Morphological Evidence for Enhanced Kisspeptin and Neurokinin B Signaling in the Infundibular Nucleus of the Aging Man

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Peptidergic neurons synthesizing kisspeptin (KP) and neurokinin B (NKB) in the hypothalamic infundibular nucleus have been implicated in negative sex steroid feedback to GnRH neurons. In laboratory rodents, testosterone decreases KP and NKB expression in this region. In the present study, we addressed the hypothesis that the weakening of this inhibitory testosterone feedback in elderly men coincides with enhanced KP and NKB signaling in the infundibular nucleus. This central hypothesis was tested in a series of immunohistochemical studies on hypothalamic sections of male human individuals that were divided into arbitrary “young” (21–49 yr, n = 11) and “aged” (50–67 yr, n = 9) groups. Quantitative immunohistochemical experiments established that the regional densities of NKB-immunoreactive (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 36% in young to 68% in aged men. Collectively, these immunohistochemical data suggest an aging-related robust enhancement in central KP signaling and a moderate enhancement in central NKB signaling. These changes are compatible with a reduced testosterone negative feedback to 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. (Endocrinology 153: 5428–5439, 2012)
Tissue preparation for immunohistochemistry

Materials and Methods

"young" (21–49 yr, n = 11) and "aged" (50–67 yr, n = 9) groups. The abundance of KP-immunoreactive (IR) and NKB-IR cell bodies, the size of NKB-IR perikarya, the regional density of KP-IR and NKB-IR fibers, the incidence of KP-IR and NKB-IR appositions onto GnRH-IR neurons, and the colocalization of KP and NKB in neuronal cell bodies and in afferents to GnRH-IR neurons were studied and compared between the two age groups.

Human subjects

The age above 50 yr was chosen to arbitrarily define aged men (19–21). Human hypothalamic tissue samples from 11 male subjects under 50 yr of age (21, 31, 33, 36, 37, 39, 40, 41, 45, 46, and 49 yr) and from 9 subjects above (50, 50, 51, 59, 62, 64, 67, 69, and 78 yr) were obtained at autopsy from the Forensic Medicine Department of the University of Debrecen, with the permission of the Regional Committee of Science and Research Ethics (DEOECD RKEB/IKEB, 3183-2010). Selection criteria included sudden causes of death, lack of history of neurological and endocrine disorders, and postmortem intervals less than 48 h.

Tissue preparation for immunohistochemistry

After dissection, the hypothalamic tissue blocks were first rinsed with tap water and then immersed into 4% formaldehyde in 0.1M PBS (pH 7.4) for 7–14 d at 4 C. Before section preparation, the fixed hypothalami were trimmed further to include the optic chiasm rostrally, the mamillary bodies caudally, and the anterior commissure dorsally (10, 21, 22). Sagittal cuts were made 2 cm lateral from the midsagittal plane on both sides, and then, the blocks were cut in halves and infiltrated with 20% sucrose for 5 d at 4 C. The right hemihypothalami were placed in a freezing mold, surrounded with Jung tissue freezing medium (diluted 1:1 with 0.9% sodium chloride solution; Leica Microsystems Nussloch GmbH, Nussloch, Germany), snap frozen on powdered dry ice, and sectioned coronally at 30 μm with a Leica SM 2000R freezing microtome (Leica Microsystems Nussloch GmbH). The sections were stored permanently in antifreeze solution [30% ethylene glycol, 25% glycerol, and 0.05 M phosphate buffer (pH 7.4)] at −20 C.

Tissue pretreatments

Before immunohistochemistry, the sections were rinsed in PBS and pretreated with a mixture of 0.5% H2O2 and 0.2% Triton X-100 for 30 min. Then, antigen retrieval with 0.1 M citrate buffer (pH 6.0) was carried out at 80 C for 30 min. In immunofluorescent experiments, the sections were additionally treated with Sudan black to reduce tissue autofluorescence from lipofuscin deposits (10, 23, 24).

Detection of KP- and NKB-synthesizing neurons using peroxidase-based immunohistochemistry

To detect KP-immunoreactivity, every 24th hemihypothalamic section from the Inf of each human individual was incubated in a sheep polyclonal antiserum (1:100,000; GQ2) against human KP-54. This antiserum recognizes human KP-54, KP-14, and KP-10 and shows virtually no cross-reactivity (<0.01%) with other related human RF-amide peptide, including prolactin-releasing peptide, neuropeptide FF, neuropeptide AF and RF-amide-related peptides (RFRP1, RFRP2, and RFRP3) (25). The GQ2 antibodies were used successfully in previous immunohistochemical experiments to study the distribution of KP neurons and their connectivity to GnRH cells in the rhesus monkey (9, 26) and the human (10, 21, 24). Incubation in the primary antiserum for 48 h at 4 C was followed by biotinylated secondary antibodies (1:500, biotin-SP-antisheep IgG; Jackson ImmunoResearch, West Grove, PA) and the ABC Elite reagent (1:1000; Vector Laboratories, Burlingame, CA) for 60 min each. The peroxidase signal was visualized with nickel-intensified diaminobenzidine chromogen and then, postintensified with silver-gold (27).

To detect NKB-synthesizing neurons, a second series of sections was incubated with a previously characterized rabbit polyclonal antiserum (1:100,000, IS-682; P. Ciolfi) against the C-terminal 28 amino acids of human pro-NKB (9, 10, 21) followed by working dilutions of biotinylated secondary antibodies (1:500, 60 min, biotin-SP-antisheep IgG; Jackson ImmunoResearch) and the ABC Elite reagent (1:1000, 60 min). The signal was visualized with silver-gold-intensified nickel-diaminobenzidine, as in case of KP-IR neurons.

Dual-immunoperoxidase detection of KP-IR and NKB-IR inputs to GnRH neurons

Another two series of sections were processed for the detection of KP immunoreactivity or NKB immunoreactivity as described above. Subsequently, GnRH neurons were detected with a previously characterized (21) guinea pig antiserum (no. 1018;
The primary antibodies were reacted with biotin-SP-antiguinea pig IgG (1:500, 60 min; Jackson ImmunoResearch), the ABC Elite reagent (1:1000, 60 min; Vector Laboratories), biotin tyramide working solution [1:1000, in 0.05 M Tris-HCl buffer (pH 7.6), containing 0.003% H₂O₂, 30 min] (28), and finally, avidin-cyanine-3 (Cy3) (1:1000, 60 min; Jackson ImmunoResearch). Then, the sections were treated for 30 min with 0.5% H₂O₂ and 0.1% sodium azide in PBS, to inactivate horseradish peroxidase. To detect NKB, the IS-682 primary antibodies were used at 1:50,000 (48 h; 4°C) and reacted with antirabbit-peroxidase (1:500, 1 h; Jackson ImmunoResearch). Then, fluorescein isothiocyanate (FITC)-tyramide [diluted 1:500 with 0.05 M Tris-HCl buffer (pH 7.6), containing 0.003% H₂O₂, 30 min] (28) was deposited on the peroxidase sites. Control experiments included the omission of the NKB primary antibody step from the dual-labeling procedure. Absence of FITC signal in these control sections indicated that no FITC-tyramide deposition was due to residual peroxidase activity on KP-IR sites.

**FIG. 1.** Aging-related expansion of KP-IR and NKB-IR neuronal elements in the Inf of the human male. KP-immunoreactivity (A–D) and NKB-immunoreactivity (E–H) were visualized using the silver-gold-intensified nickel-diaminobenzidine chromogen. The representative photomicrographs were taken from the Inf of 31-yr-old (A and C), 40-yr-old (E and G), and 67-yr-old (B, D, F, and H) 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 and D) compared with young (A and 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 and H) compared with young (E and G) men, and age-related increases also take place in the regional density of NKB-IR fibers (E–H). C, D, G, and H correspond to framed regions in A, B, E, and F, respectively. For quantitative comparisons, see Figs. 2 and 3. Scale bar, 50 μm (C, D, G, H) and 200 μm (A, B, E, F).

To maximize the sensitivity of colocalization experiments, dual-immunofluorescent studies used the tyramide signal amplification approach for the detection of both KP and NKB, as described recently (24). First, KP was detected using sequential incubations in KP antibodies (1:30,000, 48 h, 4°C), biotinylated antiserum IgG (1:500, 60 min; Jackson ImmunoResearch), the ABC Elite reagent (1:1000, 60 min; Vector Laboratories), biotin tyramide working solution [1:1000, in 0.05 M Tris-HCl buffer (pH 7.6), containing 0.003% H₂O₂, 30 min] (28), and finally, avidin-cyanine-3 (Cy3) (1:1000, 60 min; Jackson ImmunoResearch). Then, the sections were treated for 30 min with 0.5% H₂O₂ and 0.1% sodium azide in PBS, to inactivate horseradish peroxidase. To detect NKB, the IS-682 primary antibodies were used at 1:50,000 (48 h; 4°C) and reacted with antirabbit-peroxidase (1:500, 1 h; Jackson ImmunoResearch). Then, fluorescein isothiocyanate (FITC)-tyramide [diluted 1:500 with 0.05 M Tris-HCl buffer (pH 7.6), containing 0.003% H₂O₂, 30 min] (28) was deposited on the peroxidase sites. Control experiments included the omission of the NKB primary antibody step from the dual-labeling procedure. Absence of FITC signal in these control sections indicated that no FITC-tyramide deposition was due to residual peroxidase activity on KP-IR sites.

**Triple-immunofluorescent visualization of NKB, KP, and GnRH**

A series of sections was used to study the colocalization pattern of NKB and KP in neuronal afferents to GnRH neurons. Incubation in a cocktail of primary antibodies (IS-682 anti-NKB, 1:500; GQ2 sheep anti-KP, 1:1000; no. 1018 guinea pig anti-GnRH, 1:5000) for 48 h at 4°C was followed by a mixture of fluorochrome-conjugated secondary antibodies (antirabbit-FITC, 1:200; antisheep-Cy3, 1:1000; antiguinea pig-amino-methyl-coumarin acetate (AMCA), 1:100) (Jackson ImmunoResearch) for 5 h.

**Section mounting and coverslipping**

Sections processed with peroxidase-based immunohistochemistry were mounted on microscope slides from Elvanol, air dried, dehydrated with 95% (5 min), followed by 100% (2 × 5 min) ethanol, cleared with xylene (2 × 5 min), and coverslipped with DPX mounting medium (Sigma, St. Louis, MO). Immunofluorescent specimens were mounted from 0.1 M Tris-HCl buffer (pH 7.6) and coverslipped with the aqueous mounting medium Mowiol (Sigma).

**Analyses and statistics**

Representative light microscopic images were prepared with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope using the AxioVision 4.6 software (Carl Zeiss, Jena, Germany).
Zeiss, Göttingen, Germany). Confocal images were prepared with Nikon A1R (Nikon, Wien, Austria) and Radiance 2100 (Bio-Rad Laboratories, Hemel Hempstead, UK) confocal systems. For quantitative studies, the immunostained microscopic specimens as well as the digital photographs were randomized, coded, and analyzed by investigators blind to the origin of samples. Group comparisons were carried out with one-way ANOVA using the Statistica 8.0 software package (StatSoft, Inc., Tulsa, OK). Immunohistochemical samples from eight to 11 young male and five to nine aged male subjects were included in each experiment and statistical group.

**Experimental design**

**Experiment 1. Studies of the incidence of KP-IR and NKB-IR perikarya in the Inf**

The abundance of KP-IR and NKB-IR perikarya was counted in the Inf at \( \times 100 \) magnification in a 0.25-mm\(^2\) counting area, with the aid of a 5 × 5 ocular grid, as described earlier (10, 21, 24). Each individual was characterized by the highest number of IR cell bodies per counting area that was detectable in two to six sections.

**Experiment 2. Studies of the perikaryon size of NKB-IR neurons**

We measured and compared between young and aged men the profile area of KP-IR and NKB-IR neurons using an approach detailed elsewhere (21). Briefly, solitary NKB-IR perikarya were identified in digital photographs of the Inf, and the surrounding neuronal processes were erased from the images using the Adobe Photoshop CS software. Digital images of the labeled cell bodies were compiled into TIF files and opened with the ImageJ software. The profile area of NKB-IR neurons was determined as described recently (21). The profile area of each neuron was characterized with the mean profile area of 10–30 labeled perikarya.

**Experiment 3. Studies of the regional density of KP-IR and NKB-IR fibers**

The regional density of IR fibers was determined as described recently (21). First, digital images were taken 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 perikarya and proximal dendrites were erased using the Adobe Photoshop software and opened with the ImageJ software. The regional fiber density in each photograph was defined as the area occupied by IR fibers/total area. For each subject, the mean fiber density was derived from one to three digital images.

**Experiment 4. Colocalization studies of KP and NKB in the Inf**

The incidence of double-labeled KP-IR and NKB-IR perikarya was determined quantitatively from the dual-immunofluorescent specimens in which the tyramide signal amplification was used. This analysis included one to three representative confocal images per subject.

**Experiment 5. Studies of the incidence of KP-IR and NKB-IR appositions onto GnRH-IR neurons of the Inf**

Dual-immunoperoxidase-labeled sections were used (one to three from each individual) to determine the number of axonal contacts along the outlines of GnRH-IR perikarya and dendrites. Counting of the appositions was carried out using a \( \times 100 \) oil-immersion objective. Contacts were defined using consistently applied stringent criteria (21, 24, 29). Each human subject was characterized with the mean number of con-
tacts per GnRH soma and per 100 μm of GnRH dendrite length (21).

Experiment 6. Colocalization studies of KP and NKB in neuronal afferents to GnRH neurons of the Inf

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 (512 × 512 pixels, z-steps 0.6 μm) were obtained by scanning GnRH neurons in the Inf and their KP-IR and NKB-IR contacts using a ×60 oil immersion objective and a Radiance 2100 confocal system. The three fluorochromes were detected with the following laser lines and filters: 488 nm for FITC, 543 nm for Cy3, 405 nm for AMCA, with dichroic/emission filters 560/500–540 nm for FITC, 650/560–610 nm for Cy3, and 500/420–480 nm for AMCA. The separately recorded green, red, and blue channels were merged and displayed with the Laser Vox software (Bio-Rad Laboratories) running on an IBM-compatible personal computer. Appositions were validated if no gap was visible between the juxtaposed profiles in at least one optical slice. A total of 604 contacts (mixed axo-dendritic and axosomatic) were analyzed to count the percent ratios of double-labeled inputs to GnRH neurons.

Results

Experiment 1. Incidence of KP-IR and NKB-IR perikarya in the Inf

Quantitative analysis of the labeled perikarya in peroxidase-labeled specimens (using the maximal number of immunolabeled somata per 0.25-mm² counting frame for each individual) revealed the following differences. 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 (Figs. 1A and E, and 2). NKB-IR cell bodies also outnumbered KP-IR perikarya in aged men (P = 0.0005) but only 2.2-fold (Figs. 1, B and F, and 2).

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) (Figs. 1, A and B, and 2). NKB-IR cell bodies also showed higher mean incidence in aged compared with young men (P = 0.016) (Figs. 1, E and F, and 2), but the difference was only 1.5-fold.

Experiment 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 ± 10.6 μm²) compared with young (167.3 ± 9.6 μm²) men. The increase between the two age groups was statistically significant (P = 0.02).

Experiment 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 =
The density of NKB-IR axons was also high in aged men but only 2.9-fold higher than the density of KP-IR axons ($P = 0.004$) (Figs. 1, B, D, F, and H, and 3).

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 ($P = 0.032$) (Figs. 1, A–D, and 3), whereas the density of NKB-IR axons showed a 1.5-fold aging-related increase ($P = 0.018$) (Figs. 1, E–H, and 3).

**Experiment 4. Colocalization of KP and NKB in neuronal perikarya of the Inf**

The quantitative analysis of labeled cell bodies in dual-immunofluorescent specimens (Figs. 4 and 5) 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. 4A). Similarly, in aged men, $77.9 \pm 5.9\%$ of the KP-IR cell bodies contained NKB-immunoreactivity (Fig. 4B). 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 ($P = 0.001$).

**Experiment 5. Incidence of KP-IR and NKB-IR appositions onto GnRH-IR neurons**

Sections double-labeled for KP and GnRH or NKB and GnRH used the silver-gold-intensified nickel-diaminobenzidine and diaminobenzidine chromogens, in combination.

The high-power light microscopic analysis of these sections confirmed the previous observations (10, 21) that KP-IR and NKB-IR axons establish axo-somatic and axo-dendritic contacts onto GnRH-IR neurons of the Inf (Fig. 6, A–H).

The quantitative analysis of appositions (Fig. 7) established that the NKB-IR innervation is heavier compared with the KP-IR innervation. In young men, GnRH-IR cell bodies received 6-fold more NKB-IR than KP-IR appositions, and GnRH-IR dendrites received 6.4-fold more NKB-IR than KP-IR appositions (cell bodies, $P = 0.013$; dendrites, $P = 0.005$) (Figs. 6 and 7). In aged men, GnRH-IR cell bodies received 5.3-fold more NKB-IR than KP-IR appositions, and dendrites received 6.4-fold more NKB-IR appositions than KP-IR appositions (cell bodies, $P = 0.0002$; dendrites, $P = 0.0001$) (Figs. 6 and 7).

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 (Figs. 6 and 7). 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, $P = 0.006$) (Figs. 6 and 7).

**Experiment 6. Colocalization of KP and NKB in neuronal afferents to GnRH neurons**

In triple-immunofluorescent specimens, the axonal KP and NKB immunolabeling showed a low degree of overlap only. GnRH neurons were most frequently contacted by single-labeled axons both in young (data not shown) and aged (Fig. 8) men, without detectable age effects on the extent of colocalizations. KP-immunoreactivity was detected in $7.3 \pm 1.5\%$ of NKB-IR afferents in young and $9.5 \pm 3.7\%$ of NKB-IR afferents in aged men. 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.

**Discussion**

In the present study, we provide comprehensive immunohistochemical evidence for robust enhancements in KP
and moderate increases in NKB signaling in the Inf of aged men.

**Roles of mediobasal hypothalamic KP and NKB neurons in reproductive regulation**

Hypothalamic “KNDy” neurons of the mediobasal hypothalamus (7, 8, 30, 31), which cosynthesize KP, NKB, and dynorphin A, have been implicated in negative sex steroid feedback to GnRH neurons (5, 13, 32) and also proposed to profoundly influence the GnRH neurosecretory pulses (6–8, 11). Several recent models of the GnRH pulse generator (6–8) propose that the intranuclear communication of the KNDy neuronal network uses NKB signaling through its receptor, NK3, and, possibly, also dynorphin A signaling via its receptor, k-opioid receptor. In ovariectomized goats, central NKB increases and dynorphin A decreases the frequencies of multiunit activity volleys and LH secretory pulses (6). The putative pulse generator cells, in turn, appear to communicate with GnRH neurons mainly using KP signaling via its receptor, KISS1R. Accordingly, GnRH neurons express KISS1R (33–35), and the majority of GnRH neurosecretory pulses in monkeys show temporal association with KP pulses in the median eminence (36). Existing models of the pulse generator and negative sex steroid feedback are based on the similarities of recently published reports from several species and do not provide explanation to some conflicting data and unexplained inconsistencies in the literature. For example, although in ovariectomized goats, NKB enhanced the frequencies of the multiunit activity volleys and the LH secretory pulses (6), ovariectomized rats responded with reduced frequencies to the centrally administered NK3 agonist senktide (37). Intracerebroventricular injection of senktide in the latter study did not affect the rise in LH concentrations after administration of KP, and KP did not preclude the senktide-induced suppression of LH pulses, suggesting independent mechanisms of KP and NKB actions. The senktide-induced inhibition of pulsatile LH secretion could be prevented with a κ opioid receptor antagonist, indicating that NKB suppresses the frequency of the GnRH pulse generator in a dynorphin A/κ opioid receptor-dependent fashion (37). The role of KP might also be more complex than to provide a simple output signal toward GnRH neurons. In some studies, KP also appears to act on the pulse generator system and increases the frequency of neurosecretory pulses in rats (38). In men, a single injection of KP has been shown to reset the hypothalamic GnRH clock (39), whereas chronic infusion of KP stimulates LH pulsatility (40). Conflictingly, other studies found no effect of KP on the frequency of multiunit
Electrical activity volleys in ovariectomized goats (41) and rats (42).

Furthermore, the role of dynorphin A may not be universal. Although it is present in the majority of NKB (and/or KP) neurons in the ARC of sheep (5, 43), mice (7, 8), rats (44, 45), and goats (6), our recent immunohistochemical study only found low levels of dynorphin A-immunoreactivity and colocalization with KP in the Inf of young male human subjects (24). The functional significances of KP, NKB, and dynorphin A in reproductive regulation may considerably vary among species, between sexes, and at different ages. It will be a future challenge to decipher the relationship between the characteristic immunohistochemical images of KNDy neuropeptides and the patterns of GnRH pulsatility and negative feedback.

**Sex- and aging-dependent variations in KP-immunoreactivity and NKB-immunoreactivity and their colocalization pattern**

Recent work from our laboratory (10, 21, 24) provides immunohistochemical evidence that the KP and NKB systems are sexually dimorphic in the mediobasal hypothalamus of aged humans; postmenopausal women contained significantly higher numbers of KP-IR (and NKB-IR) perikarya, higher densities of KP-IR fibers, and higher incidences of KP-IR inputs to GnRH neurons than did elderly men. The high levels of KP-immunoreactivity and NKB-immunoreactivity (21) and mRNAs (46, 47), and the postmenopausal hypertrophy of KP- and NKB-synthesizing neurons in the Inf (21, 46, 47), may be mostly attributable to the removal of negative estrogen feedback from the reproductive axis, whereas testosterone can continue to suppress KP and NKB syntheses in elderly men. The two sexes also differed in the degree of neuropeptide colocalization within KP-IR and NKB-IR inputs to GnRH neurons, which was 9–10% in aged men and 26–31% in postmenopausal women (21). It is interesting to note that these colocalization percentages are below the extent of neuropeptide colocalization in neuronal cell bodies of the Inf in both men (results of the present study) and women (10). From this, it occurs that the axon projections of NKB/KP neurons do not necessarily contain detectable levels of both neuropeptides. Indeed, in the present as well as in previous (10, 21, 24) studies, we found that the Inf contains many single-labeled, in addition to dual-labeled, NKB-IR and KP-IR axons in both sexes. This discrepancy between the extent of somatic and axonal neuropeptide colocalization may also be contributed by NKB-IR and KP-IR inputs to the Inf and to GnRH neurons from outside the Inf, where KP and NKB do not colocalize (10). We also have to recognize the possibility that low neuropeptide levels in axons could remain undetected in our immunofluorescent experiments.

As we discussed in an earlier report (21), 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 critically 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.

Our present study used similar quantitative immunohistochemical metrics to address the predicted age-dependent enhancements of central KP and NKB signaling in men. The comparative experiments were carried out on postmortem 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 en-
enhanced central KP and NKB signaling in elderly men. 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. In our previous study, we observed a 22% aging-related increase in the mean profile area of NKB neurons, which was statistically significant. This observation is reminiscent to the previously reported mild (≈12%) increase in the size of unidentified neurons in the Inf of the aging men (48). We note that the reported hypertrophy of NKB mRNA expressing neurons is much more robust (≈90%) in the absence of negative feedback in postmenopausal women (46). In accordance with more dramatic changes in the Inf of the aging female, in our previous study, we observed 55–85% larger KP-IR and NKB-IR neurons in postmenopausal women than in aged men (21).

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. In addition, KP neurons also exhibited a more robust sexual dimorphism in our previous study on aged humans, in comparison with NKB neurons (21). It is possible that both the age- and the sex-dependent phenomena simply reflect a stronger down-regulation of KP than NKB by circulating sex steroids. In accordance with this concept, a recent study on mice (49) established that the KP-encoding Kiss1 gene is, indeed, more sensitive to estrogenic suppression in comparison with the NKB-encoding gene (Tac2 in rodents).

The low degree of overlap between KP and NKB neurons in aged men suggests that the aging-related changes in KP and NKB signaling may be distinct. 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 found evidence that the percent ratio of KP-IR NKB perikarya rose from 36% in young to 68% in aged men. In male rodents, testosterone regulates KP and NKB expression of the mediobasal hypothalamus negatively (8, 13), and similarly, testosterone treatment reduces KP expression in the ARC of orchidectomized monkeys (15). 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 feed-

![FIG. 8. Detection of NKB-immunoreactivity and KP-immunoreactivity in neuronal appositions onto GnRH neurons of the Inf. The simultaneous immunofluorescent detection of NKB (green), KP (red), and GnRH (blue), followed by confocal analysis, revealed direct appositions of single- and double-immunolabeled (yellow) axons onto GnRH-IR cell bodies and dendrites in the Inf of a 50-yr-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 μm (4 μm in high-power insets).](https://academic.oup.com/endo/article-abstract/153/11/5428/2424475)
and only starts to reach detectable levels with the weakening of negative feedback in aged individuals. The physiological importance of the increased neuropeptide coexpression requires clarification.

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 testosterone 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 (50). Midlife transition in aging men is characterized by decreased serum levels of free testosterone and dihydrotestosterone, increased levels of LH, FSH, and sex hormone binding globulin (16, 17). In addition, aging is associated with depressed pulsatile and elevated basal LH secretion and a decline in LH secretory burst mode (18). Elderly men also secrete LH and testosterone more irregularly and more asynchronously than do young men (51, 52). Some of these endocrine alterations result from a reduced androgen receptor-mediated negative feedback to the hypothalamus (18), 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 (18). In view of the proposed involvement of KP/NKB neurons in testosterone negative feedback to the male hypothalamus (8, 13, 15), in our present study 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 lower extents. Finally, in dual-immunofluorescent studies, the incidence of KP-IR NKB perikarya increased from 36% in young to 68% in aged men.

In summary, our data provide immunohistochemical evidence for the aging-related enhancements in central KP and NKB signaling in the Inf, which is compatible with a reduced testosterone 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.

Acknowledgments

We thank Ms. Hajni Bekó for expert technical assistance and Mr. László Barna, the Nikon Microscopy Center at the Institute of Experimental Medicine, Nikon Austria GmbH, and Auro-Science Consulting Ltd. for kindly providing microscopy support.

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This work was supported by Hungarian Scientific Research Fund Grants OTKA K83710 and K100722 and by the European Community’s Seventh Framework Program (FP7/2007-2013; Grant Agreement 245009) and by TAMOP-4.1.2-08/2/A/KMR-2009-0006. W.S.D. is funded by a National Institute for Health Research Career Development fellowship.

Disclosure Summary: The authors have nothing to disclose.

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