Morphological evidence for enhanced kisspeptin and neurokinin B signaling in the infundibular nucleus of the aging man

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Abstract

Peptidergic neurons synthesizing kisspeptin (KP) and neurokinin B (NKB) in the hypothalamic infundibular nucleus (Inf) have been implicated in negative sex steroid feedback to gonadotropin-releasing hormone (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 Inf. 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 years; N=11) and 'aged' (50-67 years; 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- 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.

Introduction

Type I gonadotropin-releasing hormone (GnRH) synthesizing neurons are protagonists in the hypothalamic control of reproduction. The pulsatile neurosecretory output from GnRH axon terminals into the hypophysial portal circulation regulates the release of the adenohypophysial gonadotropins LH and FSH which, in turn, stimulate gonadal functions (1).

Peptidergic neurons synthesizing kisspeptin (KP) (2) and neurokinin B (NKB) (3, 4) in the hypothalamic arcuate nucleus (ARC; called infundibular nucleus in the human; Inf) are important upstream regulators of GnRH neurosecretion. As shown first in sheep (5) and later in other species (6-8) including monkeys (9) and humans (10), KP and NKB are extensively colocalized in the ARC/Inf; KP/NKB neurons have been proposed to serve as pacemakers for the GnRH neurosecretory pulses (6, 7, 11) and to mediate negative sex steroid feedback to GnRH neurons (5, 12-15). In male rodents, testosterone regulates KP and NKB expression of the ARC negatively (8, 13).

Reproductive aging in men during midlife transition is characterized by decreased serum levels of free testosterone and increased levels of LH, FSH and sex hormone binding globulin, among other endocrine alterations (16, 17). 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 (18). In view of the proposed involvement of KP/NKB neurons in negative feedback (5, 12, 13), the present study was carried out to determine whether weakening of the inhibitory testosterone feedback in elderly men coincides with enhanced KP and NKB signaling in the Inf. This central hypothesis was tested in a series of quantitative immunohistochemical experiments on hypothalamic sections of 20 male human individuals that were divided into the arbitrary ‘young’ (21-49 years; N=11) and ‘aged’ (50-67 years; N=9) groups. The abundance of KP-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.

Materials and methods

Human subjects

Human hypothalamic tissue samples from eleven male subjects under 50 years of age (21, 31, 33, 36, 37, 39, 40, 41, 45, 46, 49 years) and from nine subjects above (50, 50, 51, 59, 62, 64, 67, 69, 78 years) 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 (DEOEC RKEB/IKEB: 3183-2010). Selection criteria included sudden causes of death, lack of history of neurological and endocrine disorders and *post mortem* intervals below 48h.

Tissue preparation for immunohistochemistry

Following dissection, the hypothalamic tissue blocks were first rinsed with tap water and then, immersed into 4% formaldehyde in 0.1M phosphate buffer saline (PBS; pH 7.4) for 7-14 days at 4°C. Prior to section preparation, the fixed hypothalami were trimmed further to include the optic chiasm rostrally, the mammillary bodies caudally and the anterior commissure dorsally (10, 19, 20). Sagittal cuts were made 2cm lateral from the 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 coronally at 30µm with a Leica SM 2000R freezing microtome (Leica Microsystems). The sections were stored permanently in anti-freeze solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer; pH 7.4) at -20˚C.

Tissue pretreatments

Prior to immunohistochemistry, the sections were rinsed in PBS and pretreated with a mixture of 0.5% H_2O_2 and 0.2% Triton X-100 for 30 min. Then, antigen retrieval with 0.1M 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, 21, 22).

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 (GQ2; 1:100,000) against human kisspeptin-54. This antiserum recognizes human kisspeptin-54, kisspeptin-14 and kisspeptin-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, RFRP3) (23). 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, 24) and the human (10, 20, 22). Incubation in the primary antiserum for 48 h at 4°C was followed by biotinylated secondary antibodies (biotin-SP-antisheep IgG; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:500) and the ABC Elite reagent (Vector, Burlingame, CA; 1:1000) for 60 min each. The peroxidase signal was visualized with nickel-intensified diaminobenzidine chromogen and then, post-intensified with silver-gold (25).

To detect NKB synthesizing neurons, a second series of sections was incubated with a previously characterized rabbit polyclonal antiserum (IS-682; P. Ciofi; 1:100,000) against the C-terminal 28 amino acids of human pro-NKB (9, 10, 20) followed by working dilutions of biotinylated secondary antibodies (biotin-SP-antirabbit IgG; Jackson ImmunoResearch Laboratories; 1:500; 60 min) and the ABC 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 or NKB immunoreactivities as described above. Subsequently, GnRH neurons were detected with a previously characterized (20) guinea pig antiserum (#1018; 1:10,000). The primary antibodies were reacted with biotin-SP-antiguinea pig IgG (Jackson ImmunoResearch; 1:500; 60 min) and the ABC reagent (1:1000; 60 min) and then, the peroxidase signal was developed with diaminobenzidine chromogen.

Dual-immunofluorescent visualization of NKB and KP

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 (22). First, KP was detected using sequential incubations in KP antibodies (1:30,000; 48h; 4ºC), biotinylated anti-sheep IgG (Jackson ImmunoResearch Laboratories; 1:500; 60 min), the ABC Elite reagent (Vector; 1:1000; 60 min), biotin tyramide working solution (1:1000, in 0.05M Tris-HCl buffer, pH 7.6, containing 0.003% H_2O_2; 30 min) (26) and finally, avidin-Cy-3 (Jackson ImmunoResearch; 1:1000; 60 min). 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, the IS-682 primary antibodies were used at 1:50,000 (48h; 4ºC) and reacted with anti-rabbit-peroxidase (Jackson ImmunoResearch; 1:500; 1h). Then, FITC-tyramide (26) (diluted 1:500 with 0.05M Tris-HCl buffer, pH 7.6, containing 0.003% H_2O_2; 30 min) 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 rabbit anti-NKB, 1:500; GQ2 sheep anti-KP, 1:1000; #1018 guinea pig anti-GnRH, 1:5000) for 48h at 4ºC was followed by a mixture

of fluorochrom-conjugated secondary antibodies (anti-rabbit-FITC, 1:200; anti-sheep-Cy3, 1:1000; anti-guinea pig-AMCA, 1:100; Jackson ImmunoResearch) for 5h.

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% (2X5 min) ethanol, cleared with xylene (2X5 min) and coverslipped with DPX mounting medium (Sigma, St. Louis, USA). Immunofluorescent specimens were mounted from 0.1M Tris-HCl buffer (pH 7.6) and coverslipped with the aqueous mounting medium Mowiol.

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, Göttingen, Germany). Confocal images were prepared with Nikon A1R (Nikon, 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, USA). Immunohistochemical samples from 8-11 young male and 5-9 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 were counted in the Inf at 100X magnification in a 0.25 mm^2 counting area, with the aid of a 5X5 ocular grid, as described previously (10, 20). Each

individual was characterized by the highest number of immunoreactive cell bodies per counting area that was detected in 2-6 sections.

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

We measured and compared the profile area of NKB-IR neurons between young and aged men using an approach detailed elsewhere (20). 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 for area/cell body analysis with the Image J software (http://rsbweb.nih.gov/ij/download.html). The threshold was set to highlight the labeled cell bodies in all specimens. The signal areas were measured and then, converted to μm^2 using appropriate calibration. Each human subject 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 immunoreactive fibers was determined as described recently (20). 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 ("eraser tool") from the photomicrographs. The remaining images containing neuronal fibers 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.

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

The incidence of double-labeled KP-IR and NKB-IR perikarya were determined quantitatively from the dual-immunofluorescent specimens in which the tyramide signal amplification was used. This analysis included 1-3 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 (1-3 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 63X oil-immersion objective. Contacts were defined using consistently applied stringent criteria (20, 22, 27). Each human subject was characterized with the mean number of contacts per GnRH soma and per 100μm GnRH dendrite (20).

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 (512x512 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 60x oil immersion objective and the Radiance 2100 confocal microscope. The three fluorochromes were detected with the following laser lines and filters: 488 nm for FITC, 543 nm for CY3, 405nm for AMCA, with dichroic/emission filters 560 nm/500–540 nm for FITC, 650 nm/560-610 nm for CY3, 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. 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 axo-somatic) 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 mm^2 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, E and Graph 1). NKB-IR cell bodies also outnumbered KP-IR perikarya in aged men (P=0.0005), but only 2.2-times (Figs. 1B, F and Graph 1).

Aging was associated with increased perikaryon numbers. KP-IR cell bodies showed a 2.6-times higher mean density in aged compared with young men (P=0.004)(Figs. 1A, B and Graph 1). NKB-IR cell bodies also showed higher mean incidence in aged compared with young men (P=0.016) (Figs. 1E, F and Graph 1), but the difference was only 1.5-fold.

Experiment 2. Perikaryon size of NKB-IR neurons

Although the mean profile area of NKB-IR cell bodies was 12% higher in the Inf of aged 185.5±14.5μm^2) compared with young (165.3±11.7μm^2) men, there was no significant difference (P=0.30) between the two age groups.

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=0.000003) (Figs. 1A, C, E, G and Graph 2). The density of NKB-IR axons was also high in aged men, but only 2.9-times higher than the density of KP-IR axons (P=0.004) (Figs. 1B, D, F, H and Graph 2).

Aging was associated with increased KP and NKB fiber densities. KP-IR fibers showed 3.1-times higher density in aged than in young men (P=0.032)(Figs. 1A-D and Graph 2), whereas the density of NKB-IR axons showed a 1.5-fold aging-related increase (P=0.018)(Figs. 1E-H and Graph 2).

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

The quantitative analysis of labeled cell bodies in dual-immunofluorescent specimens (Fig. 2 and Graph 3) 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 (Fig. 2A). Similarly, in aged men 77.9±5.9% of the KP-IR cell bodies contained NKB immunoreactivity (Fig. 2B). 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 (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, 20) that KP-IR and NKB-IR axons establish axo-somatic and axo-dendritic contacts onto GnRH-IR neurons of the Inf (Figs. 3 A-H).

The quantitative analysis of appositions (Graph 4) 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. 3 and Graph 4). 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 (cell bodies: P=0.0002; dendrites: P= 0.0001)(Fig. 3 and Graph 4).

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-times heavier KP-IR input to the cell bodies (P=0.004) and a 2-times 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. 3 and Graph 4). 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-times and axo-dendritic contacts 1.9-times more frequent in aged than in young individuals (axo-dendritic contacts: P=0.001; axo-somatic contacts: P=0.006)(Fig. 3 and Graph 4).

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 partial overlap only. GnRH neurons were most frequently contacted by single-labeled axons both in young (not shown) and aged (Fig. 4) 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 (P=0.66).

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, 28, 29) which co-synthesize KP, NKB and dynorphin A have been implicated in negative sex steroid feedback to GnRH neurons (5, 13, 30) 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, KOR. 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 (31-33) and the majority of GnRH neurosecretory pulses in monkeys show temporal association with KP pulses in the median eminence (34). 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, while 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 (35). The role of KP might also be more complex than to provide a simple output signal towards GnRH neurons. KP also appears to act on the pulse generator system. It increases the frequency of neurosecretory pulses in rats (36). In humans a single injection of KP has been shown to reset the hypothalamic GnRH clock in men (37), while chronic infusion of KP stimulates LH pulsatility (38). Furthermore, the role of dynorphin A may not be universal. While it is present in the majority of NKB (and/or KP) neurons in the ARC of sheep (5, 39), mice (7, 8), rats (40, 41) 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 (22). 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 and NKB immunoreactivities and their colocalization pattern

Recent work from our laboratory (10, 20, 22) provides immunohistochemical evidence that the KP and NKB systems are sexually dimorphic in the mediobasal hypothalamus of aged humans; postmenopausal women contained higher numbers of KP- (and NKB-) IR perikarya, higher densities of KP-IR fibers and higher numbers of KP-IR inputs to GnRH neurons than did elderly men. The high levels of KP and NKB immunoreactivities (20) and mRNAs (42, 43) and the postmenopausal hypertrophy of KP- and NKB-synthesizing neurons in the Inf (20, 42, 43) may be mostly attributable to the removal of

negative estrogen feedback from the reproductive axis, whereas testosterone continues to suppress KP and NKB syntheses in elderly men. As we discussed in an earlier report (20), 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 *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. 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, 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 feedback to KP and NKB neurons in aged, compared with young, men.

In Experiment 2 we addressed the possibility that the reduced negative feedback causes a similar hypertrophy of NKB neurons as does ovarian failure in postmenopausal women (20, 42). As

opposed to the robust (>90%) postmenopausal increase in the mean surface area of NKB neurons (42), in this study we only found a 12% aging-related increase in the mean profile area of NKB neurons which was not significant, unlike a similarly mild but statistically significant increase in the size of unidentified Inf neurons that was reported earlier by Rance and colleagues (44).

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 (20). 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 (45) 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 confirms our recent observation that in young men below 37 years only 33% of the NKB-IR cell bodies expressed KP immunoreactivity (22). In the present study we also demonstrated that the low percentage of NKB-IR neurons that contained KP increased to 68% in aged (≥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 testosterone 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.

In experiment

MÉg idézni: . For example, there is evidence that Kp does not affect volleys of multi-unit activity in goats (Ohkura et al., J Neuroendocrinol 21: 811-13, 2009) and rats (Kinsey-Jones et

al., Endocrinology 149: 1004-8, 2008) that could be mentioned. In addition, it might be informative to the readers to note that reference 34 demonstrated that senktide reduced frequencies in OVX rats via dynorphin (46, 47)

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 (48). 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 (49, 50). 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 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, 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.

Acknowledgements

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587 **Legends**

588

589 **Figure 1. Aging-related expansion of KP-IR and NKB-IR neuronal elements in the Inf of the**
590 **human male**

591 KP (**A-D**) and NKB (**E-H**) immunoreactivities were visualized using the silver-gold intensified nickel-
592 diaminobenzidine chromogen. The representative photomicrographs were taken from the Inf of 31 (**A,**
593 **C**), 40 (**E, G**) and 67 (**B, D, F, H**) year old individuals. Overall, NKB-IR perikarya and fibers are more
594 abundant than KP-IR elements in both young and aged subjects. KP-IR neurons show robust age-related
595 changes; the number of KP-IR cell bodies and the density of KP-IR fibers are much higher in aged (**B, D**)
596 compared with young (**A, C**) men. NKB immunoreactivity also increases with age, although changes are
597 of lower degrees. Note that the number of NKB-IR cell bodies is higher in aged (**F, H**) compared with
598 young (**E, G**) men and age-related increases also take place in the regional density of NKB-IR fibers (**E-**
599 **H**). For quantitative comparisons, see Graphs 1 and 2. Scale bar=50μm.

600

Figure 2. Effect of aging on the expression of KP immunoreactivity within NKB-IR perikarya of the Inf

Dual-immunofluorescent studies which simultaneously used two different tyramide signal amplification approaches revealed the dominance of NKB (green) over KP (red) immunoreactivity in the Inf of a young (A; 36 year old) male human individual. Note that this dominance is less obvious in the aged man (B; 67 year old) 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±5.1% of NKB-IR neurons in the young and 68.1±6.8% of NKB-IR neurons in the aged male group. See also Graph 3. Scale bar=100μm in low-power images (A and B) and 40μm in high-power insets.

Figure 3. Aging-related increments in KP-IR and NKB-IR afferent contacts onto GnRH-IR neurons of the Inf

Sections double-labeled for KP and GnRH (**A-D**) or NKB and GnRH (**E-H**) with the combined use of silver-gold-intensified nickel-diaminobenzidine (black) and diaminobenzidine (brown) chromogens demonstrate that KP-IR axons establish axo-somatic (**A, B**) and axo-dendritic (**C, D**) contacts (arrows) onto GnRH neurons of the Inf. Similar contacts can also be observed in high numbers between NKB-IR axons and GnRH-IR neurons (arrows in **E-H**). The KP-IR input is significantly heavier in aged compared with young men (compare **B** to **A** and **D** to **C**). The NKB-IR axo-somatic (**E, F**) and axo-dendritic (**G, H**) inputs also increase with age. In addition, in both age groups, GnRH-IR cell bodies and dendrites receive several times more NKB-IR (**E-H**) than KP-IR (**A-D**) inputs. (**A, C**: 31 year-old man; **E, G:** 40 year-old man; **B, D:** 62 year-old man; **F, H:** 67 year-old man). For quantitative comparisons, see Graph 4. Scale bar=50μm.

Figure 4. Detection of NKB and KP immunoreactivities 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 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 subjects. Scale bar=10μm (4μm in high-power insets).

Graph 1. Regional density of KP-IR and NKB-IR perikarya in the Inf of young and aged men

The maximal number of immunoreactive cell bodies per 0.25mm^2 counting frame (as identified from 1-6 sections per subject) was determined with the aid of an ocular frame and used as the index of regional neuron density (10, 20). 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. *$P<0.05$.

Graph 2. Density of KP-IR and NKB-IR fibers in the Inf of young and aged men

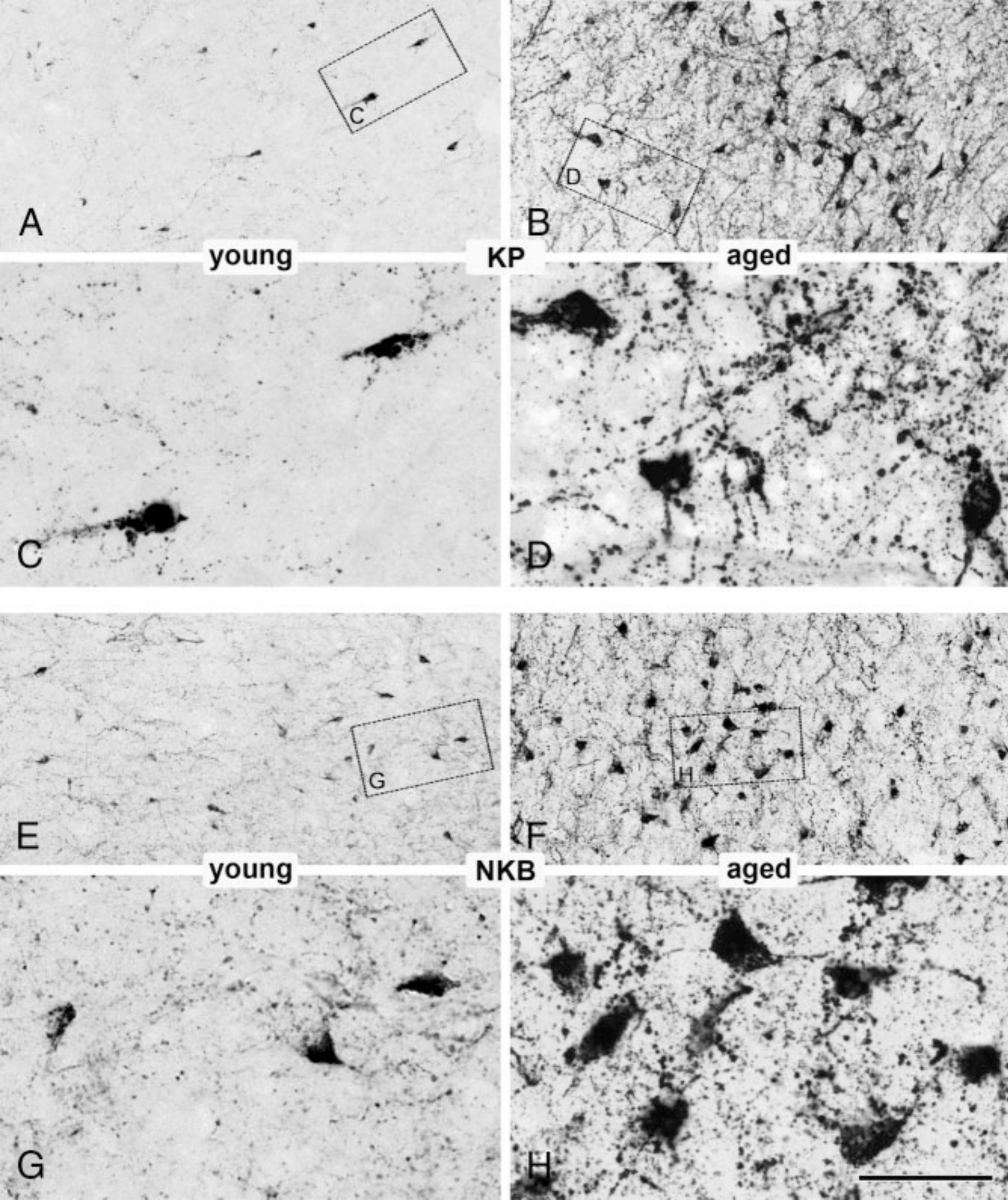
The area covered by immunoreactive fibers (divided by the total area analyzed) was determined with the Image J software in digital photographs of the Inf and used as an index of regional fiber density (presented in arbitrary units). Areas of the photomicrographs that were occupied by labeled cell bodies and their proximal dendrites were erased using the Adobe Photoshop software and excluded from the analysis. The density of NKB-IR fibers defined this way is 6-fold higher than the density of KP-IR fibers in young men and 2.9-times higher in aged men. Fiber density increases with age. KP-IR fibers show 3.1-times and NKB-IR fibers 1.5-times higher densities in aged compared with young men. *$P<0.05$.

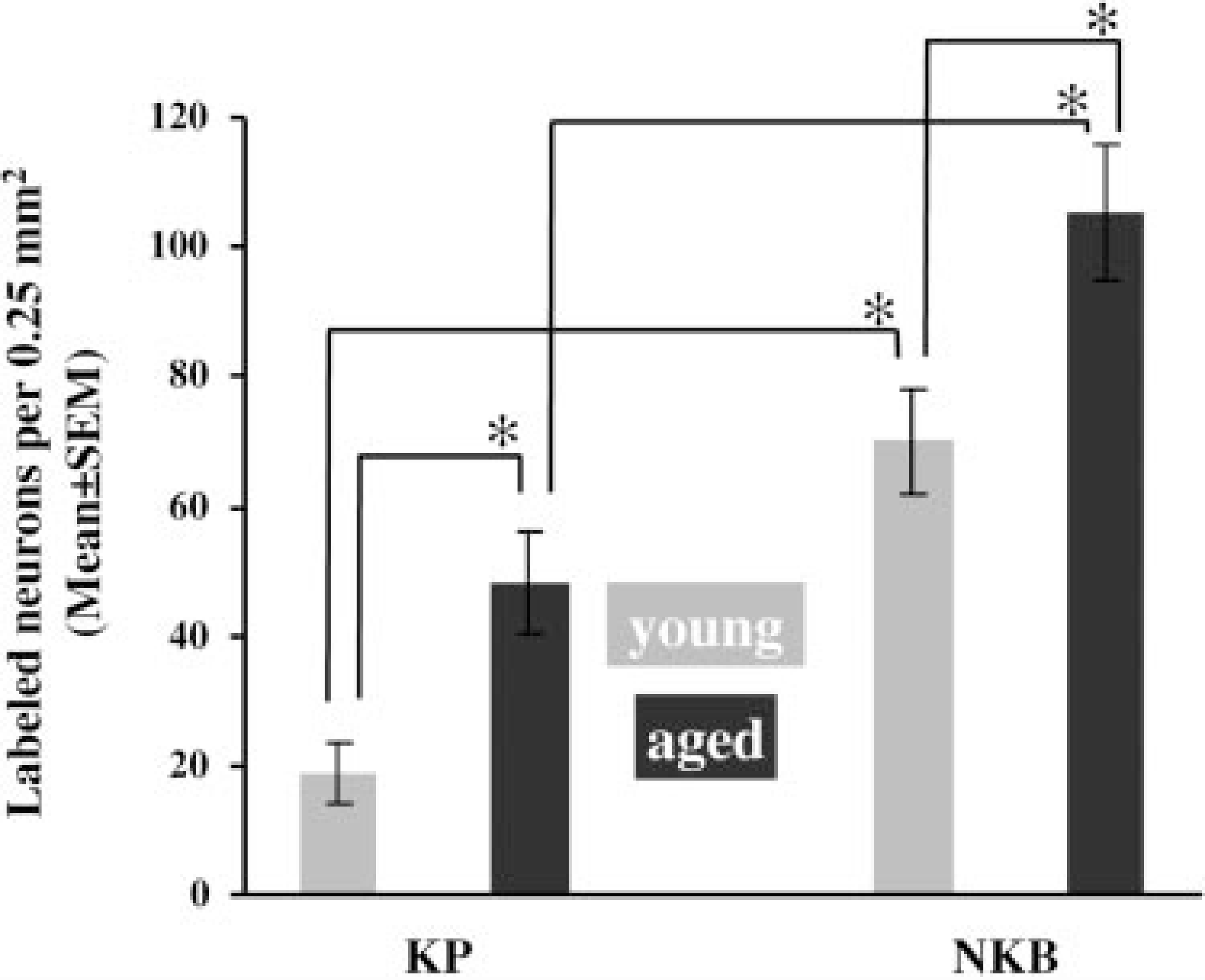
Graph 3. Effect of aging on the overlap between NKB-IR and KP-IR perikarya

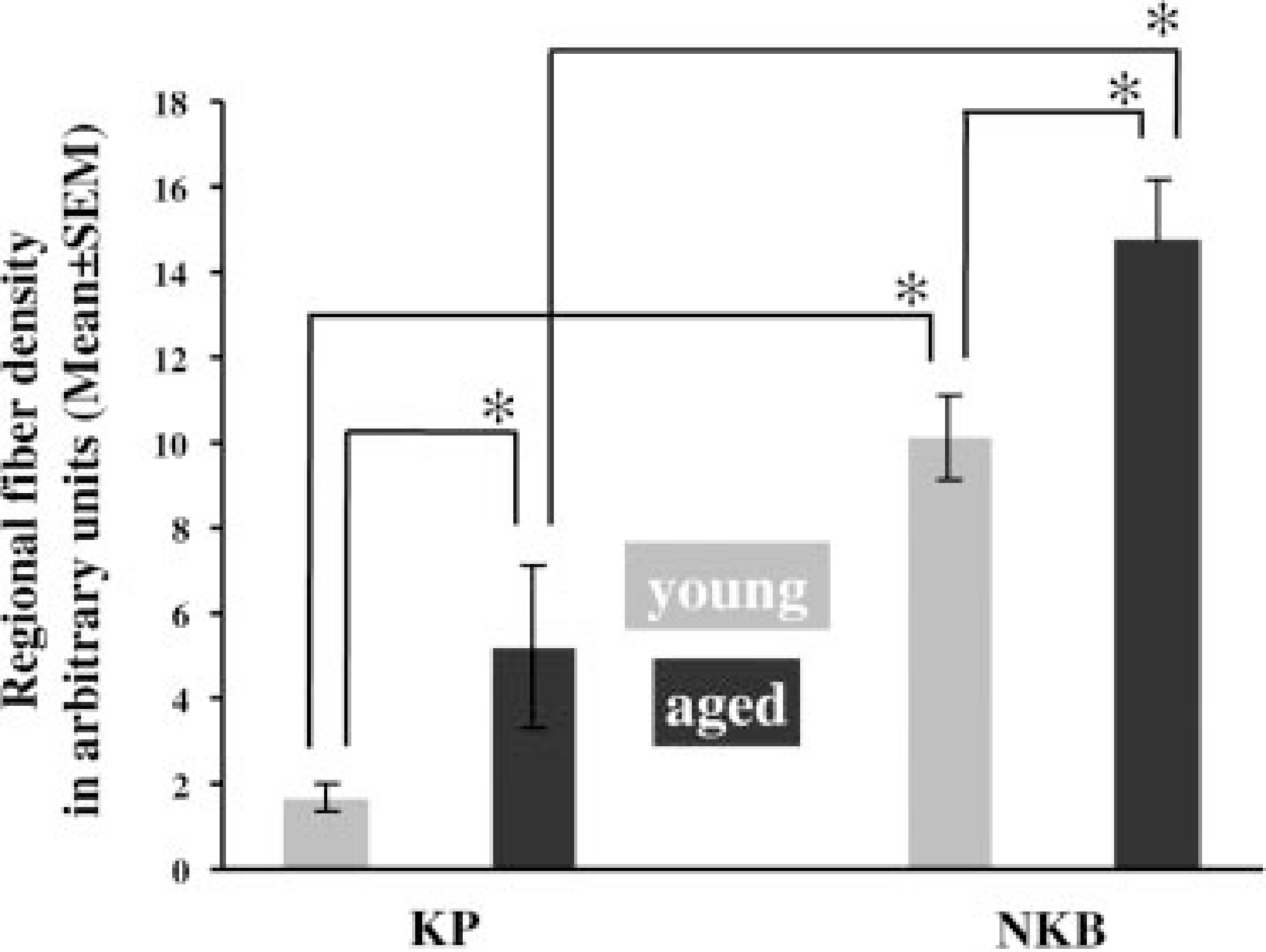
The ratios of double-labeled NKB-IR and KP-IR perikarya were determined quantitatively from dual-immunofluorescent specimens in which tyramide signal amplification approaches were applied to maximize both labeling. In young men 72.7±6.0% of KP-IR perikarya also contained NKB immunoreactivity. Similarly,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

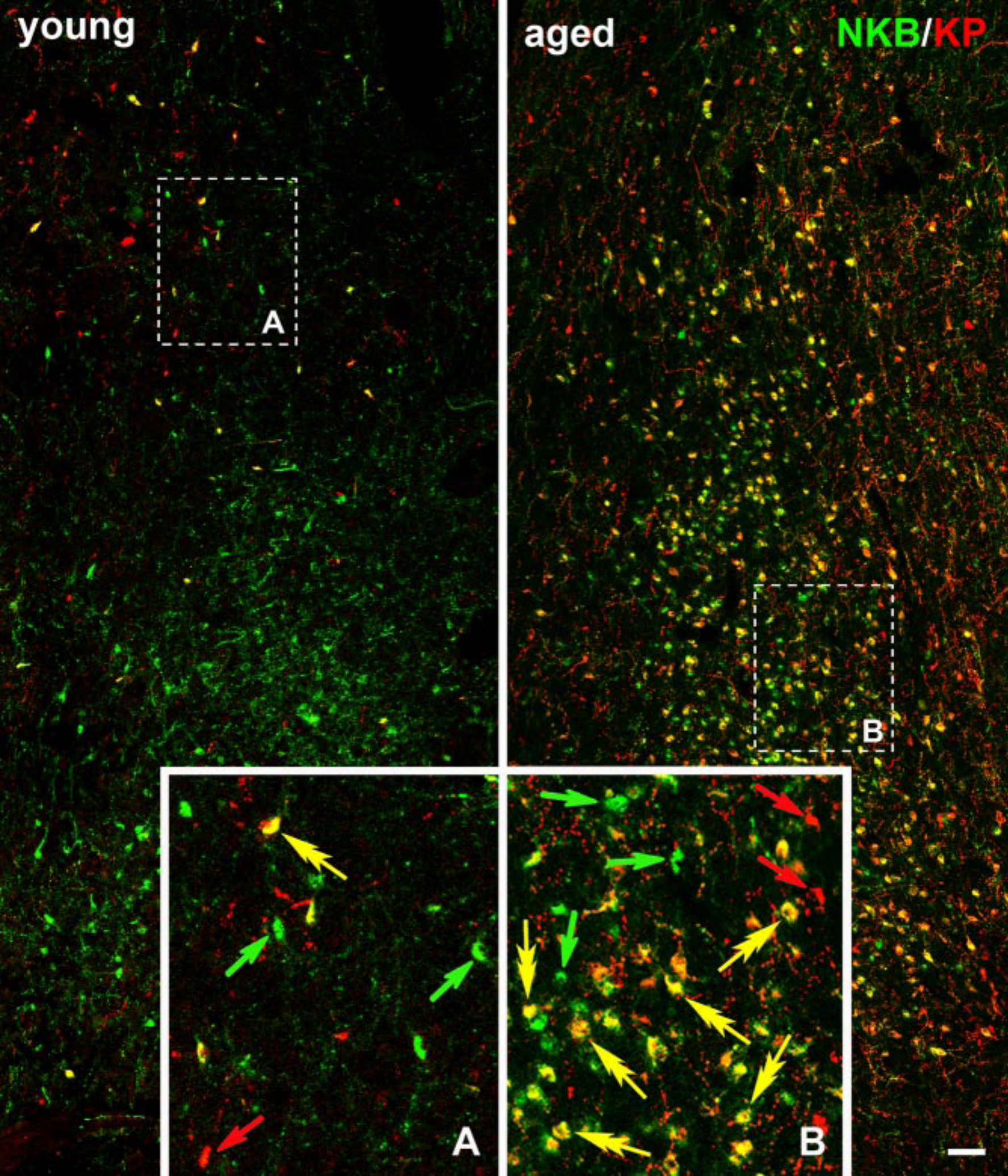
Graph 4. Effects of aging on the incidences of KP-IR and NKB-IR contacts onto GnRH-IR neurons of the Inf

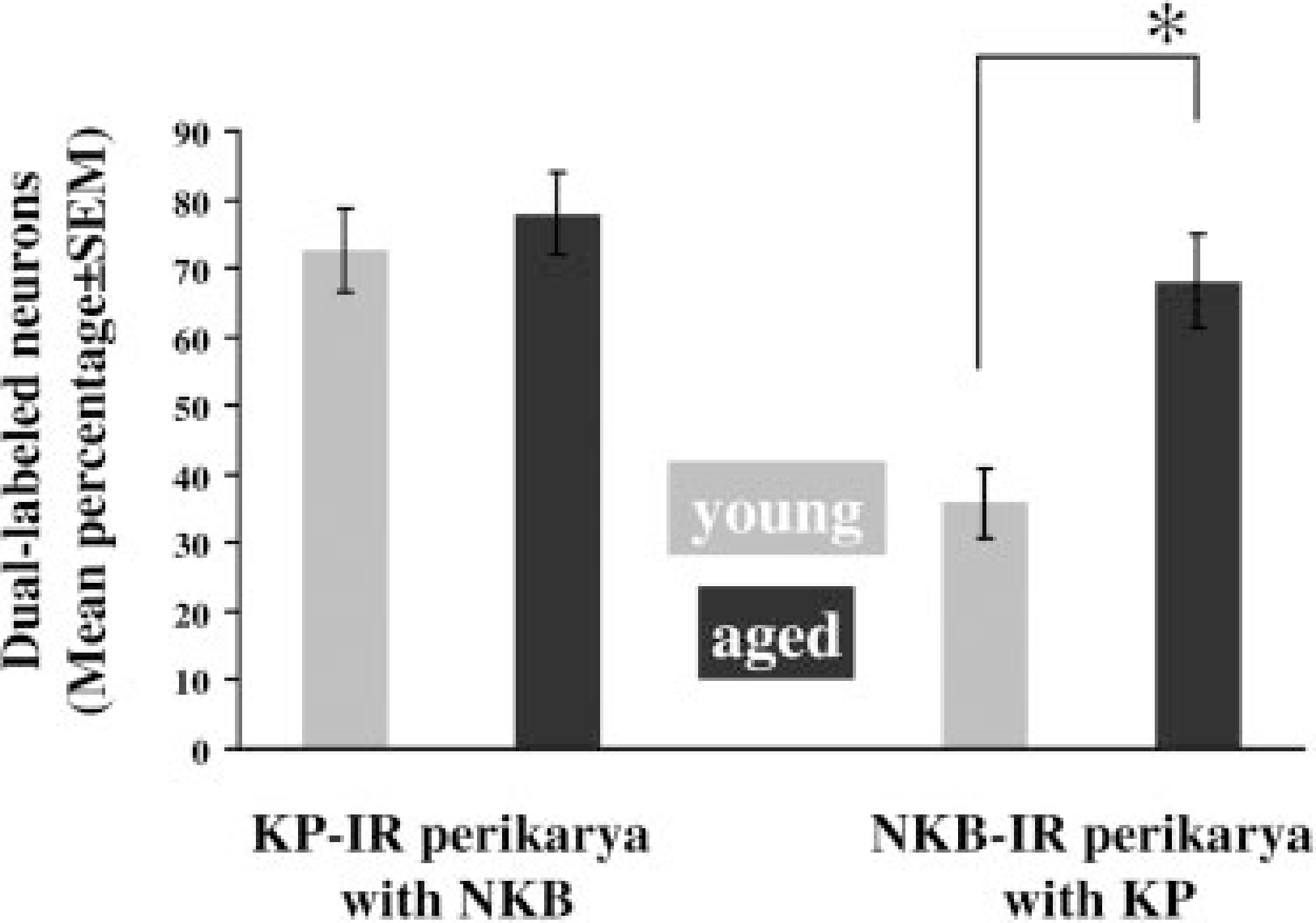
High-power light microscopic analysis of sections, dual-immunolabeled with the combined use of silver-gold-intensified nickel-diaminobenzidine and diaminobenzidine chromogens (as shown in Fig. 2), was carried out to determine the relative frequencies of KP-IR (left columns) and NKB-IR (right columns) 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 found in 1-3 sections of the Inf from each of 11 young and 9 aged individuals. Note that the number of NKB-IR contacts is considerably higher than the number of KP-IR contacts on both the somatic (**A**) and the dendritic (**B**) membranes of young as well as aged men. The innervation ratio by the two peptidergic systems is similar on GnRH cell bodies (**A**) and dendrites (**B**). The mean incidences of KP-IR as well as NKB-IR inputs to GnRH cell bodies (**A**) and dendrites (**B**) show considerable increases with age. *$P<0.05$.

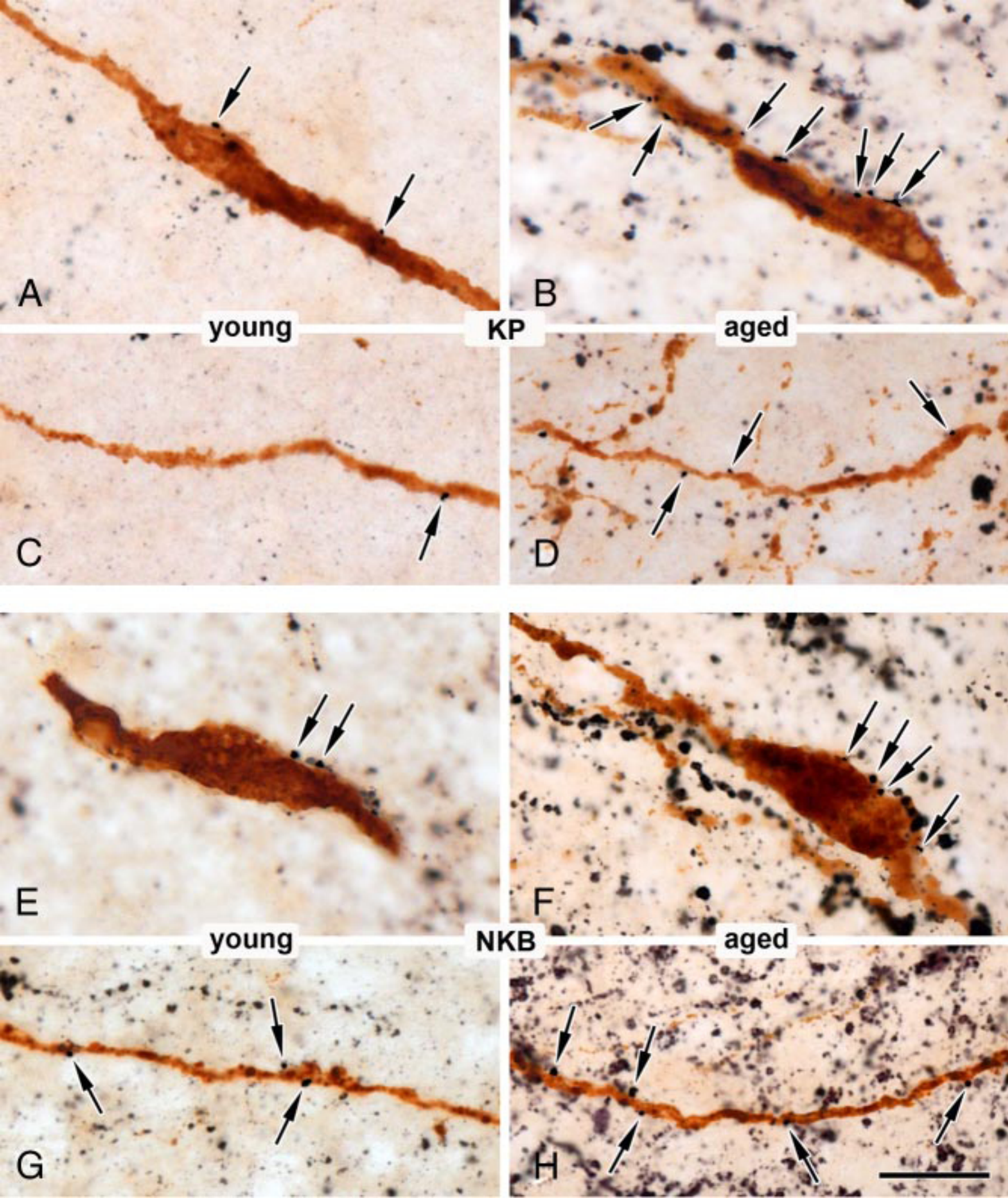

A
B
C
D
young
KP
aged
C
D
E
F
G
H
young
NKB
aged
G
H

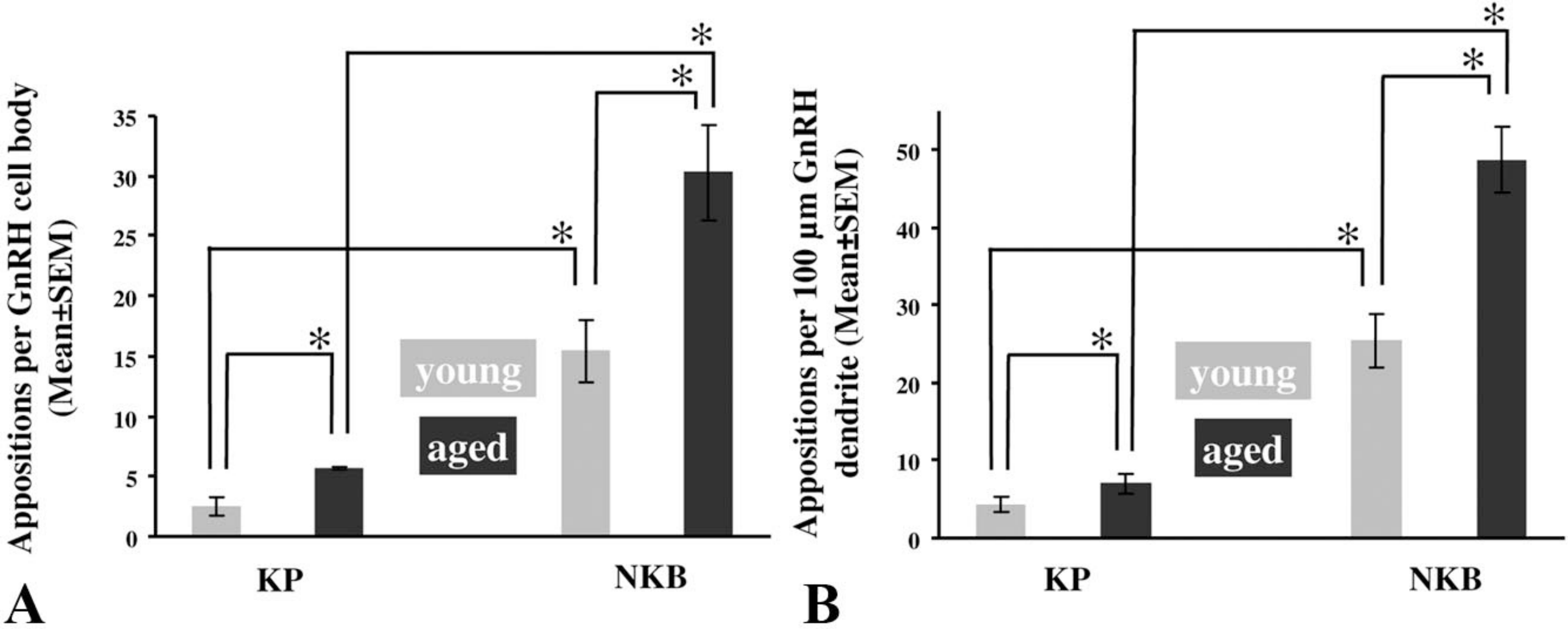

Labeled neurons per 0.25 mm2
(Mean±SEM)
120
100
80
60
40
20
0
young
aged
KP
NKB
*
*
*
*

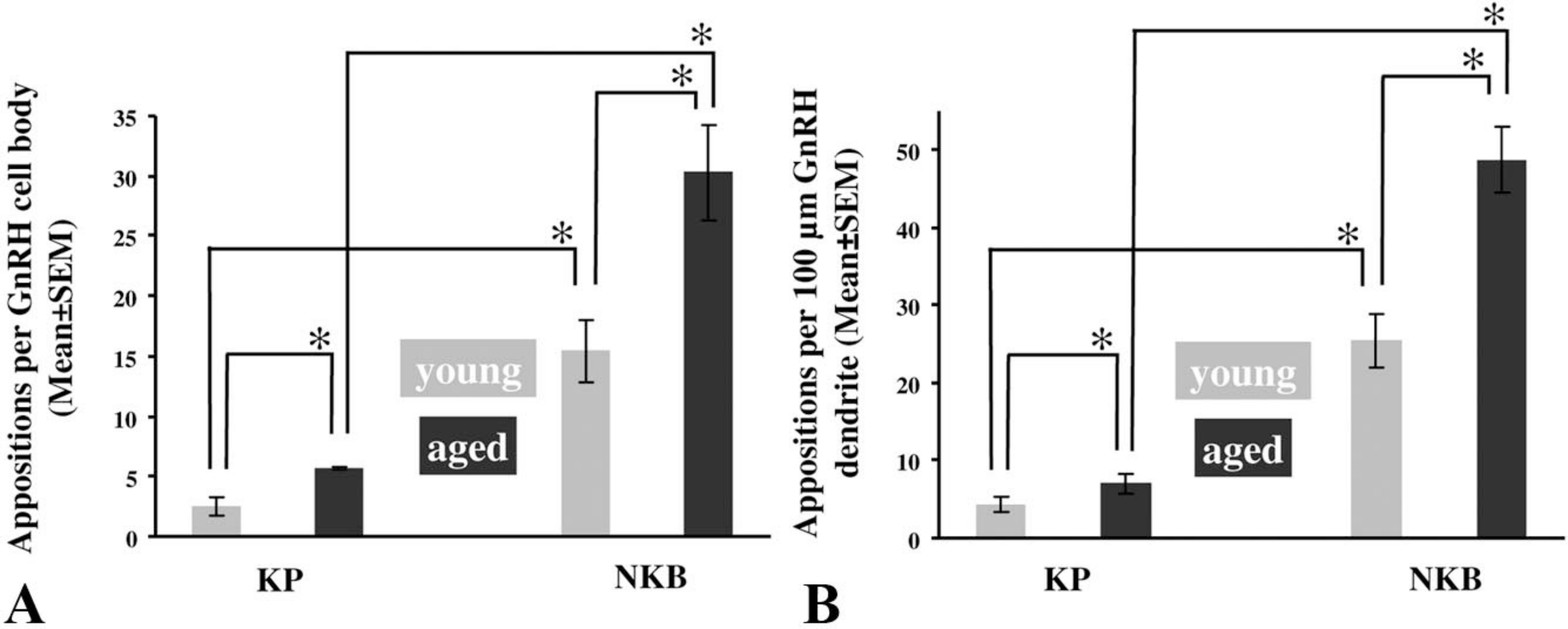

Regional fiber density
in arbitrary units (Mean±SEM)
0
2
4
6
8
10
12
14
16
18
young
aged
*
*
*
KP
NKB

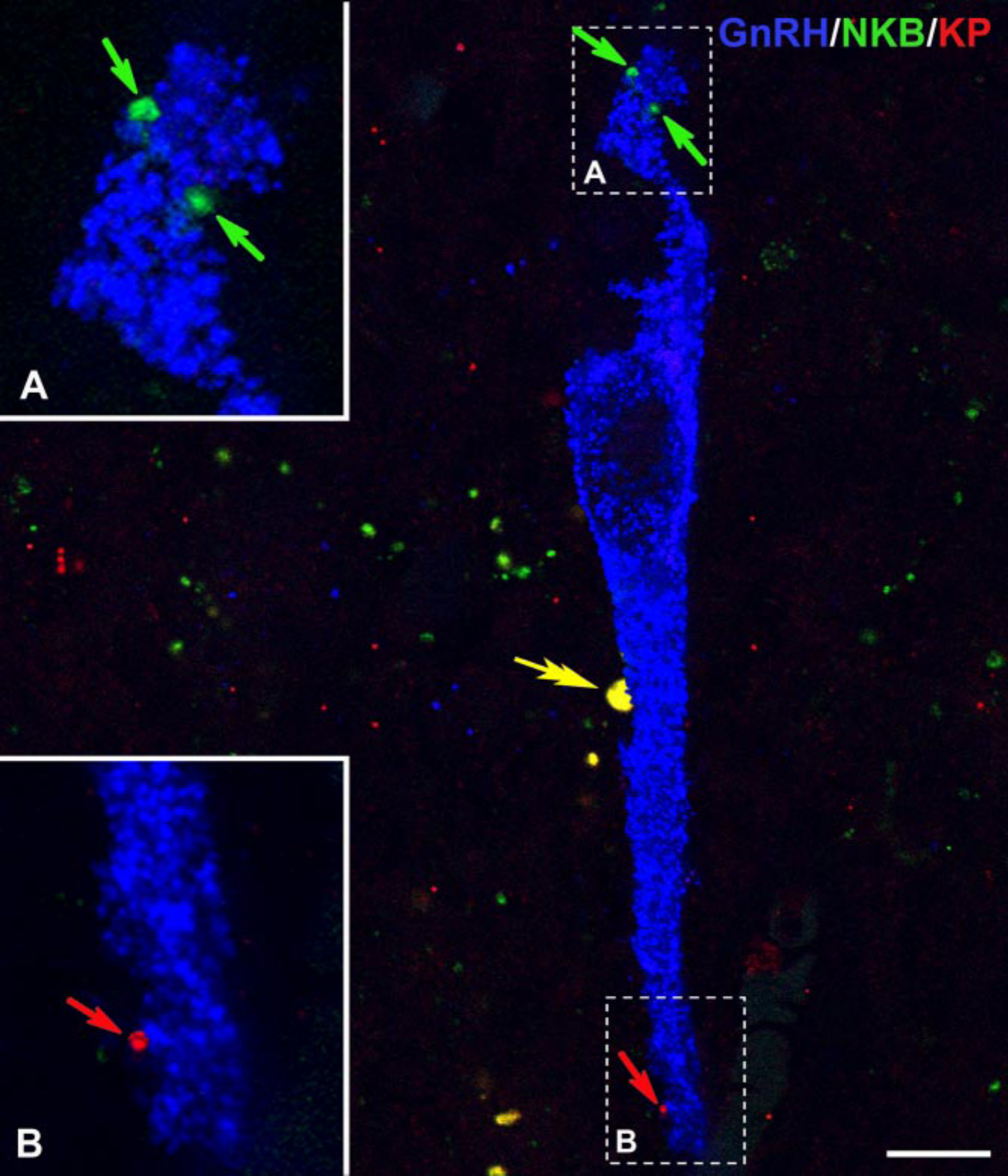

young
aged
NKB/KP
A
B
A
B


Dual-labeled neurons
(Mean percentage±SEM)
90
80
70
60
50
40
30
20
10
0
young
aged
*
KP-IR perikarya
with NKB
NKB-IR perikarya
with KP


A
B
young
KP
aged
C
D
E
F
young
NKB
aged
G
H


Appositions per GnRH cell body (Mean±SEM)
35
30
25
20
15
10
5
0
young
aged
KP
NKB
A
Appositions per 100 µm GnRH dendrite (Mean±SEM)
50
40
30
20
10
0
young
aged
KP
NKB
B


Appositions per GnRH cell body (Mean±SEM)
35
30
25
20
15
10
5
0
young
aged
KP
NKB
A
Appositions per 100 µm GnRH dendrite (Mean±SEM)
50
40
30
20
10
0
young
aged
KP
NKB
B


GnRH/NKB/KP
A
A
B
B