Novel mechanisms of peptidergic signaling in reproductive regulation

Ph.D. Thesis

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Introduction

Gonadotropin-releasing hormone (GnRH) synthesizing neurons of the hypothalamus play a crucial role in the central regulation of reproduction in all mammals. The hypophysiotropic axons of GnRH neurons secrete the GnRH into the hypophysial portal circulation at the hypothalamic median eminence (ME). From here, portal veins carry GnRH to the anterior pituitary gland. GnRH binds to 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 gonadotropins.

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. The episodic secretion of GnRH has been shown to correlate with the pulsatile secretion of LH from the adenohypophysis. Coordinated function of the cells, to allow regular pulses to be generated in terms of secretory events, could be achieved through dendro-dendritic connections. The neuronal network accounting for the establishment of the GnRH neurosecretory pulses is commonly referred to as the GnRH/LH 'pulse generator'.

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

Kisspeptin (KP) plays a particularly important role in the central regulation of reproduction. Administration of KP, KP antibodies, KP antagonists or the use of transgenic animal models deficient for KP or its receptor (KISS1R) have provided evidence that KP regulates the pulsatile and surge release of GnRH.

Neurokinin B (NKB) is another critically important neuropeptide which interacts with KP in the neuroendocrine regulation of GnRH/LH secretion. NKB may increase GnRH secretion from the hypothalamic ME where GnRH axons are apposed to NKB axons in the rat. In addition, NKB neurons also influence reproduction by acting on other NKB neurons in the arcuate nucleus (ARC) where they establish frequent contacts with one another.

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. Putative GnIH homologues, RF-amide related peptides (RFRP-1, RFRP-2, RFRP-3), have also been identified in mammals.

The GnRH neuronal system which represents the final common pathway in the neuroendocrine control of reproduction responds to feedback actions of circulating 17β-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-β (ER-β), interneurons expressing the classical estrogen receptor (ER-α) play a critically important role in sensing and conveying indirect information on circulating estrogens to the GnRH neuronal system.

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-α and respond with c-Fos expression to an acute administration of E2. RFRP axons provide inputs to GnRH neurons, suggesting direct regulation. Intracerebroventricular injection of RFRP-3 reduced plasma LH secretion in ovariectomized (OVX) hamsters and gonadally intact rats. In one study (study 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.

KP neurons of the ARC also synthesize NKB in the sheep, the goat, the mouse, monkey and the human. The recently introduced terminology of KNDy (Kisspeptin/Neurokinin B/Dynorphin) neurons refers to the co-synthesis of dynorphins (DYN) by the majority of these KP/NKB cells at least in the sheep, the rat, the mouse and the goat. 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, and possibly, also the positive feedback effects of estrogens, at least in this species. In addition, KNDy neurons also appear to constitute an important component of the GnRH pulse generator.

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. Based on our preliminary data indicating that the number of KP neurons is very low in young human males subjects, we predicted that the degree of overlap between the KNDy neuropeptides is

much lower in young male humans than suggested earlier for sheep, goats or mice. In one of our studies (study 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 infundibular nucleus (Inf) and the infundibular stalk (InfS) of young men. Furthermore quantitative immunohistochemistry was used to study various aspects of the putative morphological sex (study 3) and age (study 4) differences.

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

Materials and Methods

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- and temperature 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).

The ovariectomy was carried out under deep anesthesia. On post-ovariectomy day 9, the mice were re-anesthetized and implanted subcutaneously with a single silastic capsule containing either sunflower oil (OVX) or 100µg/ml E2 in sunflower oil (OVX+E2).

Four days later, the mice were anesthetized and killed by transcardiac perfusion with 4% paraformaldehyde (PFA). These mice were used in colocalization studies of ER-α and RFRP-1

immunoreactivities. Another mice were OVX and treated similarly to generate OVX and OVX+E2 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-β and RFRP-1 immunoreactivities.

Human 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 (study 2), we used tissues of young male individuals (age 21-37 years). To investigate the sexual dimorphism of the human hypothalamic KP and NKB neuronal systems (study 3), we used tissue samples from male subjects above 50 years of age (50-67 years) and from 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) and ‘aged’ (50-67 years) male groups were formed (study 4).

Following dissection, the human hypothalamic tissue blocks were immersion-fixed with 4% paraformaldehyde. Following fixation, the hypothalami were trimmed further to include the optic chiasma rostrally, the mammillary bodies caudally and the anterior commissure dorsally.

Prior to immunohistochemistry, human tissues were permeabilized and endogenous peroxidase activity reduced. Subsequently, antigen retrieval was carried out by using a 0.1M citrate buffer wash at 80°C. In immunofluorescent experiments, the sections were also pretreated with Sudan black to reduce tissue autofluorescence from lipofuscin deposits.

Immunohistochemical methods

Peroxidase-based immunohistochemical single-labeling

Sections processed for peroxidase-based immunohistochemistry were first incubated in the working dilution of primary antibodies. The primary antibodies were reacted with biotinylated secondary antibody and then, with the ABC Elite reagent. The peroxidase signal was developed with diaminobenzidine or with nickel-intensified diaminobenzidine chromogen. The immunostained sections were mounted on microscope slides, air-dried, dehydrated, cleared with xylene and coverslipped with DPX mounting medium.

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 (studies 1, 2). In others, the afferent connectivity between two different neuronal phenotypes was investigated (studies 3, 4). In these studies the Ni-DAB chromogen was post-intensified with silver-gold.

Innervation and colocalization studies with the combined use of silver-gold-intensified Ni-DAB and DAB chromogens

In studies to examine the KP-IR and NKB-IR afferent inputs to GnRH neurons (studies 2-4), two series of sections were processed for the detection of KP or NKB immunoreactivities. GnRH neurons were detected with a new guinea pig antiserum. The primary antibodies were reacted with biotinylated anti-guinea pig IgG and the ABC reagent and then, the peroxidase signal was developed with DAB chromogen.

In studies to reveal the presence of the estrogen receptor isoforms in RFRP neurons (study 1) ERs were detected with an antiserum raised in rabbit, followed by biotinylated secondary antibodies 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 against the C-terminus of rat RFRP-1, using the biotinylated secondary antibody-ABC technique and non-intensified DAB as the chromogen.

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 (studies 2-4), dual-immunofluorescent labeling was used. Incubation in a cocktail of primary antibodies (rabbit anti-NKB and sheep anti-KP, or rabbit anti-DYN and sheep anti-KP) was followed by a cocktail of fluorochrom-conjugated secondary antibodies (anti-rabbit-FITC, anti-sheep-Cy3).

To maximize sensitivity, in some dual-immunofluorescent studies (studies 2-4) tyramide signal amplification was used. In these experiments KP was detected first using sequential incubations in sheep KP antibodies, biotinylated anti-sheep IgG, the ABC Elite reagent, biotin tyramide working solution and finally, avidin-Cy3. Then, the sections were treated for 30 min with 0.5% H_2O_2 and 0.1% sodium azide in PBS, to inactivate horseradish peroxidase. To detect NKB or DYN, the rabbit primary antibodies were used and reacted with anti-rabbit-peroxidase. Then, FITC-tyramide was deposited on the peroxidase sites.

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, sheep anti-KP, guinea pig anti-GnRH,) was followed by a cocktail of fluorochrom-conjugated secondary antibodies (all raised in donkey; anti-rabbit-FITC, anti-sheep-Cy3, anti-guinea pig-AMCA) (studies 3, 4).

Sections processed for immunofluorescent experiments were mounted from 0.1M Tris–HCl buffer and coverslipped with the aqueous mounting medium Mowiol.

***In situ* hybridization** (study 1)

To prepare a probe to preproRFRP mRNA a 424-bp cDNA fragment (corresponding to bases 136-559 of the rat preproRFRP mRNA; AB040288) was amplified with PCR from rat hypothalamic cDNA. The linearized RFRP template was transcribed with T7 RNA polymerase in the presence of ^{35}S-UTP.

The sections hybridized with the RFRP cRNA 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, the slides were dipped into Kodak NTB autoradiographic emulsion and exposed for 2 weeks. The emulsion autoradiographs were developed using standard procedures and Kodak processing chemicals. The sections were air-dried, dehydrated, cleared with xylene, and coverslipped with DPX mounting medium.

Microscopy

The light- (study 1-4) and fluorescent (study 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. To analyze the double- and triple-immunofluorescent labeling (studies 3, 4) we used a Radiance 2100 confocal microscope. Furthermore, confocal images (studies 2, 4) were prepared with an inverted Nikon Eclipse Ti-E microscope equipped with an A1R confocal system too.

Data analysis and statistics

Quantitative analysis of ISH signal (study 1)

X-ray films were scanned for analysis. For consistency, the autoradiographic images of the most heavily labeled sections in each animal were selected for the quantitative analysis of RFRP mRNA expression. The digital image files were analyzed with the Image J software. The

autoradiographic signal in each animal was characterized with the mean of 'integrated density' measurements. 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. 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.

Analysis of quantitative immunohistochemical experiments

The digital images were processed with the Adobe Photoshop CS software. Quantitative data were expressed as mean±SEM and statistical comparisons were carried out one-way ANOVA followed by Newman-Keuls post-hoc test.

Perikaryon size (mean immunoreactive profile area)

To determine the average size of KP-IR and NKB-IR cell bodies (studies 3, 4), 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 signal areas were measured with Image J software and then, converted to μm2 using appropriate calibration. For each human subject the mean profile area of labeled perikarya was derived from an average of 10-30 labeled cells.

Regional incidence of cell bodies

The number of immunoreactive cell bodies was counted (studies 2-4) at 100X magnification within a 0.25 mm^2 counting area with the aid of a 5X5 ocular grid. Each subject was characterized by the maximal number of immunoreactive perikarya in this counting area.

Regional density of labeled fibers

Digital images were taken (studies 2-4) from the bulk of KP-IR and NKB-IR neurons in the Inf. The immunolabeled cell bodies and their proximal dendrites were erased ('eraser tool') from the photomicrographs using the Adobe Photoshop CS software. The remaining images were analyzed with the Image J software. The regional fiber density in each photograph was defined as the area occupied by immunoreactive fibers/total area. The overlap between NKB-IR and KP-IR axons or DYN-IR and KP-IR axons (study 2) was also studied qualitatively in confocal images of dual-immunofluorescent specimens.

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 (studies 2, 4) were determined quantitatively from the dual-immunofluorescent specimens in which the tyramide signal amplification was used.

Studies of neuropeptides in afferents to GnRH neurons

Dual-immunoperoxidase labeled sections were selected to determine the number of KP/NKB axonal contacts along the outlines of GnRH-IR cell bodies and dendrites (studies 2-4). 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 (studies 3, 4), multiple stacks of optical slices were obtained by scanning GnRH neurons in the Inf and their KP-IR and NKB-IR contacts using a confocal microscope.

Results

Estrogenic down-regulation of RF-amide related peptide expression via estrogen receptor-α

The expression of RFRP mRNA is negatively regulated by estrogen

Radioisotopic *in situ* hybridization studies revealed a restricted regional distribution of RFRP mRNA synthesizing neurons in the mouse hypothalamus. 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. 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.

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 revealed lower single-cell levels of RFRP mRNA expression in the OVX+E2 *vs.* the OVX group. In retrospect, this unbiased analysis identified significantly fewer 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).

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

We have carried out colocalization experiments with dual-label immunohistochemistry in an attempt to identify which ER isoform may account for the estrogenic down-regulation of RFRP mRNA expression. A heavy ER-α immunolabeling was present in the ARC and ventromedial nuclei (VMN) and in scattered cell nuclei within the dorsomedial and periventricular nuclei where most RFRP-1-IR cells occurred. A pale nuclear ER-α signal was detected in 18.7±3.8% of RFRP-1-IR cells, whereas the majority of RFRP neurons did not contain ER-α signal. Many ER-β-IR cell nuclei were detectable in the hypothalamic paraventricular nucleus (PVN), but only scattered cell nuclei were labeled for ER-β in the dorsomedial nucleus. ER-β-positive RFRP neurons were not revealed.

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

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

We propose that the different colocalization patterns in our human study and in previous animal experiments partly reflect species differences in reproductive mechanisms. In peroxidase-based immunohistochemistry, many NKB-IR perikarya were identified in the Inf. KP-IR cell bodies occurred in much lower numbers in neighboring sections. Quantitative analysis showed that the density of KP neurons was about 5 times lower than that of NKB-IR perikarya. DYN-IR perikarya were either entirely absent in some subjects or extremely rare in the Inf of others, preventing quantitative studies. In contrast, the supraoptic nucleus contained many DYN labeled perikarya, making it unlikely that the low DYN signal in the Inf reflects technical limitations.

A surprising segregation of NKB-IR and KP-IR perikarya was revealed in dual-immunofluorescent specimens. 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.

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 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. DYN-IR fibers were also detectable in the Inf, although less frequently than either NKB-IR or KP-IR axons.

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. 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. Similarly, the majority of KP-IR fibers were also devoid of dynorphin B immunoreactivity in the Inf and the InfS.

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. Both were abundantly innervated by GnRH-IR axons, 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 were surrounded by dense networks of NKB-IR fibers and innervated only moderately by KP-IR fibers. Very few DYN-IR fibers occurred in the proximity of the portal capillaries.

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

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. Quantitative analysis established that GnRH-IR perikarya and dendrites, respectively, received 6 and 5 times heavier NKB-IR than KP-IR innervation.

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

Sex difference in perikaryon size of KP and NKB neurons

The morphometric analyses of KP-IR and NKB-IR neurons in one of our studies 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. KP-IR cell bodies as well as NKB-IR cell bodies were hypertrophied and their profile area was significantly larger in the Inf of aged women

(284.2±27.3μm^2 for KP-IR and 298.1±19.7μm^2 for NKB-IR neurons) in comparison with aged men (154.8±19.2μm^2 for KP-IR and 190.4±20.4μm^2 for NKB-IR neurons).

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. NKB-IR neurons outnumbered KP-IR neurons by 120%.
- 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.
- KP-IR cell bodies showed a 170% higher mean density (incidence of cell bodies per 0.25mm^2 counting frame) in females compared with males. This robust sex difference was statistically significant.
- 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.

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. NKB-IR axons did not show this robust sex difference. Quantitative analysis of labeled axons 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.
- In the Inf of females, the densities of NKB-IR and KP-IR fibers did not differ statistically.
- KP-IR fibers showed a robust sexual dimorphism, with a 161% higher density in females vs. males. This difference was statistically significant.
- NKB-IR fibers showed only 8.4% higher mean density in females than in males and the sexes did not differ statistically, in contrast with the dramatic and significant sexual dimorphism of the KP-IR fiber network.

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 that KP-IR axons establish axo-somatic and axo-dendritic contacts on GnRH neurons of the Inf. In the present study, we observed similar axo-somatic and axo-dendritic appositions between NKB-IR axons and GnRH-IR neurons.

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 and the dendrites of GnRH-IR neurons in women compared with men.

In both sexes, GnRH-IR cell bodies and dendrites received significantly heavier NKB-IR input than KP-IR input.

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. However, there was a trend for somewhat more NKB-IR contacts in females.

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. The semiquantitative analysis of afferent contacts onto GnRH-IR cell bodies and dendrites established that 8.8±5.5% of NKB-IR afferents in males also contained KP immunoreactivity. The ratio of double-labeled afferents was 31.3±4.9% in females. Similarly, the percentage of KP-IR contacts that colocalized NKB signal was significantly higher in females (25.8±2.4%) than in males (10.2±4.6%).

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

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 in young men, and outnumbered KP-IR neurons 3.7-fold. NKB-IR cell bodies also outnumbered KP-IR perikarya in aged men, but only 2.2-fold.

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. NKB-IR cell bodies also showed higher mean incidence in aged compared with young men, but the difference was only 1.5-fold.

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±10.6μm^2) compared with young (167.3±9.6μm^2) men. This subtle increase between the two age groups was statistically significant.

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. 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.
- 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, whereas the density of NKB-IR axons showed a 1.5-fold aging-related increase.

Colocalization of KP and NKB in neuronal perikarya of the Inf

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

In young men 72.7±6.0% of KP-IR perikarya also contained NKB immunoreactivity. Similarly, in aged men 77.9±5.9% of the KP-IR cell bodies contained NKB immunoreactivity. There was a lower degree of overlap in the opposite direction. In young men only 35.8±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±6.8%. This aging-related increase in the percentage of KP-IR NKB neurons was statistically significant.

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.

The quantitative analysis of appositions 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. 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 appositions than KP-IR appositions.

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 and a 2-fold heavier KP-IR input to the dendrites of GnRH-IR neurons in the Inf of aged, in comparison with young, men. 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. 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.

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 and aged men. The quantitative analysis of the KP/NKB colocalization revealed KP immunoreactivity in 7.3±1.5% 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. NKB signal was observed in 7.9 ± 2.8% of KP-IR afferents in young and 11.6 ± 4.6% of KP-IR afferents in aged men.

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-α immunoreactivity were detectable in about 20% of RFRP neurons. The majority of RFRP neurons showed no ER-α signal and RFRP neurons did not exhibit ER-β 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. Dual-immunofluorescent 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 testosterone levels.

List of publications

List of publications underlying the thesis

1. 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

2. 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

3. 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

4. Molnár C. S., Vida B., Sipos M. T., Ciofi P., Borsay B. A., Rácz K., Herczeg L., Bloom S. R., Ghatei M. A., Dhillo W. S., Liposits Z. and Hrabovszky E. (2012) Morphological evidence for enhanced kisspeptin and neurokinin B signaling in the infundibular nucleus of the aging man *Endocrinology* 153(11):5428-39

List of other publications

1. 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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5. Derks N. M., Pintér O., Zuure W., Ledent C., Watanabe M., Molnár C. S., Wei Y., Roubos E. W., Wu S., Hrabovszky E., Zelena D., Kozicz T. (2012) Cannabinoid modulation of midbrain urocortin 1 neurones during acute and chronic stress *Journal of Neuroendocrinology* 24(11):1447-1461

6. 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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