuroanatomy and clinical perspectives, in The human hypothalamus. Swaab, 2020.
120. Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K. (2007) Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev, 53: 367-378.
121. Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M. (2007) Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology, 148: 1774-1783.
122. Porteous R, Petersen SL, Yeo SH, Bhattarai JP, Ciofi P, de Tassigny XD, Colledge WH, Caraty A, Herbison AE. (2011) Kisspeptin neurons co-express met-enkephalin and galanin in the rostral periventricular region of the female mouse hypothalamus. J Comp Neurol, 519: 3456-3469.

123. Kallo I, Mohacsik P, Vida B, Zeold A, Bardoczi Z, Zavacki AM, Farkas E, Kadar A, Hrabovszky E, Arrojo EDR, Dong L, Barna L, Palkovits M, Borsay BA, Herczeg L, Lechan RM, Bianco AC, Liposits Z, Fekete C, Gereben B. (2012) A novel pathway regulates thyroid hormone availability in rat and human hypothalamic neurosecretory neurons. PLoS One, 7: e37860.
124. Clarkson J, Herbison AE. (2011) Dual phenotype kisspeptin-dopamine neurones of the rostral periventricular area of the third ventricle project to gonadotrophin-releasing hormone neurones. J Neuroendocrinol, 23: 293-301.
125. Cravo RM, Margatho LO, Osborne-Lawrence S, Donato J, Jr., Atkin S, Bookout AL, Rovinsky S, Frazao R, Lee CE, Gautron L, Zigman JM, Elias CF. (2011) Characterization of Kiss1 neurons using transgenic mouse models. Neuroscience, 173: 37-56.
126. Norman RL, Resko JA, Spies HG. (1976) The anterior hypothalamus: how it affects gonadotropin secretion in the rhesus monkey. Endocrinology, 99: 59-71.
127. Cogen P, Antunes JL, Louis KM, Dyrenfurth I, Ferin M. (1980) The effects of anterior hypothalamic disconnection on gonadotropin secretion in the female rhesus monkey. Endocrinology, 107: 677-683.
128. Krey LC, Butler WR, Knobil E. (1975) Surgical disconnection of the medial basal hypothalamus and pituitary function in the rhesus monkey. I. Gonadotropin secretion. Endocrinology, 96: 1073-1087.
129. Plant TM, Moossy J, Hess DL, Nakai Y, McCormack JT, Knobil E. (1979) Further studies on the effects of lesions in the rostral hypothalamus on gonadotropin secretion in the female rhesus monkey (Macaca mulatta). Endocrinology, 105: 465-473.
130. Estrada KM, Clay CM, Pompolo S, Smith JT, Clarke IJ. (2006) Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J Neuroendocrinol, 18: 806-809.
131. Smith JT, Li Q, Pereira A, Clarke IJ. (2009) Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. Endocrinology, 150: 5530-5538.

132. Merkley CM, Porter KL, Coolen LM, Hileman SM, Billings HJ, Drews S, Goodman RL, Lehman MN. (2012) KNDy (kisspeptin/neurokinin B/dynorphin) neurons are activated during both pulsatile and surge secretion of LH in the ewe. Endocrinology, 153: 5406-5414.
133. Watanabe Y, Uenoyama Y, Suzuki J, Takase K, Suetomi Y, Ohkura S, Inoue N, Maeda KI, Tsukamura H. (2014) Oestrogen-induced activation of preoptic kisspeptin neurones may be involved in the luteinising hormone surge in male and female Japanese monkeys. J Neuroendocrinol, 26: 909-917.
134. Vargas Trujillo M, Kalil B, Ramaswamy S, Plant TM. (2017) Estradiol Upregulates Kisspeptin Expression in the Preoptic Area of both the Male and Female Rhesus Monkey (Macaca mulatta): Implications for the Hypothalamic Control of Ovulation in Highly Evolved Primates. Neuroendocrinology, 105: 77-89.
135. Hrabovszky E. (2014) Neuroanatomy of the human hypothalamic kisspeptin system. Neuroendocrinology, 99: 33-48.
136. Mikula S, Trotts I, Stone JM, Jones EG. (2007) Internet-enabled high-resolution brain mapping and virtual microscopy. Neuroimage, 35: 9-15.
137. Dudas B, Mihaly A, Merchenthaler I. (2000) Topography and associations of luteinizing hormone-releasing hormone and neuropeptide Y-immunoreactive neuronal systems in the human diencephalon. J Comp Neurol, 427: 593-603.
138. Hrabovszky E, Liposits Z. (2013) Afferent Neuronal Control of Type-I Gonadotropin Releasing Hormone Neurons in the Human. Front Endocrinol (Lausanne), 4: 130.
139. Moenter SM, Karsch FJ, Lehman MN. (1993) Fos expression during the estradiol-induced gonadotropin-releasing hormone (GnRH) surge of the ewe: induction in GnRH and other neurons. Endocrinology, 133: 896-903.
140. Boukhliq R, Goodman RL, Berriman SJ, Adrian B, Lehman MN. (1999) A subset of gonadotropin-releasing hormone neurons in the ovine medial basal hypothalamus is activated during increased pulsatile luteinizing hormone secretion. Endocrinology, 140: 5929-5936.
141. Barry J, Dubois MP. (1975) Immunofluorescence study of LRF-producing neurons in the cat and the dog. Neuroendocrinology, 18: 290-298.

142. Mikami S, Ichino K, Yamada S, Taniguchi K. (1985) Immunocytochemical localization of neuropeptides in the ventral hypothalamus of the cat. Nihon Juigaku Zasshi, 47: 931-942.
143. Belda M, Covenas R, Narvaez JA, Aguirre JA, Tramu G. (2000) Distribution of luteinizing hormone-releasing hormone in the upper brainstem and diencephalon of the cat: an immunocytochemical study. Brain Res Bull, 51: 281-291.
144. Stephens SBZ, Kauffman AS. (2017) Regulation and Possible Functions of Kisspeptin in the Medial Amygdala. Front Endocrinol (Lausanne), 8: 191.
145. Herbison AE. (2008) Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). Brain Res Rev, 57: 277-287.
146. Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA. (2005) Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology, 146: 2976-2984.
147. Amelkina O, Tanyapanyachon P, Thongphakdee A, Chatdarong K. (2019) Identification of feline Kiss1 and distribution of immunoreactive kisspeptin in the hypothalamus of the domestic cat. J Reprod Dev, 65: 335-343.
148. Bakker J, Baum MJ. (2000) Neuroendocrine regulation of GnRH release in induced ovulators. Front Neuroendocrinol, 21: 220-262.
149. Gudermuth DF, Newton L, Daels P, Concannon P. (1997) Incidence of spontaneous ovulation in young, group-housed cats based on serum and faecal concentrations of progesterone. J Reprod Fertil Suppl, 51: 177-184.
150. Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K. (2005) Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology, 146: 4431-4436.
151. Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, Bateman K, Goodman RL, Tilbrook AJ, Ubuka T, Bentley GE, Clarke IJ, Lehman MN. (2008) Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology, 149: 5770-5782.

152. Kallo I, Vida B, Deli L, Molnar CS, Hrabovszky E, Caraty A, Ciofi P, Coen CW, Liposits Z. (2012) Co-localisation of kisspeptin with galanin or neurokinin B in afferents to mouse GnRH neurones. J Neuroendocrinol, 24: 464-476.
153. Hrabovszky E, Molnar CS, Sipos MT, Vida B, Ciofi P, Borsay BA, Sarkadi L, Herczeg L, Bloom SR, Ghatei MA, Dhillo WS, Kallo I, Liposits Z. (2011) Sexual dimorphism of kisspeptin and neurokinin B immunoreactive neurons in the infundibular nucleus of aged men and women. Front Endocrinol (Lausanne), 2: 80.
154. Molnar CS, Vida B, Sipos MT, Ciofi P, Borsay BA, Racz K, Herczeg L, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Hrabovszky E. (2012) Morphological evidence for enhanced kisspeptin and neurokinin B signaling in the infundibular nucleus of the aging man. Endocrinology, 153: 5428-5439.
155. Krajewski SJ, Burke MC, Anderson MJ, McMullen NT, Rance NE. (2010) Forebrain projections of arcuate neurokinin B neurons demonstrated by anterograde tract-tracing and monosodium glutamate lesions in the rat. Neuroscience, 166: 680-697.
156. Matsuyama S, Ohkura S, Mogi K, Wakabayashi Y, Mori Y, Tsukamura H, Maeda K, Ichikawa M, Okamura H. (2011) Morphological evidence for direct interaction between kisspeptin and gonadotropin-releasing hormone neurons at the median eminence of the male goat: an immunoelectron microscopic study. Neuroendocrinology, 94: 323-332.
157. Borsay BA, Skrapits K, Herczeg L, Ciofi P, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Hrabovszky E. (2014) Hypophysiotropic Gonadotropin-Releasing Hormone Projections are Exposed to Dense Plexuses of Kisspeptin, Neurokinin B and Substance P Immunoreactive Fibers in the Human: A Study on Tissues from Postmenopausal Women. Neuroendocrinology.
158. Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. (2005) Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology, 146: 156-163.

159. Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. (2005) Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci U S A, 102: 2129-2134.
160. Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E. (2008) An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. Endocrinology, 149: 4151-4157.
161. True C, Verma S, Grove KL, Smith MS. (2013) Cocaine- and amphetamine-regulated transcript is a potent stimulator of GnRH and kisspeptin cells and may contribute to negative energy balance-induced reproductive inhibition in females. Endocrinology, 154: 2821-2832.
162. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. (2005) Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology, 146: 3686-3692.
163. Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. (2009) Distribution of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol, 21: 673-682.
164. Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A. (2006) Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. Neurosci Lett, 401: 225-230.
165. Tomikawa J, Homma T, Tajima S, Shibata T, Inamoto Y, Takase K, Inoue N, Ohkura S, Uenoyama Y, Maeda K, Tsukamura H. (2010) Molecular characterization and estrogen regulation of hypothalamic KISS1 gene in the pig. Biol Reprod, 82: 313-319.
166. Smith JT, Shahab M, Pereira A, Pau KY, Clarke IJ. (2010) Hypothalamic expression of KISS1 and gonadotropin inhibitory hormone genes during the menstrual cycle of a non-human primate. Biol Reprod, 83: 568-577.
167. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. (2008) Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. J Neurosci, 28: 8691-8697.

168. Zhou S, Holmes MM, Forger NG, Goldman BD, Lovern MB, Caraty A, Kallo I, Faulkes CG, Coen CW. (2013) Socially regulated reproductive development: analysis of GnRH-1 and kisspeptin neuronal systems in cooperatively breeding naked mole-rats (Heterocephalus glaber). J Comp Neurol, 521: 3003-3029.
169. Smith JT, Clay CM, Caraty A, Clarke IJ. (2007) KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology, 148: 1150-1157.
170. Karsch FJ, Dierschke DJ, Knobil E. (1973) Sexual differentiation of pituitary function: apparent difference bewteen primates and rodents. Science, 179: 484-486.
171. Pau KF, Kuehl DE, Jackson GL. (1982) Effect of frontal hypothalamic deafferentation on luteinizing hormone secretion and seasonal breeding in the ewe. Biol Reprod, 27: 999-1009.
172. Mai J, Assheuer J, Paxinos G, eds. *Atlas of the human brain*. 1997, Academic Press: San Diego.
173. Smith MS, True C, Grove KL. (2010) The neuroendocrine basis of lactation-induced suppression of GnRH: role of kisspeptin and leptin. Brain Res, 1364: 139-152.
174. Kulin HE, Reiter EO. (1976) Gonadotropin and testosterone measurements after estrogen administration to adult men, prepubertal and pubertal boys, and men with hypogonadotropism: evidence for maturation of positive feedback in the male. Pediatr Res, 10: 46-51.
175. Barbarino A, De Marinis L. (1980) Estrogen induction of luteinizing hormone release in castrated adult human males. J Clin Endocrinol Metab, 51: 280-286.
176. Stearns EL, Winter JS, Faiman C. (1973) Positive feedback effect of progestin upon serum gonadotropins in estrogen-primed castrate men. J Clin Endocrinol Metab, 37: 635-638.
177. Yeo SH, Herbison AE. (2011) Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain. Endocrinology, 152: 2387-2399.
178. Qiu J, Nestor CC, Zhang C, Padilla SL, Palmiter RD, Kelly MJ, Ronnekleiv OK. (2016) High-frequency stimulation-induced peptide release synchronizes arcuate kisspeptin neurons and excites GnRH neurons. Elife, 5.

# 8. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

## Publications related to the PhD thesis

**Journal articles:**

- **Rumpler E**, Skrapits K, Takacs S, Gocz B, Trinh SH, Racz G, Matolcsy A, Kozma Z, Ciofi P, Dhillo WS, Hrabovszky E. (2020) Characterization of kisspeptin neurons in the human rostral hypothalamus. Neuroendocrinology (DOI: 10.1159/000507891; **IF: 4.271**)
- **Rumpler E**, Takacs S, Gocz B, Baska F, Szenci O, Horvath A, Ciofi P, Hrabovszky E, Skrapits K. (2020) Kisspeptin neurons in the infundibular nucleus of ovariectomized cats and dogs exhibit unique anatomical and neurochemical characteristics. Front Neurosci, 14: 598707. (**IF: 3.707**)

## Publications not related to the PhD thesis

**Journal articles:**

- Bodo K, Boros A, **Rumpler E**, Molnar L, Borocz K, Nemeth P, Engelmann P. (2019) Identification of novel lumbricin homologues in Eisenia andrei earthworms. Dev Comp Immunol, 90: 41-46. (**IF: 3.192**)

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