# Novel mechanisms of peptidergic signaling in reproductive regulation

Ph.D. Thesis

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### **TABLE OF CONTENTS**

1. LIST OF ABBREVIATIONS	5
2. INTRODUCTION	7
2.1. Central regulation of the gonadal axis	7
2.2. Peptidergic systems and mechanisms acting upstream from the GnRH neuronal systems	9
2.3. Role of kisspeptin, neurokinin B, RF-amide related peptides in sex steroid feedback	11
2.4. Sex differences in steroid feedback mechanisms	13
2.5. Aging related alterations in the central regulation of the reproductive axis	14
3. SPECIFIC AIMS	16
4. MATERIALS AND METHODS	17
4.1. Animals	17
4.1.1. Surgery (Gonadectomy and hormone replacement of female mice)	17
4.1.2. Transcardiac perfusion	17
4.2. Human tissues	18
4.2.1. Collection of human hypothalamic tissues	18
4.3. Section preparation and pretreatments for immunohistochemistry and <i>in s</i>	itu
4.3.1 Preparation of hypothalamic sections for immunohistochemistry in studies	18
using mouse tissues	18
4.3.2. Preparation of frozen tissue sections for in situ hybridization experiments of	on
mouse tissues	19
4.3.3. Preparation of human tissue sections for immunohistochemistry	19
4.4. Immunohistochemical methods	20
4.4.1. Peroxidase-based immunohistochemical single-labeling	20
4.4.2. Silver-gold intensification of the Ni-DAB chromogen	20
4.4.3. Innervation and colocalization studies with the combined use of silver-gold	l-
intensified Ni-DAB and DAB chromogens	21
4.4.4. Dual-immunofluorescent investigations to study the colocalization of KP as	nd
NKB or KP and DYN immunoreactivities in the Inf	21
4.4.5. Triple-immunofluorescent studies to analyze the colocalization of KP and l	VKB
in neuronal afferents to human GnRH neurons	22

4.5. In situ hybridization	23
4.5.1. Preparation of hybridization probes	23
4.5.2. In situ hybridization studies with <sup>35</sup> S-labeled cRNA hybridization probes	24
4.5.3. Autoradiography	24
4.6. Microscopy and data analysis	25
4.6.1. Light- and fluorescent microscopy	25
4.6.2. Confocal laser microscopy	25
4.6.3. Data analysis and statistics	25
4.6.3.1. Quantitative analysis of ISH signal	25
4.6.3.2. Analysis of quantitative immunohistochemical experiments	26
4.6.3.2.1. Perikaryon size (mean immunoreactive profile area)	27
4.6.3.2.2. Regional incidence of cell bodies	27
4.6.3.2.3. Regional density of labeled fibers	27
4.6.3.2.4. Neuropeptide colocalization in cell bodies	28
4.6.3.2.5. Studies of neuropeptides in afferents to GnRH neurons	28
	20
5. <b>KE</b> SUL 15	30
5.1. Estrogenic down-regulation of RF-amide related peptide expression via	
estrogen receptor-α	30
5.1.1. RFRP mRNA levels of OVX mice decrease in response to E2 treatment	30
5.1.2. Nuclear estrogen receptor- $\alpha$ occurs in a small subset of RFRP-synthesizing	
neurons, whereas estrogen receptor-β is absent	32
5.2. Immunohistochemical evidence for the absence of 'KNDy neurons' in youn human males	g 33
5.2.1. Incidence of KP-, NKB- and DYN-immunoreactive perikarya in the Inf	35
5.2.2. Abundance of KP- and NKB-immunoreactive fibers in the Inf	37
5.2.3. Abundance of KP- and NKB-immunoreactive fibers in the InfS	38
5.2.4. Frequency of KP-IR and NKB-IR appositions onto GnRH-IR neurons	41
5.3. Sexual dimorphism of kisspeptin and neurokinin B systems in aged human	
individuala	
murviuuais	42
5.3.1. Perikaryon size of KP-IR and NKB-IR neurons	<b> 42</b> 42
5.3.1. Perikaryon size of KP-IR and NKB-IR neurons 5.3.2. Incidence of KP-IR and NKB-IR cell bodies in the Inf	<b> 42</b> 42 44
5.3.1. Perikaryon size of KP-IR and NKB-IR neurons 5.3.2. Incidence of KP-IR and NKB-IR cell bodies in the Inf 5.3.3. Regional density of KP-IR and NKB-IR fibers	<b> 42</b> 42 44 45
5.3.1. Perikaryon size of KP-IR and NKB-IR neurons 5.3.2. Incidence of KP-IR and NKB-IR cell bodies in the Inf 5.3.3. Regional density of KP-IR and NKB-IR fibers 5.3.4. Frequency of KP-IR and NKB-IR appositions onto GnRH-IR neurons	<b> 42</b> 42 44 45 46
5.3.1. Perikaryon size of KP-IR and NKB-IR neurons 5.3.2. Incidence of KP-IR and NKB-IR cell bodies in the Inf 5.3.3. Regional density of KP-IR and NKB-IR fibers 5.3.4. Frequency of KP-IR and NKB-IR appositions onto GnRH-IR neurons 5.3.5. Colocalization of KP and NKB in neuronal afferents to GnRH neurons	<b> 42</b> 42 44 45 46 49
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li> <li>5.3.2. Incidence of KP-IR and NKB-IR cell bodies in the Inf</li> <li>5.3.3. Regional density of KP-IR and NKB-IR fibers</li> <li>5.3.4. Frequency of KP-IR and NKB-IR appositions onto GnRH-IR neurons</li> <li>5.3.5. Colocalization of KP and NKB in neuronal afferents to GnRH neurons</li> <li>5.4. Aging related changes of the human hypothalamic kisspeptin and neurokin</li> </ul>	<b> 42</b> 42 44 45 46 49 <b>in</b>
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li> <li>5.3.2. Incidence of KP-IR and NKB-IR cell bodies in the Inf</li> <li>5.3.3. Regional density of KP-IR and NKB-IR fibers</li></ul>	42 42 44 45 46 49 in 51
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li></ul>	42 42 44 45 46 49 in 51 51
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li></ul>	42 42 44 45 46 49 in 51 51 53
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li></ul>	42 42 44 45 46 49 in 51 53 53 53
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li></ul>	42 42 44 45 46 49 in 51 53 53 54
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li></ul>	42 42 44 45 46 49 in 51 53 53 54 56
<ul> <li>5.3.1. Perikaryon size of KP-IR and NKB-IR neurons</li></ul>	42 42 44 45 46 49 in 51 53 53 54 56 59

6. DISCUSSION	61
6.1. Possible contribution of the estrogenic regulation of RF-amide related pep to estrogen feedback mechanisms	tide 61
6.1.1. The expression of RFRP mRNA is negatively regulated by estrogen	61
6.1.2. A relatively small subset of RFRP neurons expresses ER-α immunoreactivi	ty 61
6.1.3. Putative sites of action of RFRP on the reproductive axis	62
6.2. Species differences in the neuropeptide compliment of hypothalamic kisspe	eptin
6.2.1 Species differences in the colocalization of KNDy particles	03
6.2.2. Role of NKB in the regulation of the human reproductive axis	05 64
6.2.3. Role of KP in the regulation of the human reproductive axis	04
6.2.4. Absence of DVN immunoreactivity from most KP alements	00
0.2.4. Adsence of D1W immunoreactivity from most K1 etements	00
6.3. Importance of sex differences in the central regulation of human reproduc	tion
6.2.1. Son differences in parihamon size of VD and NVD neurons	68
6.2.2. History relative levels of NKP us KP and NKB neurons	00
6.2.2. Son differences in VD immunorestinity	09
6.3.4. Sex differences in NKP immunoreactivity	/1
0.5.4. Sex algerences in NKB immunoreactivity	12
6.4. Role of enhanced kisspeptin and neurokinin B signaling in aging men, as a	
cause or a consequence of reproductive decline	74
6.4.1. Aging-dependent variations in KP and NKB immunoreactivities and their	74
	/4
6.4.2. Aging-dependent changes in the central regulation of male reproduction	75
7. CONCLUSIONS	78
8 SUMMADV	80
	00
9. ÖSSZEFOGLALÁS	81
10 REFERENCES	87
	02
11. LIST OF PUBLICATIONS	. 100
11.1. List of publications underlying the thesis	. 100
11.2. List of other publications	. 101
12 ACKNOWLEDGEMENTS	103
	. 103

### 1. List of abbreviations

ARC	- arcuate nucleus
AVPV	- anteroventral periventricular nucleus
BV	- blood vessel
DAB	- diaminobenzidine
DMN	- dorsomedial nucleus
DYN	- dynorphin
E2	- 17β-estradiol
ER-a	- estrogen receptor alpha
ER-β	- estrogen receptor beta
FSH	- follicle-stimulating hormone
GABA	- gamma-aminobutyric acid
GnIH	- gonadotropin-inhibiting hormone
GnRH	- gonadotropin-releasing hormone
icv	- intracerebroventricular
Inf	- Infundibular nucleus
InfS	- Infundibular stalk
i.p.	- intraperitoneal
IR	- immunoreactive
ISH	- in situ hybridization
KISS1R	- kisspeptin receptor
KNDy	- <u>K</u> isspeptin/ <u>N</u> eurokinin B/ <u>D</u> ynorphin
KP	- kisspeptin
KOR	- dynorphin receptor
LH	- luteinizing hormone
ME	- median eminence
MPOA	- medial preoptic area
Ni-DAB	- nickel-intensified diaminobenzidine
NK3	- neurokinin B receptor
NKB	- neurokinin B
OVLT	- organum vasculosum of the lamina terminalis

OVX	- ovariectomized	
PBS	- phosphate buffered saline	
PFA	- paraformaldehyde	
PVN	- paraventricular nucleus	
RFRP	- RFamide-related peptide	
RP3V	- rostral periventricular area of the third ventricle	
SC.	- subcutan	
SSC	- standard saline citrate	
Т	- testosterone	
TBS	- Tris buffered saline	
VMN	- ventromedial nucleus	

### 2. Introduction

### 2.1. Central regulation of the gonadal axis

Gonadotropin-releasing hormone (GnRH) synthesizing neurons of the hypothalamus play a crucial role in the central regulation of reproduction in all mammals [1]. The hypophysiotropic axons of GnRH neurons secrete the GnRH decapeptide into the fenestrated capillaries of the hypophysial portal circulation at the hypothalamic median eminence (ME). From here, long portal veins carry GnRH to the anterior pituitary gland. GnRH binds to type-1 GnRH receptor on the surface of gonadotroph cells to regulate the synthesis and secretion of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

FSH and LH released into the systemic circulation, in turn, act on the gonads to stimulate gametogenesis and gonadal steroid secretion, respectively. Gonadal hormones act on both the hypothalamus and the pituitary in a classical negative feedback loop, inhibiting thereby the synthesis of GnRH and the two gonadotropin [2, 3].

GnRH neurons are derived from the nasal placod and only migrate into the brain during fetal development [4, 5]. Defects of this migratory process can cause reproductive deficiencies due to the absence of GnRH neurons in the forebrain. Hypogonadotropic hypogonadism characterizing this status often coincides with anosmia (Kallmann syndrome).

The number of GnRH neurons regulating reproduction is surprisingly low (1000-2000). By the time of birth, these cells have already settled in the hypothalamus. They are distributed in a relatively large area, without forming solid groups of neurons. In rats, GnRH cells are located in diagonal band of Broca, medial septum, medial preoptic area (MPOA), organum vasculosum of the lamina terminalis (OVLT) and medial preoptic nucleus [6]. In humans, GnRH cells are even more scattered. A relatively large subset of them can be found in the infundibular nucleus (Inf) [7], which is analogous to the arcuate nucleus (ARC) of rodents.

The cell body of GnRH neurons is relatively small (10-20 $\mu$ m) and fusiform. GnRH neurons have one or two dendrites at the poles of the elongated perikaryon and one axon

emanating from either the cell body or one of the main dendrites [8]. According to a widely accepted view, GnRH cells form a network via establishing axo-somatic and axo-dendritic connections with one another [9], making the coordinate firing and GnRH secretion possible. GnRH cells project to two circumventricular organs, the external zone of the ME and the OVLT [4, 5].

At the level of the ME, GnRH terminals release GnRH into the fenestrated capillaries of the portal vasculature in the form of secretory pulses which occur once every 30-90 minutes [2]. The episodic secretion of GnRH has been shown to correlate with the pulsatile secretion of LH from the adenohypophysis in many species [10, 11]. Coordinated function of the cells, to allow regular pulses to be generated in terms of secretory events, could be achieved through dendro-dendritic connections [12-14]. The neuronal network accounting for the establishment of the GnRH neurosecretory pulses is commonly referred to as the GnRH/LH 'pulse generator'. Similarly to observations on various laboratory animal species, the pattern of LH secretion was also found to be pulsatile in primates [15].

It has been shown in the sheep that a strong temporal correlation exists between the GnRH secretory episodes measured in the portal blood and the pulses of LH in the peripheral blood [16]. In contrast, the relationship between GnRH and FSH secretion is more complex as FSH secretion exhibits a random pattern, without any strict correlation with the GnRH/LH secretory episodes [17]. The pattern of GnRH secretion carries relevant information for adenohypophysial gonadotroph cells. The control of FSH and LH synthesis is closely linked to the transcription of the distinct  $\beta$ -subunits, *Fshb* and *Lhb* respectively. Both FSH and LH contain a common  $\alpha$ -subunit (CGA); therefore, it is FSH $\beta$  and LH $\beta$  that confer the specific actions of the gonadotropins [18]. Notably, in follicular phase, low frequency GnRH secretion preferentially stimulates FSH $\beta$  gene expression and protein synthesis. To the opposite, during the preovulatory hypersecretion ('surge') of GnRH and LH, the frequent GnRH pulses preferentially stimulate LH $\beta$  gene expression and LH secretion from the gonadotrophs [19-23].

The functions of GnRH cells are strongly modulated by neuronal afferents. Classical neurotransmitters as well as neuropeptides released from these afferents play important roles in the neuronal regulation of GnRH cells.

One of the most important neurotransmitters in the neuronal regulation of the GnRH neuron is gamma-aminobutyric acid (GABA). A significant proportion of synaptic contacts on GnRH neurons is GABAergic [24]. Both GABA<sub>A</sub> and GABA<sub>B</sub> [25] receptors have been described on GnRH cells. The direct action of GABA on GnRH neurons is mediated by postsynaptic GABA<sub>A</sub> receptors, which are ligand-gated chloride-ion channels composed of five subunits [26]. Functional GABA<sub>B</sub> receptors have also been detected in GnRH neurons [27, 28]. GABAergic afferents play important roles in the mediation of metabolic [29], circadian [30] and estrogen signals to the GnRH system [30]. Our recent work indicates that GABAergic neurons also provide a highly abundant input to GnRH neurons in the human [31].

L-glutamate is a critically important excitatory neurotransmitter in the afferent control of GnRH cells [32]. Activation of ionotropic glutamate receptors contributes to both the pulsatile [33] and the surge [34] release of GnRH. Our recent immunohistochemical work has revealed an abundant glutamatergic input to GnRH neurons of the human. While GABAergic afferents dominates over glutamatergic afferents on the perikaryon of GnRH cells, the combined incidence of two different subtypes of glutamatergic inputs exceeds the incidence of GABAergic inputs on the dendritic compartment of GnRH neurons in human [31].

# 2.2. Peptidergic systems and mechanisms acting upstream from the GnRH neuronal systems

A large number of neuropeptides can regulate GnRH secretion from the hypophysiotropic GnRH axon terminals by acting on either the somato-dendritic or the axonal compartment of the GnRH neurons.

Kisspeptin (KP), a neuropeptide encoded by the metastasis suppressor gene KiSS1, plays a particularly important role in the central regulation of reproduction. Administration of KP, KP antibodies [35-37], KP antagonists [38-40] or the use of transgenic animal models deficient for KP or its receptor (KISS1R) [41] have provided evidence that KP regulates the pulsatile [37] and surge [36, 42] release of GnRH.

KP synthesizing neurons in various mammals have been localized to two major anatomical sites, the preoptic region and the ARC. The KP cell population of the preoptic region exhibits evident anatomical variations among species. In rodents, these KP cells form a compact nucleus in the rostral periventricular area of the third ventricle (RP3V) [43-45] (also cited often as the kisspeptin cell population of the anteroventral periventricular nucleus; AVPV). Kisspeptin neurons are much more scattered and present in lower numbers in the preoptic region of the sheep [46, 47], the monkey [48] and the human [49]. The second (and largest) kisspeptin synthesizing cell group can be consistently detected within the ARC in all mammalian species studied so far [35, 36, 43, 44, 46-48, 50-73] and in the analogous Inf of primates [49, 58].

KP axons innervate the perikaryon and dendrites of GnRH neurons [53]. The majority of GnRH neurons express KISS1R [50, 51, 74] and respond to KP with increased neuronal activity [75].

Neurokinin B (NKB) is another critically important neuropeptide which interacts with KP in the neuroendocrine regulation of GnRH/LH secretion. NKB belongs to the family of tachykinins. From the three tachykinin receptors, NKB primarily activates the NK3 receptor form [76], but it has been shown recently that the reproductive effects of NKB in mice also involve the other two types of tachykinin receptors, NK1 and NK2 [77].

NKB is encoded by the *TAC3* gene in humans, and the *Tac2* gene in rodents, whereas NK3 is encoded by the *TACR3/Tacr3* gene [76]. Inactivating mutations of the neuropeptide- or the neuropeptide receptor gene are associated with hypogonadotropic hypogonadism in humans [78, 79].

NKB may increase GnRH secretion from the hypothalamic ME where GnRH axons are apposed to NKB axons [80, 81] and express NK3 immunoreactivity [80] in the rat. In addition, NKB neurons also influence reproduction by acting on other NKB neurons in the ARC where they establish frequent contacts with one another [82, 83] and express NK3 autoreceptors.

An inhibitory neuropeptide named gonadotropin-inhibiting hormone (GnIH) has been identified in the quail hypothalamus; GnIH inhibits gonadotropin release from the pituitary in a dose-dependent manner [84]. In addition to acting as a release-inhibiting hormone on gonadotrophs, GnIH also regulates fertility via influencing the neurosecretory output of hypophysiotropic GnRH neurons. Accordingly, GnIHimmunoreactive (IR) neuronal contacts [85] and GnIH receptors [86] are present on avian GnRH neurons. Putative GnIH homologues, RF-amide related peptides (RFRP-1, RFRP-2, RFRP-3), have also been identified in mammals [87]. With some anatomical species differences, the majority of neurons that synthesize preproRFRP mRNA and RFRP peptides have been localized to the dorsomedial nucleus (DMN) of the hypothalamus in hamsters, rats, mice and sheep [88] and to the intermediate periventricular nucleus in monkeys [89]. Unlike in birds where GnIH-IR axons have access to the hypophysial portal vasculature [84, 90], RFRP neurons in rodents do not project to the external zone of the ME [88] and do not accumulate Fluoro-Gold (retrograde neuronal tracer) from the systemic circulation [91], suggesting that RFRP peptides regulate fertility primarily via central mechanisms. These mechanisms include direct inhibitory actions exerted upon GnRH neurons, as indicated by RFRP-IR neuronal contacts on GnRH neurons [62, 92] and by the RFRP-3-induced hyperpolarization [93] and reduced electric activity of a large subset of GnRH neurons [94] in slice preparations of GnRH-GFP transgenic mice.

# 2.3. Role of kisspeptin, neurokinin B, RF-amide related peptides in sex steroid feedback

The GnRH neuronal system which represents the final common pathway in the neuroendocrine control of reproduction responds to feedback actions of circulating 17 $\beta$ -estradiol (E2). The feedback effect of sex steroids takes place at the level of the hypothalamus to regulate GnRH neurons as well as at the level of pituitary gonadotrophs. The fact that the maintenance of plasma level of gonadal steroids acts as a negative brake on the GnRH- gonadotropin axis is most simply demonstrated by gonadectomy, which causes GnRH and gonadotropin levels to rise. There is a difference between males and females regarding the feedback effect of sex steroids on brain, most notably the presence of 'positive feedback' mechanism in females and the absence of the same in males. This positive feedback drives the preovulatory surge in GnRH and LH secretion in female. While direct estrogen actions upon GnRH neurons can be exerted via estrogen receptor- $\beta$  (ER- $\beta$ ) [95-99], interneurons expressing the classical estrogen receptor (ER- $\alpha$ ) play a critically important role in sensing and conveying indirect information on circulating estrogens to the GnRH neuronal system [100].

KP neurons of the ARC also synthesize NKB in the sheep [47, 70], the goat [73], the mouse [101], monkey [72] and the human [49]. The recently introduced terminology of KNDy (Kisspeptin/Neurokinin B/Dynorphin) neurons [70] refers to the co-synthesis of dynorphins (DYN) by the majority of these KP/NKB cells at least in the sheep [47, 102], the rat [82], the mouse [101, 103, 104] and the goat [73]. Evidence from studies of sheep suggests that KNDy neurons of the ARC play an important role in conveying the negative feedback effects of sexual steroids onto GnRH neurons [59], and possibly, also the positive feedback effects of estrogens [105], at least in this species. In addition, KNDy neurons also appear to constitute an important component of the GnRH pulse generator [73, 101]. Recent models of the GnRH pulse generator [73, 101, 103] suggest that KNDy neurons communicate with one another via NKB and its receptor, NK3, and possibly, also DYN and its receptor, KOR. In ovariectomized goats, central NKB increases and DYN decreases the frequencies of multiunit activity volleys and LH secretory pulses [73]. Pulse generator cells, in turn, appear to communicate with GnRH neurons primarily via KP/KISS1R signaling. GnRH neurons express KISS1R [50, 51, 106] and the majority of GnRH neurosecretory pulses show temporal association with KP pulses in the ME of monkeys [107].

The closer analysis of anatomical reports describing the colocalization of 'KNDy' neuropeptides, in retrospect, reveals that neuropeptide and receptor colocalizations are often only partial and also variable in the different studies, species, sexes and age groups. In particular, NKB-IR neurons and their fibers are partly distinct from the KP-IR elements in various human models [49], thus challenging the universal validity of the KNDy neuron concept. Based on our preliminary data indicating that the number of KP neurons is very low in young human males subjects [49], we predicted that the degree of overlap between the KNDy neuropeptides is much lower in young male humans than suggested earlier for sheep [47, 70], goats [73] or mice [101]. In one of our studies (**5.2**) underlying this thesis, we investigated the universal validity of the KNDy neuron concept via the parallel immunohistochemical analysis of KP-, NKB- and DYN immunoreactivities in the Inf and the infundibular stalk (InfS) of young men.

Evidence that the RFRP neuronal system may be involved in estrogen feedback signaling to GnRH neurons has emerged from studies of hamsters. RFRP neurons in this rodent species contain ER- $\alpha$  and respond with c-Fos expression to an acute

administration of E2 [88]. RFRP axons provide inputs to GnRH neurons, suggesting direct regulation. Intracerebroventricular injection of RFRP-3 reduced plasma LH secretion in ovariectomized (OVX) hamsters [88] and gonadally intact rats [92, 108]. In one study (5.1) underlying this thesis we investigated the putative estrogen responsiveness of the RFRP neuronal system in mice, by addressing the estrogenic regulation of RFRP gene expression and the presence of the two estrogen receptor isoforms in RFRP neurons.

### 2.4. Sex differences in steroid feedback mechanisms

The pulsatile pattern of GnRH secretion into the hypophysial portal circulation is shaped by a sex steroid-sensitive neuronal circuitry that acts upstream from GnRH cells [109]. In both males [110] and females [111], gonadal steroid hormones exert homeostatic negative feedback on GnRH release via this upstream neuronal circuitry. In females, elevated estradiol in the late follicular phase of the reproductive cycle contributes to a switch from negative to positive feedback to induce a surge of GnRH from the hypothalamus. The subsequent surge of LH from the adenohypophysis triggers ovulation [109].

KP cells of the rodent RP3V exhibit a robust sexual dimorphism. They occur in higher numbers [35, 53, 69, 112] and provide input to a higher percentage of GnRH neurons in females compared with males [53]. There is strong evidence suggesting that in rodents, the kisspeptin cell population of the RP3V is critically involved in positive estrogen feedback to GnRH neurons [35, 113]. In contrast, positive feedback appears to be attributable mainly to the KP cells of the ARC in sheep [114] and primates [2, 115, 116].

The ARC also exhibits sexual dimorphism in several species. In the sheep, this nucleus contains higher NKB [117] and KP [70] cell numbers in females than in males. In rats, sex differences were reported in the projection field of NKB-IR axons in the infundibular area [81].

The sex-specific spatial and temporal patterns of hypothalamic KP expression strongly depend on the activational effects of sexual steroid hormones. Large subsets of KP neurons in both the preoptic region and the ARC contain receptors for estradiol, testosterone (T) and progesterone in various species [35, 41, 44-46, 59, 70, 118]. In rodents, androgens and estrogens upregulate KP expression in the RP3V [35, 44, 112, 118, 119] during the phase of positive estrogen feedback. In contrast, KP expression in the ARC/Inf is regulated negatively by sex steroid hormones in rodents and other species [35, 44, 112, 118-120] and so is NKB expression at this site [120-124].

The sex-dependent pattern of hypothalamic KP expression also depends on the organizational effects of T exposure in males, in addition to the circulating levels of other sex steroid hormones. There is a neonatally determined robust sexual dimorphism of the RP3V in adult rats [112] and mice [53], with higher cell numbers in females compared with males. Prenatal T exposure accounts for a similar sexual dimorphism of KP neurons in the preoptic area of the sheep [70].

In contrast, no apparent organizational effects of the perinatal T exposure on the abundance of KP neurons have been observed in the rodent ARC which contains similar KP cell numbers in intact males and in diestrous females, or in gonadectomized males and females receiving the E2 or T regimen [112]. No sex difference appears to exist in the number of NKB neurons either. However, in the rat a sexual dimorphism that develops under the organizational effects of sex steroids has been reported in the projection fields of NKB-IR axons [81]. Unlike in rodents, the ARC of the female sheep contains higher NKB [117] and KP [70] cell numbers, compared with males. Moreover, previous work from our laboratory identified higher KP neuron and fiber densities in the Inf of women *vs.* men [49]; it requires clarification to what extent this sexual dimorphism reflects the organizational effects of sex steroids during development or the difference in the adult hormonal status between men and women.

In one of the studies underlying this thesis (5.3), we have addressed several immunohistochemical correlates of the functional differences that characterize the KP and NKB neuronal systems of the Inf in men and women. Quantitative immunohistochemistry was used to study various aspects of the putative morphological sex differences.

### 2.5. Aging related alterations in the central regulation of the reproductive axis

Reproductive aging is accompanied by sex-specific neuromorphological alterations of the Inf. While gonadal functions in aging men can be quite well-preserved throughout life [125], the negative feedback response of the reproductive axis to T shows a declining trend [126]. This reduced feedback may be correlated with a mild neuronal hypertrophy in the Inf of aged men [127]. Reproductive aging is more dramatic in postmenopausal women after the depletion of ovarian follicles, leading to the loss of circulating estrogen and causing the reduction of negative estrogen feedback [128]. Previous comparison of histological samples from pre- and postmenopausal women revealed profound anatomical changes in the Inf where negative feedback is thought to take place [128]. Accordingly, *in situ* hybridization studies identified the postmenopausal hypertrophy of neurons that express ER- $\alpha$  [129], substance P [121], NKB [121], KP [58] and prodynorphin [130]. These morphometric alterations were also associated with increased NKB [121] and KP [58] and decreased prodynorphin mRNA expression [130] at this site.

Reproductive aging in men during midlife transition is characterized by decreased serum levels of free T and increased levels of LH, FSH and sex hormone binding globulin, among other endocrine alterations [131, 132]. The aging-related hypogonadism coincides with functional disturbances occurring at different levels of the reproductive axis, which include reduced androgen receptor-mediated negative feedback to the hypothalamus [126]. In view of the proposed involvement of KP/NKB neurons in negative feedback [44, 47, 118], in one study (**5.4**) included in this thesis we addressed the hypothesis that the weakening of the inhibitory T feedback in elderly men coincides with morphological signs of enhanced KP and NKB signaling in the Inf.

### 3. Specific aims

1) To investigate the putative estrogen responsiveness of the RFRP neuronal system in mice

2) To characterize the 'KNDy' neuronal system in adult human males

3) To investigate the sexual dimorphism of the human hypothalamic kisspeptin and neurokinin B neuronal systems

4) To address aging-related anatomical changes of human kisspeptin and neurokinin B neurons

### 4. Materials and Methods

### 4.1. Animals

The experiments were performed on CD1 mice that were purchased from a local colony bred at the Medical Gene Technology Unit of the Institute of Experimental Medicine (IEM). The animals were housed in light- (12:12 light-dark cycle, lights on at 07:00h) and temperature (22 <sup>Q2°</sup>C) controlled environment, with free access to standard food and tap water. All studies were carried out with permission from the Animal Welfare Committee of the Institute of Experimental Medicine of the Hungarian Academy of Sciences (No.: A5769-01) and in accordance with legal requirements of the European Community (Decree 86/609/EEC).

### *4.1.1. Surgery (Gonadectomy and hormone replacement of female mice)*

In a study to analyze the estrogenic regulation of RFRP mRNA expression in mice (5.1), we used gonadectomy, followed by hormone replacement. The ovariectomy was carried out under deep anesthesia with an intraperitoneal (i.p.) cocktail of ketamine (25 mg/kg), xylavet (5 mg/kg) and pipolphen (2.5 mg/kg) in saline. On post-ovariectomy day 9, the mice were re-anesthetized and implanted subcutaneously (*sc.*) with a single silastic capsule (Sanitech; Havant, UK; l=10 mm; ID=1.57 mm; OD=3.08 mm) containing either sunflower oil (OVX, n=5) or  $100\mu$ g/ml E2 (Sigma Chemical Company, St Louis, MO) in sunflower oil (OVX+E2, n=4).

### 4.1.2. Transcardiac perfusion

Four days later, the mice were anesthetized and killed by transcardiac perfusion with 40 ml 4% paraformaldehyde (PFA) in phosphate buffered saline solution (PBS; 0.1M; pH 7.4). The brains were removed, postfixed in 4% PFA solution for 1h at 4°C, infiltrated with 20% sucrose (in PBS) overnight, and then snap-frozen on powdered dry ice. These mice were used in colocalization studies of ER- $\alpha$  and RFRP-1 immunoreactivities (5.1). Another six mice were OVX and treated similarly to generate OVX (n=3) and OVX+E2 (n=3) groups and perfused with a mixture of 2% paraformaldehyde and 4% acrolein. These mice perfused with the acrolein-containing

fixative were used in colocalization studies of ER- $\beta$  and RFRP-1 immunoreactivities (5.1).

#### 4.2. Human tissues

### 4.2.1. Collection of human hypothalamic tissues

Human hypothalamic tissue samples were obtained from autopsies at the Forensic Medicine Department of the University of Debrecen with permission from the Regional Committee of Science and Research Ethics of the University of Debrecen (DEOEC RKEB/IKEB: 3183-2010). Selection criteria included sudden causes of death, lack of history of neurological and endocrine disorders and *post mortem* delay below 48h.

In studies to characterize the 'KNDy' neuronal system of adult humans (**5.2**), we used tissues of six young male individuals (age 21-37 years). To investigate the sexual dimorphism of the human hypothalamic KP and NKB neuronal systems (**5.3**), we used tissue samples from nine male subjects above 50 years of age (50-67 years) and from seven postmenopausal female subjects above 55 years of age (57- 70 years). To address the aging-related anatomical changes of human KP and NKB neurons, arbitrarily defined 'young' (21-49 years; N=11) and 'aged' (50-67 years; N=9) male groups were formed (**5.4**).

Following dissection, the human hypothalamic tissue blocks were rinsed briefly with running tap water and then, immersion-fixed with 4% paraformaldehyde in 0.1M PBS (pH 7.4) for 7-14 days at 4°C. Following fixation, the hypothalami were trimmed further to include the optic chiasma rostrally, the mammillary bodies caudally and the anterior commissure dorsally [99].

# 4.3. Section preparation and pretreatments for immunohistochemistry and *in situ* hybridization

# 4.3.1. Preparation of hypothalamic sections for immunohistochemistry in studies using mouse tissues

Serial 20µm (5.1) coronal sections were cut from the hypothalami with a Leica SM 2000R freezing microtome (Leica Microsystems, Nussloch Gmbh, Germany). The

sections were stored at -20°C in 24-well tissue culture plates containing anti-freeze solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer; pH 7.4).

In studies to investigate the putative colocalization of ER- $\beta$  with RFRP (5.1), the perfusion solution contained acrolein. In these cases the sections were treated with 1% sodium borohydride for 30 min and washed in PBS until the sections sank to the bottom of washing dish. Prior to immunohistochemistry, the sections were treated with 0.2% Triton X-100 and 0.5% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min. Between treatments, the sections were washed in PBS for 30 min (3 x 10 min).

# 4.3.2. Preparation of frozen tissue sections for in situ hybridization experiments on mouse tissues

For *in situ* hybridization experiments serial 20µm coronal sections were cut and stored as described above.

In studies to analyze RFRP mRNA levels in OVX and OVX+E2 mice (**5.1**), every 6th section from each paraformaldehyde-fixed mouse hypothalamus was mounted on silanized microscope slides from sterile Tris buffered saline (TBS; 50 mM; pH 7.8) with an RNase-free paint brush and air-dried. Then, the sections were processed through the following prehybridization steps: 2 min rinse in 2X standard saline citrate (SSC) solution; 10 min acetylation in 0.25% acetic anhydride (Sigma)/0.9% NaCl/0.1 M triethanolamine (pH 8.0; Sigma) [133]; a brief rinse in 2XSSC solution; dehydration in 70%, 80%, 95% and 100% ethanol (2 min each); delipidation in chloroform (5 min), then partial rehydration in 100% followed by 95% ethanol (2 min each). The slides were finally air-dried.

### 4.3.3. Preparation of human tissue sections for immunohistochemistry

Sagittal cuts were made 2cm lateral from midsagittal plane on both sides and then, the blocks were cut in halves and infiltrated with 20% sucrose for 5 days at 4°C. The right hemihypothalami were placed in a freezing mold, surrounded with Jung tissue freezing medium (Leica Microsystems, Nussloch Gmbh, Germany; diluted 1:1 with 0.9% sodium chloride solution), snap-frozen on powdered dry ice, and sectioned serially at 30µm with a freezing microtome parallel to the plane of the lamina terminalis. The sections were stored similarly to mouse sections. All experiments (**5.2**, **5.3**, **5.4**) were performed on every 24th hemihypothalamic section from each subject.

Prior to immunohistochemistry, human tissues were permeabilized and endogenous peroxidase activity reduced using a mixture of 0.2% Triton X-100 and 0.5%  $H_2O_2$  in PBS for 30 min. Subsequently, antigen retrieval was carried out by using a 0.1M citrate buffer (pH 6.0) wash at 80°C for 30 min. In immunofluorescent experiments, the sections were also pretreated with Sudan black to reduce tissue autofluorescence from lipofuscin deposits [134] [49].

### 4.4. Immunohistochemical methods

### 4.4.1. Peroxidase-based immunohistochemical single-labeling

Sections processed for peroxidase-based immunohistochemistry were first incubated in the working dilution of primary antibodies (24-72h; 4°C). The primary antibodies were reacted with biotinylated secondary antibody (1:500; 1h) and then, with the ABC Elite reagent (Vector, Burlingame, CA, 1:1000; 1h). The peroxidase signal was developed with diaminobenzidine (DAB) or with nickel-intensified diaminobenzidine (Ni-DAB) chromogen. The immunostained sections were mounted on microscope slides from Elvanol, air-dried, dehydrated with 95% (5 min), followed by 100% (2X5 min) ethanol, cleared with xylene (2X5 min) and coverslipped with DPX mounting medium (Sigma, St. Louis, USA).

### 4.4.2. Silver-gold intensification of the Ni-DAB chromogen

In some dual-peroxidase-based experiments we studied the localization of two different antigens (one nuclear and one cytoplasmic) in the same neuron (5.1, 5.2). In others, the afferent connectivity between two different neuronal phenotypes was investigated (5.3, 5.4). In these studies the Ni-DAB chromogen was post-intensified with silver-gold [135].

### 4.4.3. Innervation and colocalization studies with the combined use of silver-goldintensified Ni-DAB and DAB chromogens

In studies to examine the KP-IR and NKB-IR afferent inputs to GnRH neurons (5.2, 5.3, 5.4), two series of sections were processed for the detection of KP or NKB immunoreactivities. Subsequently, GnRH neurons were detected with a new guinea pig antiserum (#1018; see in Table 1, 1:10,000). The primary antibodies were reacted with biotinylated anti-guinea pig IgG (Jackson ImmunoResearch; 1:500; 60 min) and the ABC reagent (1:1000; 60 min) and then, the peroxidase signal was developed with DAB chromogen.

In studies to reveal the presence of the estrogen receptor isoforms in RFRP neurons (5.1) ERs were detected with an antiserum raised in rabbit (see in Table 1), followed by biotinylated secondary antibodies (Jackson ImmunoResearch Europe Ltd., Soham, Cambridgeshire, UK; 1:500) and the ABC Elite reagent for 60 min each. The signal was visualized with Ni-DAB and then post-intensified with silver-gold. Subsequently, RFRP-1 immunoreactivity was detected with mouse monoclonal antibodies (IF3; Takeda Pharmaceutical Co. Ltd, Japan; 1:20,000) against the C-terminus of rat RFRP-1 [136], using the biotinylated secondary antibody-ABC technique and non-intensified DAB as the chromogen.

## 4.4.4. Dual-immunofluorescent investigations to study the colocalization of KP and NKB or KP and DYN immunoreactivities in the Inf

In studies to examine the colocalization between KP and NKB or KP and DYN (**5.2**, **5.3**, **5.4**), dual-immunofluorescent labeling was used. Incubation in a cocktail of primary antibodies (rabbit anti-NKB, 1:1000 and sheep anti-KP, 1:1000; or rabbit anti-DYN, 1:1000 and sheep anti-KP, 1:1000; see in Table 1; 48h; 4°C) was followed by a cocktail of fluorochrom-conjugated secondary antibodies (Jackson ImmunoResearch; anti-rabbit-FITC, 1:250; anti-sheep-Cy3, 1:1000) for 5h at room temperature.

To maximize sensitivity, in some dual-immunofluorescent studies (5.2, 5.3, 5.4) tyramide signal amplification was used. In these experiments KP was detected first using sequential incubations in sheep KP antibodies (1:30,000; 48h; 4°C), biotinylated anti-sheep IgG (Jackson ImmunoResearch Laboratories; 1:500; 1h), the ABC Elite reagent (Vector; 1:1000; 1h), biotin tyramide working solution (1:1000, in 0.05M Tris-

HCl buffer, pH 7.6, containing 0.003% H<sub>2</sub>O<sub>2</sub>; 30 min) [137] and finally, avidin-Cy3 (Jackson ImmunoResearch; 1:1000; 1h). Then, the sections were treated for 30 min with 0.5% H<sub>2</sub>O<sub>2</sub> and 0.1% sodium azide in PBS, to inactivate horseradish peroxidase. To detect NKB or DYN, the rabbit primary antibodies were used at 1:50,000 (48h; 4°C) and reacted with anti-rabbit-peroxidase (Jackson ImmunoResearch; 1:500; 1h). Then, FITC-tyramide [137] (diluted 1:500 with 0.05M Tris-HCl buffer, pH 7.6, containing 0.003% H<sub>2</sub>O<sub>2</sub>; 30 min) was deposited on the peroxidase sites. Control experiments included the omission of the NKB and DYN primary antibodies. Lack of FITC labeling in these control sections indicated that no FITC-tyramide deposition is caused by residual peroxidase activity on KP-IR sites.

# 4.4.5. Triple-immunofluorescent studies to analyze the colocalization of KP and NKB in neuronal afferents to human GnRH neurons

Incubation in a cocktail of primary antibodies (rabbit anti-NKB, 1:1000; sheep anti-KP, 1:1000; guinea pig anti-GnRH, 1:3000) for 48h at 4°C was followed by a cocktail of fluorochrom-conjugated secondary antibodies (all raised in donkey; anti-rabbit-FITC, 1:250; anti-sheep-Cy3, 1:1000; anti-guinea pig-AMCA, 1:100; Jackson ImmunoResearch) for 5h (**5.3, 5.4**).

Sections processed for immunofluorescent experiments were mounted from 0.1M Tris-HCl buffer (pH 7.6) and coverslipped with the aqueous mounting medium Mowiol.

Primary antibodies	Dilution	Chromogen
5.1. Dual-label immunohistochemical experiments to colocalize estrogen receptors		
and RFRP-1 immunoreactivities		
rabbit <b>ER-</b> $\alpha$ antiserum	1:10.000	Ni-DAB
(C1355; Millipore, Temecula, CA, USA)	,	
rabbit <b>ER-</b> $\beta$ antiserum	150ng/ml	Ni-DAB
(Z8P; Zymed Laboratories; Lot 01162852)	100119,111	
mouse <b>RFRP-1</b> monoclonal antibody	1.20,000	DAB
(IF3; Takeda Pharmaceutical Co. Ltd, Japan)	1.20,000	

Table 1. Summary of the antibodies and reagent used in different experiments

5.2. 'KNDy' neuronal system in adult human males			
	sheep polyclonal antiserum to human <b>KP-54</b>	1:200,000/	Ni-DAB/
	(GQ2; gift from Dr. S.R. Bloom)	1:1,000	Cy3
	rabbit polyclonal antiserum to human <b>NKB</b>	1:100,000/	Ni-DAB/
	(gift from Dr. Philippe Ciofi)	1:1,000	FITC
	rabbit polyclonal antiserum to <b>DYN</b>	1:100,000/	Ni-DAB/
	(T-4268; Peninsula Laboratories; San Carlos, CA.)	1:1,000	FITC
	guinea-pig <b>GnRH</b> antiserum	1.50,000	DAB
	(#1018; Hrabovszky et al., 2011)	1.50,000	DITE
5.3. Sexual dimorphism of the human hypothalamic kisspeptin and neurokinin B			
	neuronal systems		
	sheep polyclonal antiserum to human KP-54	1:100,000/	Ni-DAB/
	(GQ2; gift from Dr. S.R. Bloom)	1:1,000	Cy3
	rabbit polyclonal antiserum to <b>NKB</b>	1:100,000/	Ni-DAB/
	(gift from Dr. Philippe Ciofi)	1:1,000	FITC
	guinea-pig GnRH antiserum	1:50,000/	DAB/
	(#1018; Hrabovszky et al., 2011)	1:3,000	AMCA
	5.4. Aging-related anatomical changes of human	kisspeptin an	d neurokinin B
	neurons		
	sheep polyclonal antiserum to human KP-54	1:100,000/	Ni-DAB/
	(GQ2; gift from Dr. S.R. Bloom)	1:1,000	Cy3
	rabbit polyclonal antiserum to NKB	1:100,000/	Ni-DAB/
	(gift from Dr. Philippe Ciofi)	1:500	FITC
	guinea-pig GnRH antiserum	1:10,000/	DAB/
	(#1018; Hrabovszky et al., 2011)	1:5,000	AMCA

### 4.5. In situ hybridization

### 4.5.1. Preparation of hybridization probes

To prepare a probe to preproRFRP mRNA (5.1), a 424-bp cDNA fragment (corresponding to bases 136-559 of the rat preproRFRP mRNA; AB040288, respectively) was amplified with PCR from rat hypothalamic cDNA. The amplicons

were inserted into a plasmid vector using the pGEM-T Easy Vector System from Promega (Madison, WI). The plasmids were grown in DH5α cells (Invitrogen, Carlsbad, CA, USA), isolated with the QIAGEN Plasmid Maxi kit (Qiagen; Valencia, CA, USA), linearized with *Sal*1 and purified with phenol/chloroform/isoamyl alcohol (PCI), followed by chloroform/isoamyl alcohol extractions and then precipitation with NaCl and ethanol. The linearized RFRP template was transcribed with T7 RNA polymerase in the presence of <sup>35</sup>S-UTP (NEN Life Science Products, Boston, MA, USA).

### 4.5.2. In situ hybridization studies with <sup>35</sup>S-labeled cRNA hybridization probes

In studies to detect E2 dependent changes of RFRP mRNA level (**5.1**) in the mouse hypothalamus, after prehybridization the sections were placed in humidity chamber. 50  $\mu$ l of hybridization solution was pipetted onto each section, covered with a glass coverslip and compassed with DPX. The standard hybridization reaction was carried out at 56°C for 16h (overnight). Following post-hybridization treatments including the RNase A digestion (20 µg/ml, 60 min at 37°C) of probe excess and a 30-min stringent treatment in 0.1X SSC at 60°C, the sections were rinsed briefly in 70% ethanol and air dried.

### 4.5.3. Autoradiography

The sections labeled with the RFRP probe (5.1) were first exposed to Kodak BioMax MR autoradiography films for 3 days and signals developed with standard film processing procedures.

To visualize the isotopic signal for RFRP (**5.1**), the slides were dipped into Kodak NTB autoradiographic emulsion (Kodak; Rochester, NY) and exposed for 1-2 weeks. The emulsion autoradiographs were developed using standard procedures and Kodak processing chemicals. The sections were air-dried, dehydrated with 95%, followed by 100% ethanol (5 min each), cleared with xylene (2X5 min), and coverslipped with DPX mounting medium.

### 4.6. Microscopy and data analysis

### 4.6.1. Light- and fluorescent microscopy

The light- (5.1, 5.2, 5.3, 5.4) and fluorescent (5.2) microscopic images were captured with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope using the AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany).

### 4.6.2. Confocal laser microscopy

To analyze the double- and triple-immunofluorescent labeling (**5.3**, **5.4**) we used a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK). Multiple stacks of optical slices (512x512 pixels, z-steps 0.6  $\mu$ m) were obtained using a 60x oil immersion objective. The fluorochromes were detected with the following laser lines and filters: 488 nm for FITC, 543 nm for Cy3 and Alexa594, 405nm for AMCA, with dichroic/emission filters 560 nm/500–540 nm for FITC, 650 nm/560-610 nm for Cy3 and Alexa594, 500 nm/420-480 nm for AMCA. The separately recorded green, red and blue channels were merged and displayed with the Laser Vox software (Bio-Rad) running on an IBM-compatible personal computer.

Furthermore, confocal images (**5.2**, **5.4**) were prepared with an inverted Nikon Eclipse Ti-E microscope equipped with an A1R confocal system (Nikon, Japan) too.

#### 4.6.3. Data analysis and statistics

#### 4.6.3.1. Quantitative analysis of ISH signal

X-ray film images were used for the quantitative analysis of RFRP mRNA levels in OVX and OVX+E2 mice (**5.1**). The films were scanned using a HP ScanJet 4600 Flatbed Scanner equipped with a transparent material adapter. For consistency, the autoradiographic images of the most heavily labeled four sections in each animal were selected for the quantitative analysis of RFRP mRNA expression. The digital image files were saved with TIF extension and opened for analysis with the Image J software (public domain at http://rsb.info.nih.gov/ij/download/src/). During measurements, a

threshold was set and held constant across all sections to highlight the entire positive hybridization signal area. The autoradiographic signal in each animal was characterized with the mean of the four bilateral 'integrated density' measurements (sum of pixel density values in the highlighted signal area; mean gray value X area). The OVX and the OVX+E2 groups were compared using one-way ANOVA.

The integrated density of X-ray film autoradiographs depends both on the number of labeled neurons in the signal area and the single-cell levels of RFRP mRNA expression in individual RFRP neurons. These two parameters were analyzed further using the computerized image analysis of emulsion autoradiographs from the same sections that had been selected for film analysis. Each animal was characterized with eight digital photomicrographs from the dorsomedial nuclei of these four sections. We used a consistent sampling method to reach the densest concentration of RFRP neurons in each microscope field. The eight microscopic images of each animal were scanned with an AxioCam MRc 5 digital camera, using a 20X objective lens. The TIF files were analyzed by an investigator blind to treatments. The files were opened with Image J and the threshold was set to only highlight the silver grains in the sections. Then, all neurons found bilaterally in the eight image files of each animal were identified and selected individually, using the lasso tool of the Image J software. The integrated density of highlighted pixels covered by silver grains (Mean gray value X Area) was determined for each neuron. Each animal was finally characterized with the mean integrated density over individual RFRP neurons, as determined from all cells found in the eight photomicrographs. The number of silver grain clusters identified as RFRP neurons in the OVX and the OVX+E2 groups as well as the mean integrated density of RFRP neurons in the two treatment groups were compared with one-way ANOVA.

### 4.6.3.2. Analysis of quantitative immunohistochemical experiments

The digital images were processed with the Adobe Photoshop CS software (Adobe Systems, San José, CA, USA) at a 300 dpi resolution. Quantitative data were expressed as mean±SEM and statistical comparisons were carried out one-way ANOVA followed by Newman-Keuls post-hoc test using the Statistica 8.0 software package (StatSoft, Inc, Tulsa, USA).

For quantitative immunohistochemical studies on humans (5.2, 5.3, 5.4), the immunostained microscopic specimens as well as the digital photographs were randomized, coded and analyzed by investigators blind to the origin of samples.

### *4.6.3.2.1. Perikaryon size (mean immunoreactive profile area)*

To determine the average size of KP-IR and NKB-IR cell bodies (**5.3**, **5.4**), 10-30 solitary neurons, which showed no overlap with one another, were identified in digital images of the Inf from each individual. To exclude immunoreactive neuronal processes from the area analyzed, the tissue area surrounding each immunolabeled neuron was erased using the Adobe Photoshop CS software. The digital images of selected cell bodies were compiled into TIF files and opened for area/cell body analysis with the Image J software. A threshold was determined and set to only highlight the labeled cell bodies in all specimens. The signal areas were measured this way and then, converted to  $\mu m^2$  using appropriate calibration. For each human subject the mean profile area of labeled perikarya was derived from an average of 10-30 labeled cells.

### 4.6.3.2.2. Regional incidence of cell bodies

The number of immunoreactive cell bodies was counted (5.2, 5.3, 5.4) at 100X magnification within a 0.25 mm2 counting area with the aid of a 5X5 ocular grid, as described previously [49]. Each subject was characterized by the maximal number of immunoreactive perikarya in this counting area (determined from 2-6 sections).

#### 4.6.3.2.3. Regional density of labeled fibers

Digital images were taken (5.2, 5.3, 5.4) from the bulk of KP-IR and NKB-IR neurons in the Inf. The files were opened with the Adobe Photoshop CS software. The immunolabeled cell bodies and their proximal dendrites were erased ('eraser tool') from the photomicrographs. The remaining images were compiled into TIF files and opened with the Image J software. The regional fiber density in each photograph was defined as the area occupied by immunoreactive fibers/total area. For each subject, the mean fiber density was derived from 1-3 digital images. The overlap between NKB-IR and KP-IR

axons or DYN-IR and KP-IR axons (5.2) was also studied qualitatively in confocal images of dual-immunofluorescent specimens.

Projections of NKB-, KP-, DYN- and GnRH-IR axons around the portal blood vessels of the InfS were analyzed in the **5.2** experiment. Based on previous immunohistochemical results in the median eminence of different species [72] [81], we assumed that fibers containing KNDy peptides around the portal vasculature arise mostly from the ARC/Inf. First, sections labeled with peroxidase-based immunohistochemistry were used to study the relationship of fibers with the superficial and deep capillary plexuses of the human postinfundibular eminence [138]. Then, the extents of overlap between NKB and KP immunoreactivities and between DYN and KP immunoreactivities were assessed from dual-immunofluorescent specimens.

#### 4.6.3.2.4. Neuropeptide colocalization in cell bodies

In studies to colocalize KP and NKB in the Inf the incidence of double-labeled KP-IR and NKB-IR perikarya (**5.2**, **5.4**) were determined quantitatively from the dualimmunofluorescent specimens in which the tyramide signal amplification was used. This analysis included 1-3 representative confocal images per subject.

### 4.6.3.2.5. Studies of neuropeptides in afferents to GnRH neurons

Dual-immunoperoxidase labeled sections were selected (1-2 from each individual) to determine the number of KP/NKB axonal contacts along the outlines of GnRH-IR cell bodies and dendrites (5.2, 5.3, 5.4). Counting of the appositions was carried out using a 63X oil-immersion objective and contacts defined using stringent criteria. The axon and the GnRH profile had to be in the same focus plane without any visible intervening gap, and uncertain instances of partial overlap were not considered. For each subject, the mean number of contacts per GnRH soma and 100µm GnRH dendrite was calculated.

In studies to colocalize KP and NKB in neuronal afferents to GnRH neurons of the Inf (**5.3**, **5.4**), one section from the triple-immunofluorescent specimens of the Inf was selected from each individual to analyze single- and double-labeled KP-IR and NKB-IR neuronal appositions onto GnRH neurons. Multiple stacks of optical slices (512x512)

pixels, z-steps 0.6  $\mu$ m) were obtained by scanning GnRH neurons in the Inf and their KP-IR and NKB-IR contacts using a 60x oil immersion objective and a Radiance 2100 confocal microscope. Appositions were validated if no gap was visible between the juxtaposed profiles in at least one optical slice.

### 5. Results

# 5.1. Estrogenic down-regulation of RF-amide related peptide expression via estrogen receptor- $\alpha$

#### 5.1.1. RFRP mRNA levels of OVX mice decrease in response to E2 treatment

Radioisotopic *in situ* hybridization studies revealed a restricted regional distribution of RFRP mRNA synthesizing neurons in the mouse hypothalamus, in accordance with the results of earlier studies [88]. The majority of labeled neurons were observed in the dorsomedial nucleus (DMN), an area ventral to it and in the periventricular nucleus of the caudal hypothalamus. The distribution patterns were identical in the OVX and OVX+E2 groups, but the signal was much weaker in the latter (photographic insets in Figure 1). Quantitative analysis established that a 4-day E2 treatment of OVX mice significantly decreased the integrated density of X-ray film images, used as a signal measure (P=0.012; Fig. 1).



Figure 1. In situ hybridization detection of RFRP mRNA expression in ovariectomized mice treated *sc*. with oil vehicle (OVX group; N=5) or 17 $\beta$ -estradiol (OVX+E2 group; N=4) for 4 days reveals identical signal distribution in X-ray film autoradiographs, with reduced expression levels in the latter treatment group (photographic insets). Results of quantitative image analysis, shown in the diagram, confirm that the RFRP mRNA signal (integrated density; mean gray value X signal area, expressed as percentage of the OVX group) in the DMN is lower in OVX+E2 than in OVX mice. \*P<0.05.

Silver grain clusters in the emulsion autoradiographs were analyzed to determine if E2 treatment decreased the number of detectable RFRP neurons, the single-cell levels of RFRP mRNA, or both. The integrated density analysis of silver grains over individual neurons (Fig. 2) revealed lower single-cell levels of RFRP mRNA expression in the OVX+E2 *vs.* the OVX group (P=0.021). In retrospect, this unbiased analysis identified significantly fewer (P=0.026) silver grain clusters (RFRP neurons) in OVX+E2 mice (26.4±2.6 neurons/animal; Mean±SEM), compared with OVX controls (39.0±3.6 neurons/animal).



Figure 2. Results of the single-cell image analysis, shown in the diagram, reveal reduced single-cell levels of RFRP mRNA expression (integrated density; mean gray value X area of silver grains above individual neurons), in ovariectomized mice treated *sc.* with 17 $\beta$ -estradiol for 4 days (OVX+E2 group; N=4) *vs.* their oil-treated controls (OVX group; N=5). Integrated density values are expressed as percentage of the OVX group. \*P<0.05. Individual RFRP neurons are indicated by arrows in representative emulsion autoradiographs.

5.1.2. Nuclear estrogen receptor- $\alpha$  occurs in a small subset of RFRP-synthesizing neurons, whereas estrogen receptor- $\beta$  is absent

The use of silver-gold-intensified Ni-DAB chromogen enabled the sensitive visualization of ER- $\alpha$  (Fig. 3, A-G). A heavy ER- $\alpha$  immunolabeling was present in the ARC and ventromedial nuclei (VMN) (Fig. 3A) and in scattered cell nuclei within the dorsomedial and periventricular nuclei where most RFRP-1-IR cells occurred (Fig. 3, A and B). A pale nuclear ER- $\alpha$  signal was detected in 18.7±3.8% of RFRP-1-IR cells (Fig. 3, C-E, H), whereas the majority of RFRP neurons did not contain ER- $\alpha$  signal (Fig. 3, C, F, G). Using the Z8P antiserum against ER- $\beta$ , many ER- $\beta$ -IR cell nuclei were detectable in the hypothalamic paraventricular nucleus (PVN) (Fig. 3I), but only scattered cell nuclei were labeled for ER- $\beta$  in the dorsomedial nucleus (Fig. 3J). ER- $\beta$ -positive RFRP neurons were not revealed (Fig. 3J).



Figure 3. Use of dual-label immunohistochemistry provides evidence for the presence of ER-a within a small subset (18.7%) of RFRP-1-IR neurons and the absence of ER-β in ovariectomized mice. Use of the silver-gold-intensified Ni-DAB chromogen reveals a high density of darkly labeled ER- $\alpha$ -IR cell nuclei (black signal) in the ARC and VMN (A). Heavily labeled cell nuclei are also present in the dorsomedial nucleus (DMN) where the majority of RFRP-1-IR neuronal cell bodies (brown cytoplasmic labeling) occurs (B). As shown in the high-power images (C-G) of framed areas in B, typical RFRP neurons are either devoid of the nuclear ER- $\alpha$  signal (white arrows; C, F, G) or exhibit only weak ER-a labeling (black arrows; C, D, E). Very few RFRP neurons show medium (H) and none show high labeling intensity. Arrowheads in C point to ERα-IR cell nuclei in non-RFRP neurons. The application of silver-gold-intensified Ni-DAB to detect ER- $\beta$  immunoreactivity reveals a large number of labeled nuclei in the hypothalamic paraventricular nucleus (PVN; I) but only a few labeled cells in the DMN (arrowhead in J). RFRP-1-IR cells in the DMN (arrows) do not contain ER-β signal (arrowhead) in the high-power inset. Scale bars=50µm in A, B, I, J and 10µm in C-H and in the high-power inset in J.

# 5.2. Immunohistochemical evidence for the absence of 'KNDy neurons' in young human males

The comparative analysis of NKB, KP and DYN immunoreactivities in immunoperoxidase-labeled sections of the Inf (Fig. 4) and the InfS (Fig. 6) revealed strikingly different labeling intensities for KNDy neuropeptides. In general, NKB-IR elements showed much higher abundance than KP-IR elements. DYN immunoreactivity, both in perikarya and fibers, was relatively sparse and weak.



Figure 4. The silver-gold intensified Ni-DAB chromogen was used to visualize NKB (A, B), KP (C, D) and DYN (E-G) immunoreactivities in adjacent sections of the Inf from a 31-year-old male subject. NKB-IR perikarya as well as nerve fibers (A, B) occur in much higher numbers than do KP-IR elements (C, D). For results of the quantitative analyses which reveal about five-fold differences for both perikaryon and fiber densities, see Figure 5 and 7, respectively. Arrowheads point to NKB-IR cell bodies in B and a KP-IR cell body in D. Note that NKB-IR neurons receive numerous afferent contacts (arrows in B) from NKB-IR varicose axons; analogous juxtapositions in other species were proposed to underlie the main peptidergic signaling mechanism among the putative pulse-generating KNDy neurons. Few of any DYN-IR cell bodies are detectable in the Inf (none visible in this specific case) and only scattered DYN-IR fibers occur (E, F). This low level of the DYN signal does not appear to reflect a technical limitation of the immunohistochemical approach, given that IR perikarya and fibers are abundant elsewhere in the hypothalamus, including the supraoptic nucleus (SO; G). Scale bar=100µm for A, C, E and 9µm elsewhere.

#### 5.2.1. Incidence of KP-, NKB- and DYN-immunoreactive perikarya in the Inf

In peroxidase-based immunohistochemistry, many NKB-IR perikarya were identified in the Inf (Fig. 4, A and B). KP-IR cell bodies occurred in much lower numbers in neighboring sections (Fig. 4, C and D). Quantitative analysis showed that the density of KP neurons was about 5 times lower than that of NKB-IR perikarya (Fig. 5; P=0.01 by ANOVA). DYN-IR perikarya were either entirely absent in some subjects (Fig. 4, E and F) or extremely rare in the Inf of others, preventing quantitative studies. In contrast, the supraoptic nucleus contained many DYN labeled perikarya (Fig. 4G), making it unlikely that the low DYN signal in the Inf reflects technical limitations.



Figure 5. The maximal number of immunoreactive cell bodies per  $0.25 \text{mm}^2$  counting frame (1-6 per subject) was determined with the aid of an ocular frame and used as the index of the density of labeled perikarya. Results show that in young male human individuals, the mean incidence of NKB-IR cell bodies is about 5 times higher than the incidence of KP-IR cell bodies. \*P<0.05 by ANOVA.

A surprising segregation of NKB-IR and KP-IR perikarya was revealed in dualimmunofluorescent specimens (Fig. 6, A and B). Tyramide signal amplification was crucial for sensitive detection of NKB/KP dual-labeled cell bodies which represented only 32.9±4.7% of the NKB-IR and 75.2±6.6% of the KP-IR perikarya (Fig. 6, A and B). Tyramide signal amplification was capable of visualizing only a few DYN-IR perikarya (not shown).


Figure 6. Results of immunofluorescent studies reveal a significant degree of mismatch between KP and NKB immunoreactivities (A-E) and KP and DYN (or dynorphin B) immunoreactivities (F-K) in the Inf (A, B, F, G) and the InfS (C-E, H) of the human. The dual-immunofluorescent visualization of NKB (green) and KP (red) immunoreactivities in the Inf (A, B) not only confirms the dominance of NKB-IR (green) over KP-IR (red) cell bodies (arrowheads) and axons (arrows) in the Inf, but also reveals a considerable degree of segregation between the two different perikaryon (arrowheads)- and fiber (arrows) populations. Yellow double-arrows and arrowheads point to dual-labeled fibers and cell bodies, respectively. Single-labeled NKB-IR axons are also typical around the portal blood vessels (BV) of the postinfundibular eminence in the InfS (C-E), in addition to single-labeled KP-IR fibers (red) and NKB/KP-duallabeled (yellow) axons. The dual-immunofluorescent visualization of KP and DYN (F-H) illustrates the absence of DYN immunoreactivity from KP-IR cell bodies in the Inf (red arrowheads in F). With the exceptions of a few scattered dual-labeled fibers (yellow double-arrows) in the Inf (F, G) and the InfS (H), the majority of KP-IR (red) and DYN-IR (green) axons are separate. Negative colocalization results are reproducible in the Inf (I) and the InfS (J) using an antiserum against a different prodynorphin cleavage product, dynorphin B. Note the high density of dynorphin B-IR fibers in the neighboring ventromedial nucleus (VMH) in K. High-power micrographs (A, B, D-K) represent single optical slices (0.7µm). Scale bar=100µm for C, 5µm for D and 10µm for A, B, E-K.

### 5.2.2. Abundance of KP- and NKB-immunoreactive fibers in the Inf

The incidence of immunolabeled fibers in the Inf followed a similar trend as labeled perikarya. The most frequently encountered phenotype was, again, IR for NKB. These axons established many appositions (Fig. 4B) to NKB-IR cell bodies and their dendritic processes. Quantitative analysis of the area covered by immunohistochemical signal established that the mean incidence of NKB-IR fibers was about 5 times higher than that of KP-IR fibers (Figure 7; P=0.0001 by ANOVA). DYN-IR fibers were also detectable in the Inf, although less frequently than either NKB-IR or KP-IR axons (Fig. 4, E and F).



Figure 7. The area covered by immunoreactive fibers (divided by the total area analyzed) was determined with the ImageJ software in digital photographs of the Inf and used as a fiber density measure. Areas that were occupied by labeled cell bodies and their thick proximal dendrites were deleted from the photographs with the eraser tool of the Adobe Photoshop software and thus, excluded from the analysis. The density of NKB-IR fibers (expressed in arbitrary units) is 5.4 times higher than the density of KP-IR fibers. \*P<0.0005 by ANOVA.

In immunofluorescent specimens, many NKB-IR fibers without KP immunolabeling as well as KP-IR fibers without NKB labeling could be seen in the Inf, in addition to dual-labeled axons (Fig. 6, A and B). DYN-IR fibers showed a high intensity of labeling only if the tyramide signal amplification approach was also used. Most of them were distinct from KP-IR axons, although dual-labeled KP/DYN-IR fibers occasionally occurred (Fig. 6, F and G). Similarly, the majority of KP-IR fibers were also devoid of dynorphin B immunoreactivity in the Inf and the InfS (Fig. 6, I and J), whereas this second antiserum performed well in regions rich in DYN fibers, including the ventromedial nucleus (Fig. 6K).

### 5.2.3. Abundance of KP- and NKB-immunoreactive fibers in the InfS

The InfS was associated with the superficial and the deep capillary plexuses of the postinfundibular eminence [138]. Both were abundantly innervated by GnRH-IR axons (brown color in Fig. 8, A-F), suggesting they contribute to the GnRH supply of adenohypophysial gonadotrophs. The relative abundance of the different types of

labeled fibers around the two capillary plexuses was reminiscent of the observations in the Inf. Accordingly, portal blood vessels (BV) were surrounded by dense networks of NKB-IR fibers (Fig. 8, A-C) and innervated only moderately by KP-IR fibers (Fig. 8, D-F). Very few DYN-IR fibers occurred in the proximity of the portal capillaries (Fig. 8G, I, J). This low level of DYN signal did not reflect a technical limitation, given that the magnocellular neurosecretory tract was immunolabeled heavily in the same sections (Fig. 8H).

The analysis of immunofluorescent specimens confirmed that NKB dominates over KP around the portal vasculature and NKB-IR fibers often lack KP labeling (Fig. 6, C-E). Similarly to the Inf, the InfS contained both single-labeled and double-labeled KP-IR fibers (Fig. 6, C-E). In sections dual-labeled for KP and DYN, labeled fibers were mostly distinct, although rare colocalization cases were also detectable (Fig. 6H).



Figure 8. Results of immuno-peroxidase studies illustrate the differential innervation of the portal capillary plexus by NKB-, KP- and DYN-IR fibers in a 31-year-old man. A-J: The black silver-gold intensified Ni-DAB chromogen was used to detect NKB (A-C), KP (D-F) and DYN (G-J) immunoreactivities in adjacent sections of the InfS. Note that GnRH has also been visualized in A-F with brown DAB. The postinfundibular eminence with its deep (B, E, I) and superficial (C, F, J) plexuses of portal blood vessels (BV) is surrounded by GnRH-IR hypophysiotropic axons (brown color in A-F). Out of the axons immunoreactive for the three KNDy peptides (black color), those with NKB immunoreactivity represent the most frequently encountered phenotype (A) and densely innervate both the deep (B) and the superficial (C) portal capillaries; this innervation raises the possibility of NKB release into the hypophysial portal circulation. The KP-IR innervation of the portal BVs is of much lower density (D-F). Although DYN-IR axons are readily detectable in the InfS (G) and contribute to the magnocellular axon tract (H), they only occur rarely around the portal BVs (H-I). Scale bar=100 $\mu$ m for A, D, G and 10 $\mu$ m elsewhere.

### 5.2.4. Frequency of KP-IR and NKB-IR appositions onto GnRH-IR neurons

Sections double-labeled with the silver-gold-intensified Ni-DAB and DAB chromogens were used to obtain quantitative estimates about NKB-IR and KP-IR inputs to GnRH-IR neurons. Microscopic analysis confirmed that NKB-IR and KP-IR axons provide axo-somatic and axo-dendritic inputs to GnRH neurons in the Inf (Fig. 9). Quantitative analysis (Fig. 10) established that GnRH-IR perikarya and dendrites, respectively, received 6 and 5 times heavier NKB-IR (Fig. 9A) than KP-IR (Fig. 9B) innervation (GnRH perikarya: P=0.004; GnRH dendrites: P=0.005, by ANOVA).



Figure 9. NKB-IR axons (black color in A) show a much higher abundance in the Inf and establish considerably more axo-somatic and axo-dendritic juxtapositions (arrows) onto GnRH-IR neurons (brown color) than do KP-IR axons (black color in B). Scale bar=20µm.



Figure 10. High-power light microscopic analysis of sections, dual-immunolabeled with the combined use of silver-gold-intensified Ni-DAB and DAB chromogens, was carried out to determine the relative incidences of NKB-IR and KP-IR neuronal appositions to the somata (left columns) and the dendrites (right columns) of GnRH-IR neurons. The counts were obtained from all GnRH-IR cell bodies and dendrites identified in 1-3 infundibular sections of each individual. The number of NKB-IR contacts is about 6 times higher on the somatic and 5 times higher on the dendritic compartment of GnRH neurons than those established by KP-IR axons. \*P<0.01 by ANOVA.

# 5.3. Sexual dimorphism of kisspeptin and neurokinin B systems in aged human individuals

### 5.3.1. Perikaryon size of KP-IR and NKB-IR neurons

KP-IR cell bodies (P=0.01) as well as NKB-IR cell bodies (P=0.002) were hypertrophied and their profile area was significantly larger in the Inf of aged women  $(284.2\pm27.3\mu m^2 \text{ for KP-IR and } 298.1\pm19.7\mu m^2 \text{ for NKB-IR neurons})$  in comparison with aged men  $(154.8\pm19.2\mu m^2 \text{ for KP-IR and } 190.4\pm20.4\mu m^2 \text{ for NKB-IR neurons})$  (Fig. 11, C, D, G, H and Fig. 12).



Figure 11. KP (A-D) and NKB (E-H) immunoreactivities were visualized using the silver-gold intensified Ni-DAB chromogen. The photomicrographs were taken from the Inf of 59- (A, C) and 62- (E, G) year-old male and 66-year-old (B, D, F, H) female individuals. Both KP-IR (D) and NKB-IR (H) cell bodies are larger in postmenopausal women compared with aged men (C and G, respectively). Neuronal hypertrophy is likely to reflect lack of estrogen negative feedback in postmenopausal women. KP neurons also show robust sex differences in the number of KP-IR cell bodies and the density of KP-IR fibers are much higher in aged women (B) compared with men (A). The number of NKB-IR cell bodies is also higher in women (F) compared with men (E), but the difference is less dramatic than in case of KP-IR cell bodies. No obvious sexual dimorphism exists in the regional density of NKB fibers (E-H). For quantitative comparisons, see Figures 12-14. Scale bar=200µm in A, B, E, F and 50µm in C, D, G, H.



Figure 12. The mean surface area covered by the silver-gold-intensified Ni-DAB chromogen was used as an index of perikaryon size. The area of immunolabeled cell bodies differs between aged men and women in case of both peptides. The difference likely reflects a robust neuronal hypertrophy in postmenopausal females. \*P<0.05.

## 5.3.2. Incidence of KP-IR and NKB-IR cell bodies in the Inf

Quantitative analysis of the labeled cell bodies revealed the following results:

- In males, NKB-IR cell bodies showed a significantly higher incidence compared with KP-IR cell bodies (P=0.0005). NKB-IR neurons outnumbered KP-IR neurons by 120% (Fig. 11, A, E and Fig. 13).
- In females, the mean incidence of NKB-IR cell bodies was only 23% higher than that of KP-IR perikarya. This subtle difference was not statistically significant (P=0.28) (Fig. 11, B, F and Fig. 13).
- KP-IR cell bodies showed a 170% higher mean density (incidence of cell bodies per 0.25mm<sup>2</sup> counting frame) in females compared with males. This robust sex difference was statistically significant (P=0.002) (Fig. 11, A, B and Fig. 13).
- NKB-IR cell bodies showed a 51% higher mean incidence in females *vs.* males. This sex difference was much less conspicuous than for KP-IR cell bodies, but statistically significant (P=0.006) (Fig. 11, E, F and Fig. 13).



Figure 13. The maximal number of immunoreactive cell bodies per  $0.25 \text{mm}^2$  counting frame (1-6 per subject) was determined with the aid of an ocular frame and used as the index of regional neuron density. The abundance of KP neurons determined this way is much higher in aged women compared with aged men, whereas the number of NKB cell bodies is only slightly (but significantly) higher in females. Note also that men, unlike women, contain 2.2 times as many NKB-IR as KP-IR cell bodies. \*P<0.05.

### 5.3.3. Regional density of KP-IR and NKB-IR fibers

The Inf of aged men exhibited a few KP-IR fibers only, in contrast with dense fiber networks in the Inf of postmenopausal women (Fig. 11, A-D). NKB-IR axons did not show this robust sex difference (Fig. 11, E-H). Quantitative analysis of labeled axons (area covered by immunohistochemical signal per total area) established the following results:

- In the Inf of males, the mean density of NKB-IR axons was 280% higher than that of KP-IR fibers. The difference was statistically significant (P=0.004) (Fig. 11, A, C, E, G and Fig. 14).
- In the Inf of females, the densities of NKB-IR and KP-IR fibers did not differ statistically (P=0.41) (Fig. 11, B, D, F, H and Fig. 14).
- KP-IR fibers showed a robust sexual dimorphism, with a 161% higher density in females vs. males. This difference was statistically significant (P=0.02) (Fig. 11, A-D and Fig. 14).

NKB-IR fibers showed only 8.4% higher mean density in females than in males and the sexes did not differ statistically (P=0.60) (Fig. 11, E-H and Fig. 14), in contrast with the dramatic and significant sexual dimorphism of the KP-IR fiber network.



Figure 14. The density of NKB-IR fibers was 2.8-fold higher than the density of KP-IR fibers in aged males, but not different in aged females. The density of KP-IR fibers showed a dramatic sexual dimorphism and was 2.5-fold higher in aged females vs. males. The mean density of NKB-IR fibers did not differ significantly between sexes. \*P<0.05.

## 5.3.4. Frequency of KP-IR and NKB-IR appositions onto GnRH-IR neurons

For the sections double-labeled for KP and GnRH or NKB and GnRH were used silver-gold-intensified Ni-DAB and DAB chromogens, in combination. The high-power light microscopic analysis of these sections confirmed the previous observation from our laboratory [49] that KP-IR axons establish axo-somatic and axo-dendritic contacts on GnRH neurons of the Inf (Fig. 15, A-D). In the present study, we observed similar axo-somatic and axo-dendritic appositions between NKB-IR axons and GnRH-IR neurons (Fig. 15, E-H).



Figure 15. Sections double-labeled for KP and GnRH (A-D) or NKB and GnRH (E-H) with the combined use of silver-gold-intensified Ni-DAB and DAB chromogens demonstrate that KP-IR axons establish axo-somatic (A, B) and axo-dendritic (C, D) contacts (arrows) on GnRH neurons of the Inf. Similar contacts can also be observed in high numbers between NKB-IR axons and GnRH neurons (arrows in E-H). The KP-IR input is significantly heavier on GnRH-IR neurons in women than in men (compare B to A and D to C). In both sexes, GnRH-IR cell bodies and dendrites receive more NKB-IR (E-H) than KP-IR (A-D) input. In contrast with the dramatic sexual dimorphism of KP-IR inputs, no significant sex difference can be observed in the incidence of NKB-IR axo-somatic (E, F) and axo-dendritic (G, H) juxtapositions to GnRH-IR neurons. (A, C: 59-year-old male; E, G: 52-year-old male; B, D: 57-year-old female; F, H: 64-year-old female). Scale bar=20µm.

The semiquantitative analysis of KP-IR contacts on GnRH-IR cell bodies and dendrites revealed a significantly heavier KP-IR input to the cell bodies (P=0.03) and the dendrites (P=0.021) of GnRH-IR neurons in women compared with men (Fig. 16).

In both sexes, GnRH-IR cell bodies and dendrites received significantly heavier NKB-IR input than KP-IR input (male cell bodies: P=0.002; male dendrites: P=0.002; female cell bodies: P=0.02; female dendrites: P=0.02) (Figure 16).



Figure 16. The relative frequencies of KP-IR (left column pairs) and NKB-IR (right column pairs) neuronal appositions onto the somata (A) and dendrites (B) of GnRH-IR neurons. The innervation values are highly similar on GnRH cell bodies (A) and dendrites (B). Note that the number of NKB-IR contacts is significantly higher than the number of KP-IR contacts on both the somatic (A) and the dendritic (B) membranes and in both males and females. The KP-IR input to GnRH cell bodies (A) and dendrites (B) is highly sexually dimorphic. The mean incidence of KP-IR contacts is 88.2% higher on GnRH-IR cell bodies and 177.4% higher on GnRH-IR dendrites in postmenopausal females vs. aged males. In contrast, there is no statistical difference between the two sexes in the incidences of NKB-IR contacts, although the means are somewhat higher in females. \*P<0.05.

In contrast with the dramatic sexual dimorphism of KP-IR inputs, no significant sex difference was observed in the incidence of NKB-IR axo-somatic and axo-dendritic juxtapositions to GnRH-IR neurons (axo-dendritic contacts: P=0.24; axo-somatic contacts: P=0.36) (Figure 16). However, there was a trend for somewhat more NKB-IR contacts in females.

The counts were obtained from all GnRH-IR cell bodies and dendrites that were identified in 1-3 sections of the Inf from each of 8 male and 6 female individuals.

#### 5.3.5. Colocalization of KP and NKB in neuronal afferents to GnRH neurons

The triple-immunofluorescent specimens contained numerous KP-IR and NKB-IR fibers. The sensitivity of the approach was not sufficient to visualize high numbers of KP-IR and NKB-IR cell bodies. The axonal KP and NKB immunolabeling showed a partial overlap only and GnRH neurons were most frequently contacted by single-labeled axons both in males and females (Fig. 17). The semiquantitative analysis of afferent contacts onto GnRH-IR cell bodies and dendrites (Fig. 18) established that  $8.8\pm5.5\%$  of NKB-IR afferents in males also contained KP immunoreactivity. The ratio of double-labeled afferents was  $31.3\pm4.9\%$  in females (P=0.01). Similarly, the percentage of KP-IR contacts that colocalized NKB signal was significantly higher (P=0.033) in females (25.8±2.4\%) than in males (10.2±4.6\%).



Figure 17. The simultaneous immunofluorescent detection of NKB (green color), KP (red color) and GnRH (blue color), followed by confocal analysis, revealed direct appositions of single- and double-immunolabeled (yellow color) axons onto GnRH-IR cell bodies and dendrites. Note in stacks of optical slices that NKB/KP dual-IR appositions (yellow) are more typical in postmenopausal female (B and high-power image of the framed dendritic region B') than in aged male (A and high-power inset A') subjects. Red and green arrows indicate afferent contacts that are single-labeled for KP and NKB, respectively, whereas yellow double-arrows indicate double-labeled appositions. Scale bars=10µm (Bars in A and A' also refer to B and B', respectively).



Figure 18. As revealed on triple-immunofluorescent specimens, GnRH neurons receive KP-IR, NKB-IR and double-labeled neuronal contacts. The percentage of KP-IR contacts that colocalized NKB signal is significantly higher in females ( $25.8\pm2.4\%$ ) than in males ( $10.2\pm4.6\%$ ). Similarly, while only  $8.8\pm5.5\%$  of NKB-IR afferents in males contain KP immunoreactivity, the extent of this colocalization is  $31.3\pm4.9\%$  in females. \*P<0.05.

## 5.4. Aging related changes of the human hypothalamic kisspeptin and neurokinin B neuronal systems

## 5.4.1. Incidence of KP-IR and NKB-IR perikarya in the Inf

NKB-IR cell bodies showed a significantly higher incidence than KP-IR cell bodies (P=0.00004) in young men, and outnumbered KP-IR neurons 3.7-fold (Fig. 19, A, E and Fig. 20). NKB-IR cell bodies also outnumbered KP-IR perikarya in aged men (P=0.0005), but only 2.2-fold (Fig. 19, B, F and Fig. 20).

Aging was associated with increased perikaryon numbers. KP-IR cell bodies showed a 2.6-fold higher mean density in aged compared with young men (P=0.004) (Fig. 19, A, B and Fig. 20). NKB-IR cell bodies also showed higher mean incidence in aged compared with young men (P=0.016) (Fig. 19, E, F and Fig. 20), but the difference was only 1.5-fold.



Figure 19. KP (A-D) and NKB (E-H) immunoreactivities were visualized using the silver-gold intensified Ni-DAB chromogen. The representative photomicrographs were taken from the Inf of 31- (A, C), 40- (E, G) and 67- (B, D, F, H) year-old individuals. Overall, NKB-IR perikarya and fibers are more abundant than KP-IR elements in both young and aged subjects. KP-IR neurons show robust age-related changes; the number of KP-IR cell bodies and the density of KP-IR fibers are much higher in aged (B, D) compared with young (A, C) men. NKB immunoreactivity also increases with age, although changes are of lower degrees. Note that the number of NKB-IR cell bodies is higher in aged (F, H) compared with young (E, G) men and age-related increase also takes place in the regional density of NKB-IR fibers (E-H). Scale bar=50µm.



Figure 20. The maximal number of immunoreactive cell bodies per  $0.25 \text{mm}^2$  counting frame was determined with the aid of an ocular frame and used as the index of regional neuron density. Note that NKB-IR neurons outnumber KP-IR neurons 3.7-fold in young and 2.2-fold in aged men. The abundance of KP neurons determined with this approach is 2.6 times higher in aged than in young men. Similarly, the number of NKB-IR cell bodies increases with age about 1.5-fold. \*P<0.05.

### 5.4.2. Perikaryon size of NKB-IR neurons

The mean profile area of NKB-IR cell bodies was 22.1% higher in the Inf of aged  $(204.2\pm10.6\mu m^2)$  compared with young  $(167.3\pm9.6\mu m^2)$  men. This subtle increase between the two age groups was statistically significant (P = 0.02).

### 5.4.3. Regional density of KP-IR and NKB-IR fibers

Quantitative analysis of the relative density of immunolabeled fibers revealed the following differences:

- The mean density of NKB-IR axons was 6.1-fold higher than that of KP-IR fibers in the Inf of young men (P=0.000003) (Fig. 19, A, C, E, G and Fig. 21). The density of NKB-IR axons was also high in aged men, but only 2.85-fold higher than the density of KP-IR axons (P=0.004) (Fig. 19, B, D, F, H and Fig. 21).
- Aging was associated with increased KP and NKB fiber densities. KP-IR fibers showed 3.1-fold higher density in aged than in young men (P=0.032)

(Fig. 19, A-D and Fig. 21), whereas the density of NKB-IR axons showed a 1.5-fold aging-related increase (P=0.018) (Fig. 19, E-H and Fig. 21).



Figure 21. The area covered by immunoreactive fibers (divided by the total area analyzed) was determined. The density of NKB-IR fibers was 6.1-fold higher than the density of KP-IR fibers in young males, and 2.85-fold larger in aged females. The density of KP-IR fibers also showed a dramatic age-related difference and was 3.1-fold higher in aged vs. young males. The mean density of NKB-IR fibers also differs significantly between the age groups. \*P<0.05.

### 5.4.4. Colocalization of KP and NKB in neuronal perikarya of the Inf

The quantitative analysis of labeled cell bodies in dual-immunofluorescent specimens (Fig. 22 and Fig. 23) confirmed the dominance of NKB-IR over KP-IR cell bodies in both young and aged men.

In young men 72.7 $\pm$ 6.0% of KP-IR perikarya also contained NKB immunoreactivity (Fig. 22A). Similarly, in aged men 77.9 $\pm$ 5.9% of the KP-IR cell bodies contained NKB immunoreactivity (Fig. 22B). There was a lower degree of overlap in the opposite direction. In young men only 35.8 $\pm$ 5.1% of the NKB-IR neurons contained KP immunoreactivity and most of the perikarya were single-labeled. In aged men, the ratio of double-labeled NKB neurons increased to 68.1 $\pm$ 6.8%. This aging-related increase in the percentage of KP-IR NKB neurons was statistically significant (P=0.001).



Figure 22. Dual-immunofluorescent studies, which simultaneously used two different tyramide signal amplification approaches, revealed the dominance of NKB immunoreactivity (green) over KP immunoreactivity (red) in the Inf of a young (36-year-old) (A) male human individual. Note that this dominance is less obvious in the aged man (67-year-old) (B) whose Inf exhibits greatly enhanced KP-immunoreactivity. Red and green arrows in high-power insets indicate single-labeled perikarya. Yellow double arrows point to NKB/KP neurons, which constitute  $35.8 \pm 5.1\%$  of NKB-IR neurons in the young and  $68.1\pm 6.8\%$  of NKB-IR neurons in the aged male group. Scale bar, 100 µm (low-power insets; A and B) and 40 µm (high-power insets).



Figure 23. The ratios of double-labeled NKB-IR and KP-IR perikarya were determined quantitatively from dual-immunofluorescent specimens, in which tyramide signal amplification approaches were applied to maximize both labeling. In young men,  $72.7 \pm 6.0\%$  of KPIR perikarya also contained NKB-immunoreactivity. Similarly, in aged men,  $77.9 \pm 5.9\%$  of the KP-IR cell bodies contained NKB immunoreactivity. In contrast, in young men, only  $35.8 \pm 5.1\%$  of the NKB-IR neurons contained KP-immunoreactivity, and most of the perikarya were single-labeled. In aged men, the ratio of the double-labeled NKB neurons increased to  $68.1 \pm 6.8\%$ . \*P < 0.05.

## 5.4.5. Incidence of KP-IR and NKB-IR appositions onto GnRH-IR neurons

For the sections double-labeled for KP and GnRH or NKB and GnRH were used the silver-gold-intensified Ni-DAB and DAB chromogens, in combination. The high-power light microscopic analysis of these sections confirmed that KP-IR and NKB-IR axons establish axo-somatic and axo-dendritic contacts onto GnRH-IR neurons of the Inf (Fig. 24).



Figure 24. Sections double-labeled for KP and GnRH (A-D) or NKB and GnRH (E-H) with the combined use of silver-gold-intensified Ni-DAB and DAB chromogens demonstrate that KP-IR axons establish axo-somatic (A, B) and axo-dendritic (C, D) contacts (arrows) on GnRH neurons of the Inf. Similar contacts can also be observed in high numbers between NKB-IR axons and GnRH neurons (arrows in E-H). The KP-IR input is significantly heavier on GnRH-IR neurons from aged than young men (compare B to A and D to C). In both age groups, GnRH-IR cell bodies and dendrites receive more NKB-IR (E-H) than KP-IR (A-D) input. Similar to the age-related difference of KP-IR inputs, statistically significant age-related difference can be observed in the incidence of NKB-IR axo-somatic (E, F) and axo-dendritic (G, H) juxtapositions to GnRH-IR neurons. (A, C: 31-year-old male; E, G: 40-year-old male; B, D: 62-year-old male; F, H: 67-year-old male) Scale bar=50μm.

The quantitative analysis of appositions (Fig. 25) established that the NKB-IR innervation is heavier compared with the KP-IR innervation. In young men, GnRH-IR cell bodies received 6 times more NKB-IR than KP-IR appositions and GnRH-IR dendrites received 6.4 times more NKB-IR than KP-IR appositions (cell bodies: P=0.013; dendrites: P=0.005) (Fig. 24 and 25). In aged men, GnRH-IR cell bodies received 5.3 times more NKB-IR than KP-IR appositions and dendrites received 6.4 times more NKB-IR than KP-IR appositions (cell bodies received 5.3 times more NKB-IR than KP-IR appositions (cell bodies received 6.4 times more NKB-IR than KP-IR appositions (cell bodies received 5.4 times more NKB-IR than KP-IR appositions (cell bodies received 6.4 times more NKB-IR than KP-IR appositions (cell bodies received 6.4 times more NKB-IR than KP-IR appositions (cell bodies received 6.4 times more NKB-IR than KP-IR appositions (cell bodies received 6.4 times more NKB-IR than KP-IR appositions (cell bodies received 6.4 times more NKB-IR than KP-IR appositions (cell bodies received 6.4 times more NKB-IR appositions than KP-IR appositions (cell bodies: P=0.0002; dendrites: P=0.0001) (Fig. 24 and Fig. 25).

Both the KP-IR and the NKB-IR contacts showed significant aging-dependent increases. The quantitative analysis of KP-IR appositions revealed a 2.2-fold heavier KP-IR input to the cell bodies (P=0.004) and a 2-fold heavier KP-IR input to the dendrites (P=0.007) of GnRH-IR neurons in the Inf of aged, in comparison with young, men (Fig. 24 and Fig. 25). In addition, the percentage of GnRH neurons receiving at least one KP-IR axo-somatic apposition increased from 53.7% in young to 84.9% in aged men (P=0.04). Less dramatic, though significant, aging-related increases were observed in the incidences of NKB-IR axo-somatic and axo-dendritic appositions onto GnRH-IR neurons. Axo-somatic contacts were 2-fold and axo-dendritic contacts 1.9-fold more frequent in aged than in young individuals (axo-dendritic contacts: P=0.001; axo-somatic contacts: P=0.006) (Fig. 24 and Fig. 25).



Figure 25. High-power light microscopic analysis of sections, dual-immunolabeled with the combined use of silver-gold-intensified Ni-DAB and DAB chromogens (see Figs. 24), was carried out to determine the relative frequencies of KP-IR (left column pairs) and NKB-IR (right column pairs) neuronal appositions onto the somata (A) and dendrites (B) of GnRH-IR neurons. The counts were obtained from all GnRH-IR cell bodies and dendrites that were identified in 1-3 sections of the Inf from each of 11 young and 9 aged male individuals. The innervation values are highly similar on GnRH cell bodies (A) and dendrites (B). Note that the number of NKB-IR contacts is significantly higher than the number of KP-IR contacts on both the somatic (A) and the dendritic (B) membranes and in both young and aged males. The KP-IR input to GnRH cell bodies (A) and dendrites (B) is different in the two age groups; the mean incidence of KP-IR contacts is 121.9% higher on GnRH-IR cell bodies and 62.2% higher on GnRH-IR cell bodies in aged *vs.* young males. The mean incidence of NKB-IR contacts is 396% higher on GnRH-IR cell bodies and 91.4% higher on GnRH-IR dendrites in aged than in young males. \*P<0.05.

#### 5.4.6. Colocalization of KP and NKB in neuronal afferents to GnRH neurons

In triple-immunofluorescent specimens, the axonal KP and NKB immunolabeling showed a partial overlap only. GnRH neurons were most frequently contacted by single-labeled axons both in young (not shown) and aged (Fig. 26) men. The quantitative analysis of the KP/NKB colocalization revealed KP immunoreactivity in  $7.3\pm1.5\%$  of NKB-IR afferents in young men. The ratio of double-labeled afferents was similarly low (9.5±3.7%) in aged men and there was no significant age effect on the

colocalization percentage (P=0.66). NKB signal was observed in  $7.9 \pm 2.8\%$  of KP-IR afferents in young and  $11.6 \pm 4.6\%$  of KP-IR afferents in aged men.



Figure 26. The simultaneous immunofluorescent detection of NKB (green), KP (red), and GnRH (blue), followed by confocal analysis, revealed direct appositions of singleand double-immunolabeled (yellow) axons onto GnRH-IR cell bodies and dendrites in the Inf of a 50-year-old subject. Red and green arrows indicate afferent contacts that are single-labeled for KP and NKB, respectively. Dual-labeled afferents indicated by the yellow double arrow represent less than 10% of all KP-IR and NKB-IR inputs. Note that these percentages are similarly low in young and aged subjects. Insets A and B correspond to framed regions shown in the low-power central micrograph. Scale bar, 10  $\mu$ m (4  $\mu$ m in high-power insets).

## 6. Discussion

## 6.1. Possible contribution of the estrogenic regulation of RF-amide related peptide to estrogen feedback mechanisms

In one study (5.1) underlying this thesis we provide evidence that E2 down-regulates RFRP mRNA expression of OVX mice. In addition, we show that nuclear ER- $\alpha$  occurs in a subset (18.7±3.8%) of RFRP neurons, whereas ER- $\beta$  is absent from these cells.

### 6.1.1. The expression of RFRP mRNA is negatively regulated by estrogen

In our quantitative in situ hybridization studies (5.1) we found that a 4-day E2 regimen down-regulates RFRP mRNA levels in the DMN of ovariectomized mice. This observation suggests that in the presence of high E2, RFRPs may exert a reduced inhibition on the reproductive axis via actions on GnRH neurons, gonadotrophs, or both. We propose that the withdrawal of the inhibitory RFRP tone from the reproductive axis in proestrus when E2 level is high may play a physiological role in positive estrogen feedback. In contrast with the estrogenic regulation of RFRP mRNA expression we report here in mice, a recent quantitative PCR study has found no difference between RFRP mRNA levels of ovariectomized rats and rats killed at the time of a luteinizing hormone surge induced by exogenous E2 and progesterone administration [139]. It requires clarification to what extent this discrepancy is due to differences between species, animal treatments, or sensitivity of the applied RNA quantification methods. In ewes, morphological evidence exists that the active reproductive status coincides with a reduced RFRP inhibitory tone, as reflected by decreased numbers of RFRP-3-IR cell bodies and fiber contacts on GnRH neurons during breeding season [62]. Interestingly however, E2 treatment did not affect RFRP mRNA levels of ovariectomized ewes in the same study [62].

### 6.1.2. A relatively small subset of RFRP neurons expresses ER-α immunoreactivity

We have carried out colocalization experiments (5.1) with dual-label immunohistochemistry in an attempt to identify which ER isoform may account for the estrogenic down-regulation of RFRP mRNA expression. These experiments found low levels of ER- $\alpha$  signal in a relatively small subset (18.7±3.8%) of RFRP neurons, raising the possibility that E2 may act via ER- $\alpha$  to regulate RFRP mRNA expression directly. Alternatively, the estrogenic regulation of RFRP neurons may also be indirect, considering the relatively low levels of ER- $\alpha$  in RFRP cells. On the other hand, we found no evidence for ER- $\beta$  in RFRP neurons using the same antiserum and optimized detection method that successfully detected ER- $\beta$  in GnRH neurons of the rat in our previous studies [97, 98] and ER- $\beta$  in the paraventricular nucleus of the mouse in the present study.

The RFRP synthesizing neurons are found in the dorsomedial nucleus in Syrian hamsters, rats and mice [88]. However, differences in reproductive physiology of rodent species may relate to the RFRP neuronal system. While in our present study (**5.1**), we only found low levels of ER- $\alpha$  in 18.7±3.8% of RFRP neurons in mice, 40% of RFRP neurons express ER- $\alpha$  in the Syrian hamsters [88]. In Syrian hamsters, melatonin-sensitive neurons of the dorsomedial hypothalamic nucleus play a critical role in the seasonal onset of reproductive quiescence in the short-day photoperiod, which can be prevented by the lesion of the DMN [140, 141]. The issue of whether or not RFRP neurons are specifically involved in the seasonal regulation of reproduction in seasonal breeders requires clarification.

### 6.1.3. Putative sites of action of RFRP on the reproductive axis

While it is generally agreed that GnIH/RFRP neurons innervate GnRH cells in birds, rodents, sheep and primates [62, 85, 88, 91-93], an unresolved paradox relates to the putative adenohypophysial site of action of RFRP peptides in various species. GnIH/RFRP-IR terminals do occur in the neurosecretory zone of adenohypophysis in birds [84, 90], sheep [142] and monkeys [89], but not in rodents [88, 91]. Furthermore, RFRP synthesizing neurons in rats do not accumulate Fluoro-Gold from the systemic circulation, indicating that they have no access to the hypophysial portal circulation to act on the adenohypophysis [91]. Indeed, the RFRP antagonist RF9 is not capable of eliciting LH secretion from static pituitary cultures [42]. Conversely, in support of the direct adenohypophysial effect of RFRP-3, a dose-dependent reduction in GnRH-stimulated luteinizing hormone secretion has been reported in adenohypophysial cultures of various species, including rodents [142-144].

In summary, in this study (5.1) we demonstrate that E2 down-regulates RFRP mRNA expression of OVX mice. In view that these neurons communicate with GnRH cells and RFRP can directly inhibit the electric activity of GnRH neurons [94], we propose that the negative regulation of RFRP expression may contribute to estrogen feedback to the reproductive axis. The observation of the weak ER- $\alpha$  signal in a small subset (18.7%) of RFRP neurons may indicate that E2 regulates RFRP gene expression directly, although the possibility that the action is indirect also needs to be considered.

# 6.2. Species differences in the neuropeptide compliment of hypothalamic kisspeptin neurons

Results of our human immunohistochemical studies (**5.2**) provide evidence for severe limitations of the 'KNDy neuron' terminology and concept. Specifically, our observations indicate that in young male humans the majority of NKB-IR neurons in the Inf, their processes and contacts onto GnRH neurons do not contain detectable amounts of KP immunoreactivity. Furthermore, KP-IR neuronal elements without NKB labeling occur frequently, in addition to NKB/KP dual-phenotype structures. Finally, we observed that most KP-IR neurons and fibers are devoid of DYN immunoreactivity in this human model.

## 6.2.1. Species differences in the colocalization of KNDy peptides

We propose that the different colocalization patterns in our human study (**5.2**) and in previous animal experiments partly reflect species differences in reproductive mechanisms [145]. These may include the absence of DYN signal from most KP neurons and fibers in humans, which is in contrast with the extensive coexpression in the rodent [82, 101, 103], sheep [47] and goat [73] ARC. Another putative species difference is the large excess of NKB-IR over KP-IR perikarya in this human study, as opposed to the two-fold excess of NKB over KP perikarya in the ARC that has been reported for male mice [103].

### 6.2.2. Role of NKB in the regulation of the human reproductive axis

The tachykinin peptide NKB plays a crucial role in human reproduction and inactivating mutations of the NKB and NK3 encoding genes cause normosmic hypogonadotropic hypogonadism [78, 79]. Although the first reports did not indicate fertility deficits in the NK3 mutant mice [146], later analysis focusing on the reproductive phenotype noticed subfertility [147], suggesting functional similarities in NKB/NK3 signaling between the human and mouse species.

Conflicting results of previous experiments suggest that the net effect of NKB on LH secretion depends on animal species and endocrine paradigms. Intraperitoneal or icv NKB administration to male mice had no effect on serum LH [148], whereas the icv injection of the selective NK3 agonist senktide reduced LH secretion in ovariectomized rats treated with a low dose of estradiol [149]. Reduced LH secretion in response to senktide was also observed in ovariectomized and in ovariectomized and estradiol treated rats [150] and in ovariectomized mice [101], whereas another study on rats found stimulatory effect on LH secretion in the presence of physiological levels of estradiol [151]. Senktide also stimulated LH secretion in castrated male monkeys [72], and in the follicular, but not in the luteal, phase in ewes [152], whereas reduced net LH secretion was observed in ovariectomized goats [73].

Multiunit activity recorded in the ARC is considered to be an electrophysiological correlate of the GnRH pulse generator activity. The coordinated bursts of neuronal firing occur in synchrony with the LH secretory pulses in various animal species [73, 153], including primates [154]. Senktide dose-dependently suppressed the frequency of pulsatile LH secretion and inhibited hypothalamic multiunit activity volleys in ovariectomized rats, independently of gonadal steroid levels [150]. In contrast, a robust increase in the frequency of multiunit activity volleys was observed in ovariectomized goats [73].

NKB mainly acts upstream from GnRH neurons. For example, the stimulatory effects of intravenous NKB and senktide on LH secretion could be abolished by the GnRH receptor antagonist acyline [72]. One major target site for NKB actions appears to be on NKB (KNDy) neurons of the ARC/Inf. Accordingly, we found numerous NKB-IR afferent contacts on human NKB neurons; similar contacts were reported previously in rats [82] and sheep [47, 83]; NK3 receptors are present on these cells in

64

rodents and sheep [82, 101, 155] and KP (KNDy) neurons in the ARC of male mice respond with c-Fos expression [103] and depolarization [103] to senktide.

There is little consensus regarding the possibility that NKB also influences GnRH neurons directly. In sheep, GnRH neurons do not express NK3 immunoreactivity [155]. In mice, while single-cell microarray and RT-PCR studies provided proof for NK3 mRNA expression in GnRH neurons [156], in situ hybridization studies were unable to confirm this finding [103]. Also, senktide did not activate mouse KNDy neurons in vitro [103]. In rats, while immunohistochemical studies found evidence for NK3 immunoreactivity in only 16% of GnRH-IR cell bodies [80], the receptor was more abundant on GnRH-IR axon terminals in the median eminence [80] where frequent appositions between GnRH-IR and NKB-IR axons occurred [80, 81]. Although NKB in itself did not alter GnRH release from hypothalamic explants of male rats, it abrogated the KP-induced release of GnRH, suggesting a complex mode of action which is likely parallel with, and not upstream from, the KP action [157]. Recent functional evidence from KISS1R-KO mice indicates that intact KP/KISS1R signaling is required for the suppression of LH secretion by senktide. This finding provides support for the concept that the dominant action of NKB is upstream from KP neurons, instead of being exerted directly on GnRH cells [157].

Results of the present human study (5.2) indicate that NKB-IR axons abundantly innervate human GnRH neurons and the incidence of these contacts is several times higher than those of KP-IR axons. It will require clarification whether this anatomical pathway uses NKB/NK3 signaling. Alternatively, neurotransmitter(s) other than NKB may act in this communication. This neurotransmitter is unlikely to be KP or DYN, in view of their relative paucity in NKB neuron, at least in young male human individuals. In addition to innervating GnRH-IR cell bodies and dendrites, NKB-IR axons also represented the most abundant KNDy peptide around the portal capillary plexuses of the human postinfundibular eminence [138]. This hypothalamic site that lies outside the blood-brain barrier may represent an important site of interaction between NKB-IR and GnRH-IR axons. It also remains possible that NKB is released into the hypophysial portal circulation to influence adenohypophysial functions. In vitro evidence from rats, indeed, indicates that NKB can induce prolactin secretion from perifused pituitary cells [158].

### 6.2.3. Role of KP in the regulation of the human reproductive axis

In humans, KP/KISSR1 signaling plays a pivotal role in reproduction. Loss of function mutations of the genes encoding KISSR1 [159-161] and KP [162] result in hypogonadotropic hypogonadism. In recent models of the GnRH pulse generator, KP was proposed to provide the main output signal of the pulse generator neuronal network toward GnRH neurons. It is generally believed that independently from the species, KP acts directly on GnRH neurons that express KISS1R mRNA [50, 51, 106] and in mice indeed these neurons respond with depolarization to KP [51, 75, 163].

As in laboratory animals, KP increases LH secretion in men [39, 164, 165] and women [166, 167], most potently during the preovulatory phase of the menstrual cycle in the latter. It is interesting to note that the continuous intravenous infusion of KP enhanced the LH pulse frequency in men [39], indicating that KP not only acts on GnRH neurons, but also upstream from the pulse generator network. This finding suggests a species difference from the mouse in which KNDy neurons do not appear to synthesize KISS1R [168] and express only NK3 [101] and KOR [101] mRNAs.

### 6.2.4. Absence of DYN immunoreactivity from most KP elements

The opioid peptide DYN is rather ubiquitous and may have multiple sites of action upon the reproductive axis. These sites are likely upstream from the GnRH neuron that does not appear to express KOR in rats [169]. DYN is critically involved in progesterone negative feedback to GnRH neurons in ewes; the majority of DYN cells in the ARC of ovariectomized ewes contain progesterone receptor [170] and progesterone treatment increases preprodynorphin mRNA expression in the ARC and DYN levels in the cerebrospinal fluid [171].

DYN is an important regulator of the pulse generator system. In sheep, KOR antagonists stimulate the episodic secretion of LH during the luteal phase [172]. In ovariectomized goats, central administration of DYN decreases and KOR antagonist increases the frequency of the multiunit activity volleys and that of the LH secretory pulses [73]. Opioid peptides also regulate negatively the pulsatile release of prolactin and LH in humans; this inhibitory tone can be suspended by the blockade of opioid receptors with naloxone [173, 174].

The concept and terminology of the 'KNDy neurons' rely on the similar results of colocalization experiments from several animal species. DYN has been detected in NKB (and/or KP) neurons in the ARC of sheep [47, 83], mice [101, 103], rats [81, 82] and goats [73]. Moreover, the DYN receptor KOR is present in subsets of KNDy neurons in the ARC of mice [101, 103]. Our present immunohistochemical study to address the presence of DYN immunoreactivity in human KP neurons was also supported by previous reports in which preprodynorphin mRNA expression was detected in the human Inf [130] and the monkey ARC [120].

The somewhat unexpected absence of DYN immunoreactivity in most KP-IR somata and fibers of young male humans questions the universal importance of DYN peptides within NKB and KP neurons of the Inf and reveals an important difference from the rodent, sheep and goat species [47, 70, 73, 81, 101, 103, 105]. It is worthy to note that species also vary considerably regarding the sex steroid regulation of DYN in the ARC/Inf. Preprodynorphin expressing neurons showed reduced number in postmenopausal women [130] and in ovariectomized ewes [171]. There was no change in its mRNA expression in postmenopausal monkeys [120], whereas preprodynorphin mRNA was increased in the absence of sex steroids in mice [101].

The absence of DYN immunoreactivity from most KP-IR neurons and their fibers we report in this study (**5.2**) is unlikely to be entirely caused by the limited sensitivity of the applied immunohistochemical method because i) DDY-IR cell bodies (e.g. magnocellular perikarya in the supraoptic nucleus) and fibers (e.g. a dense fiber plexus in the ventromedial nucleus) were readily detectable elsewhere in the hypothalamus, ii) substantial colocalization with KP was also undetectable using the highly sensitive tyramide signal amplification method to visualize DYN or iii) using an antiserum against a different prodynorphin cleavage product, dynorphin B.

In conclusion, the immunohistochemical observations we made on hypothalamic tissue samples of young male human subjects question the universal validity of the KNDy neuron concept and terminology and suggest that the abundance of these peptides and their overlap are species-, sex- and age-dependent.

## 6.3. Importance of sex differences in the central regulation of human reproduction

### 6.3.1. Sex difference in perikaryon size of KP and NKB neurons

The studies of a putative sex difference in the perikaryon size of KP-IR and NKB-IR neurons (mean cross-sectional area of IR neurons) based on previous work indicating that the profile area of neurons in the Inf is significantly greater in postmenopausal women than in aged men [127]. This morphometric sex difference is thought to result primarily from postmenopausal alterations of the female Inf. In 1966, Sheehan and Kovacs reported neuronal hypertrophy in a subregion of the Inf termed subventricular nucleus. The hypertrophied neurons contained enlarged nuclei and nucleoli and a prominent Nissl substance. They occurred in female individuals above 50 years of age and similarly, in women with post-partum hypopituitarism [175]. Sheehan concluded that neuronal hypertrophy is a consequence of ovarian failure [176]. A series of in situ hybridization studies from Rance and co-workers established later that the hypertrophied neurons express ER-a [129], substance P [121], NKB [121], KP [58] and prodynorphin [130] mRNAs. It was concluded that the neuronal hypertrophy is not a compensatory response to neuronal cell death, because the number of neurons in the Inf was similar in pre- and postmenopausal women [177]. Ovariectomy was also capable of inducing the hypertrophy of KP mRNA expressing neurons in the Inf of monkeys [58], providing experimental evidence to the concept that the enlargement of neuronal perikarya is a consequence of the loss of estrogen.

The morphometric analyses of KP-IR and NKB-IR neurons in one of our studies (5.3) revealed a robust sex difference in the size (mean profile area) of KP-IR as well as NKB-IR neurons in the Inf of aged men and women. The mean cross-sectional area we determined for postmenopausally hypertrophied neurons ( $284.2\pm27.3\mu m^2$  for KP-IR and  $298.1\pm19.7\mu m^2$  for NKB-IR neurons) agrees well with the profile area of KP mRNA expressing hypertrophied neurons ( $280.9\pm17.3\mu m^2$ ), as measured previously in toluidine-blue stained samples [58]. Moreover, profile area values we determined for aged men ( $154.8\pm19.2\mu m^2$  for KP-IR and  $190.4\pm20.4\mu m^2$  for NKB-IR neurons) are also comparable to the mean cross-sectional area of toluidine blue-stained hypertrophied neurons in the Inf of older men ( $176.6\pm1.7\mu m^2$ ) [127], in spite of differences in the applied histochemical approaches.

The explanation for the sex difference we report in this study (**5.3**) for the immunoreactive profile areas of KP-IR and NKB-IR neurons represents a sex difference in aging-related neuronal hypertrophy. In women, this reaches a higher magnitude due to ovarian failure after menopause, whereas in men the presence of testosterone continues to inhibit the synthetic activity of KP and NKB neurons in the Inf.

### 6.3.2. Higher relative levels of NKB vs. KP immunolabeling

An important observation in this study (5.3) was the heavier NKB than KP immunolabeling in the Inf, in particular, in aged men. This difference represents a biological rather than technical phenomenon, given that both KP and NKB immunoreactivities showed obvious sex-dependent patterns instead of random variations across tissue specimens. The observation that the Inf contained 2.2 times as many NKB-IR as KP-IR cell bodies, 2.8 times higher NKB-IR as KP-IR fiber densities and 3-5 times as many NKB-IR as KP-IR neuronal contacts on GnRH neuron, indicate that a large subset of NKB neurons do not synthesize detectable amounts of kisspeptin in the Inf of aged men. This conclusion gains additional support from immunofluorescent results which confirmed a substantial level of mismatch between KP-IR and NKB-IR as well as KP-IR afferent contacts onto GnRH neurons. The sensitivity of the immunofluorescent detection method did not allow us to determine the exact percentage of overlap between KP and NKB synthesizing neuronal cell bodies in the two sexes.

The dominance of NKB over KP labeling was also present in postmenopausal females, although the differences did not reach the same magnitudes as in men. Notably, samples from postmenopausal women showed only 1.2 times higher NKB-IR than KP-IR neurons, only 1.6-2.2 times higher NKB-IR than KP-IR afferent contacts on GnRH neurons, and no significant difference in the overall density of NKB-IR vs. KP-IR fibers in the Inf.

It is worthy of note that the sexual dimorphism of KP and NKB neurons in the ARC/Inf region is not unique to humans. The ARC of the sheep contains higher NKB [117] and KP [70] cell numbers in females than in males. In rats, sex differences were reported in the projection fields of NKB-IR axons within the infundibular area [81].

The partial segregation of the KP from the NKB immunolabeling in one of our studies in humans (5.2), with the dominance of the latter, reveals a difference from the female sheep [47] and rodents [101] where the majority of KP neurons in the ARC also contain NKB (and dynorphins). Of note, a more recent study of male mice identified about twice as many NKB mRNA expressing as KP mRNA expressing neurons in the ARC [103]. Together with the previous report on females [101], this observation indicates a sex difference in the extent of coexpression. This possible sexual dimorphism is reminiscent to our observation that NKB-KP ratio is higher in aged human males than in females. A different colocalization pattern has been observed recently in the ARC of adults in castrated male monkey model in which no NKB neurons without KP labeling were observed and only 40-60% of KP-IR neurons contained NKB immunoreactivity [72].

Two possibilities may explain the sex difference in relative levels of NKB vs. KP immunolabeling in our study (5.3). First, the putative organizational effects of a developmental sex steroid exposure may generate a lower degree of colocalization in males than in females which may be maintained throughout life. Second, the different levels of negative sex steroid feedback between aged men and women may differentially influence the expression patterns of the two peptides. The dramatically low KP levels in aged men may reflect the higher reactivity of the KISS1 gene to the suppressive effects of T. KP [58, 120, 178] as well as NKB [120, 179] mRNAs have lower premenopausal than postmenopausal level in primates, indicating that sex steroids negatively regulate the expression of both neuropeptides. These data are also in accordance with the negative regulation of KP and NKB expression by sex steroids in various animal species [35, 44, 101, 112, 118-124, 178]. In our immunohistochemical studies of aged women, we observed high level of KP immunoreactivities that was similar to the level of NKB-IR labeling. Therefore, we propose that the postmenopausal disruption of negative estrogen feedback exerts more dramatic effects on KISS1 compared with NKB expression.

It is interesting to note that NKB-IR appositions occurred more frequently than KP-IR contacts on GnRH neurons. There was no sex difference in the incidence of NKB-IR contacts on GnRH neurons, although NKB neurons were present in higher numbers in the Inf of aged women than men. It is possible that NKB-IR systems outside the Inf, that are not sexually dimorphic, contribute significantly to these afferent contacts. The putative origins of such inputs may include NKB neurons in the periventricular nucleus and the bed nucleus of the stria terminalis [49]. It is also possible that the number of NKB-synthesizing neurons is similar in the Inf of the two sexes, but different peptide synthesis and release dynamics cause a sex differences in the number of detectable cell bodies, without affecting NKB immunoreactivity in fibers and their terminals on GnRH neurons.

## 6.3.3. Sex differences in KP immunoreactivity

KP is a strong stimulator of luteinizing hormone release in both women and men [164, 166], with a sexual dimorphism in KP responsiveness of the reproductive axis. While systemic KP-10 administration stimulates gonadotropin secretion in adult men, it only elicits luteinizing hormone and follicle stimulating hormone secretion in the preovulatory phase, but not during most of the follicular phase, of the menstrual cycle in women [180].

Kisspeptin represents the main output signal from KP/NKB/DYN (KNDy) neurons in a recently proposed model of the GnRH pulse generator [101]. KP neurons in the human can influence GnRH neurons via axo-somatic, axo-dendritic and axo-axonal communication [49]. Our immunohistochemical results revealed dramatic sex differences in the number of KP-IR cell bodies, the density of KP-IR fibers, the incidence of KP-IR contacts on GnRH neurons and the percent degrees of KP/NKB colocalization in neuronal afferents to GnRH neurons. All these parameters were significantly higher in aged women compared with aged men, which is compatible with a higher KP-mediated direct excitatory drive on the reproductive axis in postmenopausal women in comparison with aged men.

It remains to be established if developmental exposure to sex steroids contributes to the sexual dimorphism of the hypothalamic KP system. Such organizational effects exist in the ARC of the sheep which contains higher numbers of KP neurons in females than males [70]. Most measurable parameters of the sexual dimorphism we report in the present study (**5.3**) may be explained by the increased expression of KP mRNA and the increased number of neurons expressing KP in women after menopause [58]. Similarly, the different incidence of KP-IR neuronal contacts on GnRH cells and the different

colocalization percentages in these inputs between the two sexes may also reflect a different level of biosynthesis which may cause subthreshold levels of KP in a subset of KP axons, in particular, in males. It requires clarification if the sex difference in the number of KP-IR neuronal appositions onto GnRH neurons reflects the numerical difference in synaptic inputs or the lower detectability of KP in a subset of KP/NKB afferent axons in aged men. The former hypothesis gains support from the observation that the number of excitatory and inhibitory synapses in the ARC can be dynamically regulated by sex steroids [181, 182]. The second possibility would be compatible with high level of KP immunoreactivity in postmenopausal females which coincided with a higher percentage of NKB-IR contacts that contained KP in our postmenopausal female samples. Our immunohistochemical observation that the KP-IR innervation of human GnRH neurons is heavier in women is in accordance with the previous findings in mice which revealed a higher percentage of GnRH neurons that received KP-IR afferent contacts in the rostral preoptic area of females [53].

### 6.3.4. Sex differences in NKB immunoreactivity

Similarly to KP, NKB also acts upstream from the GnRH neuron. In rats, NKB might directly influence GnRH secretion from the hypothalamic median eminence where GnRH-IR axons are apposed to NKB-IR axons [80, 81] and express NK3 [80]. Our present study (5.3) revealed frequent axo-dendritic and axo-somatic contacts between NKB-IR axons and GnRH neurons of the human. It requires clarification if NKB can postsynaptically influence GnRH secretion at these contact sites, given that NK3-IR or NK3 mRNA has not been detected in a majority of the cell bodies of GnRH neurons, in rats [80], ewe [155] and mice [103]. A more likely site of NKB action is on other NKB synthesizing neurons of the ARC/Inf which express NK3 and establish frequent contacts with one another [82, 101, 155]. A recent model of the GnRH pulse generator proposed an intranuclear interplay between three colocalized neuropeptides, kisspeptin, NKB and dynorphins, which uses NK3 and the dynorphin receptor KOR, but not the KP receptor [101]. Our present study (5.3) only revealed a significant sex difference in the incidence of NKB-IR cell bodies but not in the density of NKB-IR fibers and inputs to GnRH neurons. We have to note that sex differences of NKB immunoreactivity were relatively subtle, in comparison with the robust sexual dimorphism of KP immunoreactivity. If
NKB contributes basic mechanisms of pulsatility in the Inf, the NKB-dependent signaling appears to be relatively well preserved in aged males as well as females. On the other hand, the existing sex differences of the NKB system may be physiologically important in the generation of the different pulsatility patterns between aged males and females.

As discussed above for KP, sex differences in NKB immunoreactivity can either be partly or entirely due to the different sex steroid exposure of NKB neurons in aged males and females. Notably, NKB neurons of the rat [82] and the sheep [117] contain ER- $\alpha$  and estrogen treatment reduces NKB mRNA expression in the ARC of the rat [123], mouse [124], sheep [183] and monkeys [122]. Long-term, but not short-term, ovariectomy also increased NKB mRNA expression in the ARC of monkeys, similarly to the postmenopausal increase observed in old females [120].

Our results that NKB-IR neurons outnumbered KP-IR neurons by 120% in aged males suggest that many NKB neurons do not synthesize detectable amount of kisspeptin. We have also observed many single-labeled KP-IR and NKB-IR axons in the Inf, which serves as further support for the higher level of segregation of KP and NKB synthesis in the human Inf. These observations are in contrast with the high degree of match between these neuropeptides in sheep [47] and rodents [101] and also indicate that the presence of the other putative neuromessengers of the GnRH pulse generator in KP and NKB neurons of the human Inf need to be carefully readdressed to reveal potential species differences. Future colocalization experiments will be required to establish the putative coexpression of dynorphins, NK3 and the DYN receptor KOR in human KP and NKB neurons. It is worth of note in this context that although prodynorphin expression has been detected in hypertrophied neurons of postmenopausal women with *in situ* hybridization [130] and in the ARC of monkeys with real-time PCR [120], our preliminary immunohistochemical studies found little evidence for DYN A and DYN B immunoreactivities in the Inf of post mortem tissue samples. The regulation of prodynorphin expression in the ARC/Inf of different species is also somewhat controversial. The number of neurons expressing prodynorphin was reduced in postmenopausal women [130], whereas showed no significant postmenopausal change in monkeys [120] and was upregulated in the absence of sex steroids (in parallel with KiSS-1 and preproNKB gene expression) in mice [101].

In human, most of sex differences might be explained with the lack of estrogen negative feedback in aged women, whereas T can continue to suppress KP, and to a lesser extent, NKB synthesis in men. Overall, sex differences in reproductive physiology of aged humans were reflected in the dramatic sexual dimorphism of the KP system, with significantly higher incidences of KP-IR neurons, fibers and inputs to GnRH neurons in aged females vs. males.

# 6.4. Role of enhanced kisspeptin and neurokinin B signaling in aging men, as a cause or a consequence of reproductive decline

In this study (5.4) we provide comprehensive immunohistochemical evidence for robust enhancements in KP and moderate increases in NKB signaling in the Inf of aged men.

## 6.4.1. Aging-dependent variations in KP and NKB immunoreactivities and their colocalization pattern

As described above, the possibility exists that some differences between aged male and female individuals reflect putative organizational effects of sex steroids during early development. To identify these developmental effects, it will be crucially important to compare samples from young male and female individuals in both of which negative feedback is similarly intact. Overall, the higher relative levels of KP and NKB in the Inf of postmenopausal women, compared with aged men, are likely to reflect a much higher central KP signaling and a moderately increased central NKB signaling in aged females.

This study (5.4) used quantitative immunohistochemical metrics to address the predicted age-dependent enhancements of central KP- and NKB-signaling in men. Comparative experiments were carried out on post mortem hypothalamic samples of men that were categorized into the arbitrary 'young' and 'aged' groups. The quite robust age-related expansion of the KP system we observed, together with a similar, albeit less dramatic, expansion of the NKB system, are in accordance with enhanced central KP and NKB signaling in elderly men (5.4). Notably, aged men exhibited much higher densities of IR perikarya, fibers and higher numbers of afferent contacts onto GnRH neurons, in comparison with young men. Interestingly, in dual-immunofluorescent

studies we also found evidence that the percent ratio of KP-IR NKB perikarya rose from 36% in young to 68% in aged men. In male rodents, T regulates KP and NKB expression of the mediobasal hypothalamus negatively [103, 118] and similarly, T treatment reduces KP expression in the ARC of orchidectomized monkeys [184]. Therefore, the aging-related enhancements of the immunohistochemical signals for KP and NKB are likely to represent the consequences of a reduced negative sex steroid feedback to KP and NKB neurons in aged, compared with young, men.

We addressed the possibility that the reduced negative feedback causes a similar hypertrophy of NKB neurons as does ovarian failure in postmenopausal women [121]. As opposed to the robust (>90%) postmenopausal increase in the mean surface area of NKB neurons [121], in this study (**5.4**) we only found a 22% aging-related increase in the mean profile area of NKB neurons which was significant. Similar mild (~12%) increase in the size of unidentified Inf neurons was reported earlier by Rance and colleagues [127].

The heavier KP and NKB inputs to GnRH neurons in aged men may convey an enhanced stimulation to the reproductive axis. It is worthy of note that the KP system showed an overall higher sensitivity to the effects of aging than the NKB system. It is possible that both the age- and the sex-dependent phenomena simply reflect a stronger down-regulation of KP than NKB by circulating sexual steroids. In accordance with this concept, a recent study on mice [185] established that the KP-encoding Kiss1 gene is, indeed, more sensitive to estrogenic suppression in comparison with the NKB-encoding gene (Tac2 in rodents).

In this study (5.4) we also demonstrated that the low percentage of NKB-IR neurons that contained KP increased to 68% in aged ( $\geq$ 50 years) subjects. This aging-related change suggests that KP expression is kept repressed within a large population of the putative 'NKB/KP' neurons by T in young men and only starts to reach detectable levels with the weakening of negative feedback in aged individuals. The physiological importance of this mechanism requires clarification.

#### 6.4.2. Aging-dependent changes in the central regulation of male reproduction

Although aging-related changes in reproductive functions are less dramatic in males than in females because of the sustained T production in the former, clinical symptoms of hypogonadism, including decreased morning erections, erectile dysfunction and decreased frequency of sexual thoughts, commonly occur in elderly men [186]. Midlife transition in aging men is characterized by decreased serum levels of free T and dihydrotestosterone, increased levels of LH, FSH and sex hormone binding globulin [131, 132]. In addition, aging is associated with depressed pulsatile and elevated basal LH secretion, and a decline in LH secretory burst mode [126]. Elderly men also secrete LH and T more irregularly and more asynchronously than do young men [187, 188]. Some of these endocrine alterations result from a reduced androgen receptor-mediated negative feedback to the hypothalamus [126] which likely involves KP and NKB neurons of the Inf. It has been established that the central mechanisms of androgen receptor-mediated negative feedback can modulate GnRH/LH secretory frequency and duration, pulsatile LH secretion, the incremental LH response to GnRH, total LH secretion and regularity of the LH secretory process [126]. In view of the proposed involvement of KP/NKB neurons in T negative feedback to the male hypothalamus [103, 118, 184], in this study (5.4) we predicted that aging would be associated with enhanced central KP- and NKB-signaling in the Inf. The results of our quantitative immunohistochemical studies provided evidence that the regional densities of NKB-IR perikarya and fibers, and the incidence of afferent contacts they formed onto GnRH neurons, exceeded several times those of the KP-IR elements, more in young men. Robust aging-dependent enhancements were identified in the regional densities of KP-IR perikarya and fibers, and in the incidence of appositions they established onto GnRH neurons. NKB-IR neurons, fibers and axonal appositions to GnRH neurons also increased with age, but to a lower extent. Finally, in dual-immunofluorescent studies, the incidence of KP-IR NKB perikarya increased from 36% in young to 68% in aged men.

In summary, these data provide immunohistochemical evidence for the aging-related enhancements in central KP- and NKB signaling in the Inf which is compatible with a reduced T negative feedback upon KP and NKB neurons. The heavier KP and NKB inputs to GnRH neurons in aged, compared with young, men may play a role in the enhanced central stimulation of the reproductive axis. It requires clarification to what extent the enhanced KP and NKB signaling upstream from GnRH neurons is an adaptive response to hypogonadism, or alternatively, a consequence of a decline in the androgen sensitivity of KP and NKB neurons.

## 7. Conclusions

In studies underlying this thesis we have provided new information about peptidergic signaling to hypothalamic GnRH neurons of mice and humans.

We have provided evidence that subcutaneous administration of E2 for 4 days significantly down-regulated RFRP mRNA expression. In ovariectomized mice, low level of nuclear ER- $\alpha$  immunoreactivity were detectable in about 20% of RFRP neurons. The majority of RFRP neurons showed no ER- $\alpha$  signal and RFRP neurons did not exhibit ER- $\beta$  immunoreactivity. These new data raise the possibility that RFRP neurons are part of the neuronal circuitry that mediates the effects of estrogen to GnRH neurons.

We tested the validity and limitations of the KNDy neuron concept in the human with immunohistochemical analysis. We have described the regional densities of NKB-IR perikarya and fibers, and the incidence of afferent contacts they formed onto GnRH neurons, which were about 5 times higher than those of the KP-IR elements. Dualimmunofluorescent studies confirmed that considerable subsets of the NKB-IR and KP-IR cell bodies and fibers are separate and only about 30% of NKB-IR perikarya and 75% of KP-IR perikarya were dual-labeled. Furthermore, very few DYN-IR cell bodies could be visualized in the Inf. DYN-IR fibers were also rare, and with few exceptions, distinct from the KP-IR fibers. The abundance and colocalization patterns of the three immunoreactivities showed similar trends in the InfS around portal blood vessels. These new data indicate that the colocalization patterns obtained in laboratory animals are not necessarily the same in the human. For example, NKB neurons of young human males contain KP immunoreactivity relatively rarely. Also, the finding of DYN in KP/NKB neurons that was initially reported in sheep and rodents, might be irrelevant in the human. Similar species differences call for new models to explain the mechanism of negative feedback and the functioning of the GnRH pulse generator in the human.

We have studied the sex-dependent changes of KP/NKB system in the Inf with immunohistochemistry. We have described that the number of KP cell bodies, the density of KP fibers and the incidence of their contacts on GnRH neurons were much higher in aged women compared with men. The number of NKB cell bodies was only slightly higher in women and there was no sexual dimorphism in the regional density of NKB fibers and the incidence of their appositions onto GnRH cells. The incidences of NKB cell bodies, fibers and appositions onto GnRH neurons exceeded several-fold those of KP-IR elements in men. More NKB than KP inputs to GnRH cells were also present in women. Immunofluorescent studies identified only partial overlap between KP and NKB axons. KP and NKB were colocalized in higher percentages of afferents to GnRH neurons in women compared with men. Against a long-held view that the human hypothalamus does not exhibit robust sex differences, these data clearly show the sexual dimorphism of these two peptidergic systems that are critically involved in the regulation of human reproduction. Future studies will need to determine to what extent these differences are due to the organizational and activational effects of sex steroids, respectively.

Finally we have also investigated the age-related morphological alterations of the KP/NKB neuronal elements in human. Quantitative immunohistochemical experiments established that the regional densities of NKB-IR perikarya and fibers, and the incidence of afferent contacts they formed onto GnRH neurons, exceeded several times those of the KP-IR elements. Robust aging-dependent enhancements were identified in the regional densities of KP-IR perikarya and fibers, and the incidence of afferent contacts they established onto GnRH neurons. The abundance of NKB-IR perikarya, fibers and axonal appositions to GnRH neurons also increased with age, albeit to lower extents. In dual-immunofluorescent studies, the incidence of KP-IR NKB perikarya increased from ~40% in young to ~70% in aged men. Alterations in the morphology and peptide expression of human KP and NKB neurons may either result from or cause late-onset male hypogonadism, which often characterizes middle-age transition in men. Animal studies will need to determine if these changes are age-related primary hypothalamic events, or alternatively, the consequences of declining T levels.

### 8. Summary

Gonadotropin-releasing hormone (GnRH)-synthesizing neurons of the hypothalamus represent the final common output way of the hypothalamus in the neuroendocrine control of reproduction. The functions of GnRH neurons are influenced by specific afferents that include several important peptidergic systems. In studies underlying my PhD work we investigated various aspects of this peptidergic regulation acting upstream from the GnRH neuronal system of mice and humans. Our work focused on RF-amide related peptide (RFRP), kisspeptin (KP) and neurokinin B (NKB) neurons, acting as afferents to GnRH neurons. We have investigated the neuropeptide content, anatomical distribution, sex-dependent differences and aging-related changes of these neurons and their connections with GnRH cells. Our studies used morphological approaches, including immunohistochemistry and *in situ* hybridization.

Specific results of our experiments reveal various new aspects of the peptidergic regulation to GnRH cells. They reveal a mild estrogenic regulation of RFRP expression in mice which might be exerted directly on RFRP neurons through estrogen receptor alpha. In studies of human histological samples, we provide novel evidence that human KP and NKB neurons largely differ from their rodent counterparts. Their neuropeptide content and connectivity to GnRH neurons varies considerably between sexes, and at different ages.

These data add to our understanding of the peptidergic mechanisms that regulate the hypothalamic GnRH pulse generator.

## 9. Összefoglalás

A hipotalamikus gonadotropin-releasing hormone (GnRH)-t termelő idegsejtek képviselik a szaporodás neuroendokrin szabályozásának végső, hipotalamikus kimeneti jelét. A GnRH sejtek működését specifikus afferens kapcsolataik szabályozzák, beleértve számos fontos peptiderg rendszert. PhD munkám alapjául szolgáló tanulmányokban ezen, GnRH rendszer felett ható peptiderg szabályozást vizsgáltuk egerekben és emberekben. Munkánkban a GnRH sejtek bemeneteit képező RF-amide related peptide (RFRP), kisspeptin (KP) és neurokinin B (NKB) idegsejtekre főkuszáltunk. Tanulmányoztuk ezen idegsejtek neuropeptid tartalmát, anatómiai eloszlását, nem- és életkor-függő változásait, valamint a GnRH sejtekkel való kapcsolatait. Tanulmányainkban elsősorban morfológiai megközelítéseket, immunhisztokémiát és *in situ* hibridizációt alkalmaztunk.

Konkrét vizsgálati eredményeink a GnRH sejtek peptiderg szabályozásának számos új aspektusát fedték fel. Bebizonyították, hogy egerekben az RFRP expresszióját az ösztrogén befolyásolja. A hatás RFRP neuronokon az alfa típusú ösztrogén receptor közvetítésével direkt is megvalósulhat. Emberi szövetminták tanulmányozásával kimutattuk, hogy a KP és NKB idegsejtek nagymértékben különböznek a rágcsáló megfelelőiktől. Neuropeptid tartalmuk és a GnRH neuronokkal való kapcsolataik számottevően különböznek az eltérő nemű és életkorú emberekből származó mintákban.

Disszertációm alapjául szolgáló megfigyeléseink hozzájárulhatnak a hipotalamikus GnRH pulzus generátor peptiderg szabályozásának jobb megértéséhez.

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## **11. List of publications**

#### 11.1. List of publications underlying the thesis

- Molnár C.S., Kalló I., Liposits Z., Hrabovszky E. (2011) Estradiol down-regulates RF-amide related peptide (RFRP) expression in the mouse hypothalamus *Endocrinology* 152(4):1684-90
- Hrabovszky E., Molnár C. S., Sipos M. T., Vida B., Ciofi P., Borsay B. A., Sarkadi L., Herczeg L., Bloom S. R., Ghatei M. A., Dhillo W. S., Kalló I. and Liposits Z. (2011) Sexual dimorphism of kisspeptin and neurokinin B immunoreactive neurons in the infundibular nucleus of aged men and women *Frontiers in Endocrinology* 2:80
- Hrabovszky E., Sipos M. T., Molnár C. S., Ciofi P., Borsay B. A., Gergely P., Herczeg L., Bloom S. R., Ghatei M. A., Dhillo W. S. and 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(10):4978-4989
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#### 11.2. List of other publications

- Kocsis Z. S., Molnár C. S., Watanabe M., Daneels G., Moechars D., Liposits Z. and Hrabovszky E. (2010) Demonstration of vesicular glutamate transporter-1 in corticotroph cells in the anterior pituitary of the rat Neurochemistry International 56(3):479-86
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- Alvarez-Crespo M., Skibicka K. P., Farkas I., Molnár C. S., Egecioglu E., Hrabovszky E., Liposits Z., Dickson S. L. (2012) The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence *PLoS One* 7(10):e46321

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