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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

ERIK HRABOVSZKY[1], MÁTÉ T. SIPOS[1], CSILLA S. MOLNÁR[1], PHILIPPE CIOFI[2], BEÁTA Á. BORSAY[3], PÉTER GERGELY[3], LÁSZLÓ HERCZEG[3], STEPHEN R. BLOOM[4], MOHAMMAD A. GHATEI[4], WALJIT S. DHILLO[4], ZSOLT LIPOSITS[1,5]

Abbreviated title: KNDy neuron concept challenged

[1]Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, 1083 Hungary
[2]INSERM U862, Neurocentre Magendie, Bordeaux, France
[3]Department of Forensic Medicine, Faculty of Medicine of the University of Debrecen, Debrecen, 4012 Hungary
[4]Department of Investigative Medicine, Hammersmith Hospital, Imperial College London, London, United Kingdom
[5]Department of Neuroscience, Faculty of Information Technology, Pázmány Péter Catholic University, Budapest, 1083 Hungary

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Corresponding authors:
Erik Hrabovszky, MD, PhD, DSci
Department of Endocrine Neurobiology
Institute of Experimental Medicine
Hungarian Academy of Sciences
43 Szigony St.
Budapest, 1083 Hungary
Phone: 36-1-2109400, ext.: 255
Fax: 36-1-2109943
E-mail: hrabovszky.erik@koki.hu

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Abstract

Previous immunohistochemical and *in situ* hybridization studies of sheep, goats and rodents indicated that kisspeptin (KP), neurokinin B (NKB) and dynorphin A (DYN) are extensively colocalized in the hypothalamic arcuate nucleus (ARC), thus providing a basis for the 'KNDy neuron' concept; in both sexes, KNDy neuropeptides have been implicated in the generation of gonadotropin releasing hormone (GnRH) neurosecretory pulses and in the negative feedback effects of sexual steroids to the reproductive axis.

To test the validity and limitations of the KNDy neuron concept in the human, we carried out the comparative immunohistochemical analysis of the three neuropeptides in the infundibular nucleus (Inf=ARC) and stalk (InfS) of young male human individuals (<37 years).

Results of 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, were about 5 times as high as 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 33% 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.

Together, these results indicate that most NKB neurons in the Inf do not synthesize detectable amounts of KP and DYN in young male human individuals. These data call for a critical use of the 'KNDy neuron' terminology while referring to the putative pulse generator system of the mediobasal hypothalamus. We conclude that the functional importance of these three neuropeptides in reproductive regulation considerably varies among species, between sexes and at different ages.

Introduction

Information accumulated from immunohistochemical and *in situ* hybridization studies has recently formed the basis for the kisspeptin/neurokinin B/dynorphin A ('KNDy') neuron concept and terminology (1-4). As shown first for the sheep (5), many of these neurons with cell bodies located in the hypothalamic arcuate nucleus (ARC; called infundibular nucleus in humans; Inf) co-synthesize kisspeptin (KP), neurokinin B (NKB) and dynorphin A (DYN). They have been implicated in negative sex steroid feedback to gonadotropin-releasing hormone (GnRH) neurons (5-7) and proposed to also serve as pacemakers for the GnRH neurosecretory pulses (3, 4, 8, 9). Recent models of the GnRH pulse generator (3, 4, 8) suggest that KNDy neurons communicate with one another via NKB and its receptor, NK3, and possibly, also DYN and its receptor, KOR. In ovariectomized goats, central NKB increases and DYN decreases the frequencies of multiunit activity volleys and LH secretory pulses (8). Pulse generator cells, in turn, appear to communicate with GnRH neurons primarily via KP/KISS1R signaling. GnRH neurons express KISS1R (10-12) and the majority of GnRH neurosecretory pulses show temporal association with KP pulses in the median eminence of monkeys (13).

The general consensus that KP, NKB, DYN, NK3 and KOR are expressed by the same neurons relies on combined neuroanatomical data from sheep (1, 5), rats (14), mice (3, 4, 15), goats (8), monkeys (16) and humans (17, 18). However, closer analysis of these reports, in retrospect, reveals that neuropeptide and receptor colocalizations are often only partial and also variable in the different studies, species, sexes and age groups. Notably, in our recent immunohistochemical study of aged human individuals, we have detected robust sex differences in the abundance of KP-IR (17, 18) and NKB-IR (18) neuronal elements in the Inf. These studies have also revealed that the incidences of NKB-IR cell bodies, fibers and appositions onto GnRH neurons exceed several-fold those of KP-IR elements, with particularly robust differences in males (18). These results suggested that NKB-IR neurons and their fibers are partly distinct from the KP-IR elements in these human models, thus challenging the universal validity of the

KNDy neuron concept. Moreover, in triple-immunofluorescent studies of aged human males, GnRH neurons tended to receive primarily single-labeled afferent inputs from these peptidergic systems, with KP/NKB double-labeled axons representing only 10.2% of all KP-IR afferents and NKB/KP double-labeled axons about 8.8% of all NKB-IR afferents (18). From the above findings and preliminary observations indicating that KP immunolabeling is even weaker in young than in aged men, we predicted that the degree of overlap between the KNDy neuropeptides is much lower in young male humans than suggested earlier for female sheep (1, 5), goats (8) or mice (3).

In the present study we investigated the universal validity of the KNDy neuron concept via the parallel immunohistochemical analysis of NKB-, KP- and DYN immunoreactivities in the Inf and the infundibular stalk (InfS) of young men. Specifically, i) we compared the immunoreactive perikaryon and fiber densities in the Inf and the InfS, ii) we addressed the colocalization of KP with NKB and KP with DYN in perikarya and fibers, and finally, iii) we compared quantitatively the incidences of NKB-IR *vs.* KP-IR afferent contacts onto GnRH-IR neurons.

Materials and methods

Human subjects

Human hypothalamic 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 36h. Tissue specimens from six young male individuals (age 21-37 years) were used.

Section preparation

Following dissection, the hypothalamic tissue blocks were rinsed with running tap water and then, immersion-fixed with 4% formaldehyde in 0.1M phosphate buffer saline (PBS; pH 7.4) for 7-14 days. After fixation, the blocks were trimmed in a way to include the optic chiasma rostrally, the mammillary

bodies caudally and the anterior commissure dorsally (17-19). Bilateral sagittal cuts were placed 2cm lateral from the midline. The blocks were bisected into right and left halves and then, infiltrated with 20% sucrose for 5 days at 4°C. The right hemihypothalami were sectioned coronally at 30μm with a Leica SM 2000R freezing microtome (Leica Microsystems). All experiments were performed on every 24th hemihypothalamic section from each subject.

Pretreatments

The tissues were permeabilized and endogenous peroxidase activity reduced using a mixture of 0.2% Triton X-100 and 0.5% H_2O_2 in PBS for 30 min. Antigen epitopes were unmasked by incubating sections in 0.1M citrate buffer (pH 6.0) at 80 °C for 30 min (18). Dual-immunofluorescent experiments also used a Sudan black pretreatment against autofluorescence (17, 18).

Immunohistochemical detection of KP

To detect KP immunoreactivity, sections were incubated in a sheep polyclonal antiserum against human kisspeptin-54 (GQ2; 1:200,000). This antiserum recognizes human KP-54, KP-14 and KP-10, exhibits less than 0.01% cross-reactivity *in vitro* with other related human RF amide peptides (20), and was used successfully in previous immunohistochemical experiments on primate hypothalami (16-18, 21). Incubation in the primary antibodies for 48 h at 4C was followed by biotinylated secondary antibodies (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 reaction was visualized with nickel-intensified diaminobenzidine chromogen (19) and then, post-intensified with silver-gold (22).

Immunohistochemical detection of NKB and DYN

Another two parallel series of sections were used to visualize NKB- and DYN, respectively. NKB neurons were detected with rabbit polyclonal antibodies against the C-terminal 28 amino acids of human NKB (IS-682, P. Ciofi; 1:100,000) (17), whereas DYN neurons were labeled with rabbit polyclonal antibodies against amino acids 1-17 of porcine (and human) DYN (T-4268; Peninsula Laboratories; San Carlos, CA; 1:100,000). The primary antibodies were reacted with biotinylated antirabbit IgG (Jackson

ImmunoResearch Laboratories; 1:500; 1h) and then, ABC Elite reagent (1:1000; 1h). The peroxidase signal was developed with silver-gold-intensified nickel-diaminobenzidine. For control purposes, mapping studies of DYN were replicated using a previously characterized second antiserum (IS-35; 1:200,000) against a different prodynorphin-derived peptide, dynorphin B (23).

Dual-immunoperoxidase detection of KP and GnRH or NKB and GnRH

Two section series were processed for the immunohistochemical detection of KP and NKB, respectively, as above. Then, they were processed to detect GnRH using a guinea pig primary antiserum (#1018; 1:5000) (18) which was reacted with biotinylated antiguinea pig IgG (Jackson ImmunoResearch Laboratories; 1:500; 1h) and ABC Elite solution (Vector; 1:1000; 1h). The peroxidase signal was visualized with brown diaminobenzidine.

Fluorescent immunohistochemistry

Other series of sections were processed for the dual-immunofluorescent studies of the colocalization between NKB and KP or DYN and KP. Incubation in a cocktail of primary antibodies (rabbit anti-NKB, 1:1000 and sheep anti-KP, 1:1000; or rabbit anti-DYN, 1:1000 and sheep anti-KP, 1:1000; 48h; 4ºC) was followed by a cocktail of fluorochrom-conjugated secondary antibodies (Jackson ImmunoResearch; anti-rabbit-FITC, 1:250; anti-sheep-Cy3, 1:1000) for 5h at room temperature.

To maximize sensitivity, other dual-immunofluorescent studies used tyramide signal amplification. In these experiments KP was detected first using sequential incubations in KP antibodies (1:30,000; 48h; 4ºC), biotinylated antigoat IgG (Jackson ImmunoResearch Laboratories; 1:500; 1h), the ABC Elite reagent (Vector; 1:1000; 1h), biotin tyramide working solution (1:1000, in 0.05M Tris-HCl buffer, pH 7.6, containing 0.003% H_2O_2; 30 min) (24) and finally, avidin-Cy-3 (Jackson ImmunoResearch; 1:1000; 1h). 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 at 1:50,000 (48h; 4ºC) and reacted with antirabbit-peroxidase (Jackson ImmunoResearch; 1:500; 1h). Then, FITC-tyramide (24) (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 and DYN primary antibodies. Lack of FITC labeling in these control sections indicated that no FITC-tyramide deposition is caused by residual peroxidase activity on KP-IR sites.

Section mounting and coverslipping

Following section mounting, immuno-peroxidase labeled sections were coverslipped with DPX (Fluka Chemie; Buchs, Switzerland) and immunofluorescent specimens with Mowiol.

Digital photography

The light and fluorescent microscopic images were scanned with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope using the AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany). The light and fluorescent microscopic images were scanned 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 an inverted Nikon Eclipse Ti-E microscope equipped with an A1R confocal system (Nikon, Austria). The digital images were processed with the Adobe Photoshop CS software (Adobe Systems, San José, CA, USA) at a 300 dpi resolution. Quantitative data were expressed as mean±SEM and statistical comparisons used one-way ANOVA.

Experiment 1. Studies of the incidences and overlaps of IR cell bodies in the Inf

The number of immunoreactive cell bodies was counted at 100X magnification within a 0.25 mm^2 counting area with the aid of a 5X5 ocular grid, as described previously (17, 18). Each subject was characterized by the maximal number of immunoreactive perikarya in this counting area (determined from 2-6 sections.)

The overlaps between NKB- and KP immunoreactivities and between DYN- and KP immunoreactivities were first assessed in confocal images of dual-immunofluorescent specimens. The percentages of single-labeled and double-labeled NKB-IR and KP-IR perikarya were also determined

quantitatively from the specimens in which the tyramide signal amplification was used, using 1-3 representative confocal images per subject.

Experiment 2. Studies of the regional abundance of IR fibers in the Inf

The regional density of immunoreactive fibers was determined as described recently (18). First, digital images were taken from the bulk of kisspeptin-IR and NKB-IR neurons in the Inf. The files were opened with the Adobe Photoshop CS software. The immunolabeled cell bodies were erased ("eraser tool") from the photomicrographs. The remaining images were compiled into TIF files and opened with the Image J software (public domain at http://rsbweb.nih.gov/ij/download.html). The regional fiber density in each photograph was defined as the area occupied by immunoreactive fibers/total area. For each subject, the mean fiber density was derived from 1-3 digital images. The overlap between NKB-IR and KP-IR axons or DYN-IR and KP-IR axons was also studied qualitatively in confocal images of dual-immunofluorescent specimens.

Experiment 3. Studies of immunoreactive fibers in the InfS

Projections of NKB-, KP-, DYN- and GnRH-IR axons around the portal blood vessels of the InfS were analyzed in this experiment. Based on previous immunohistochemical results in the median eminence of different species (16, 23), we assumed that fibers containing KNDy peptides around the portal vasculature arise mostly from the ARC/Inf. First, sections labeled with peroxidase-based immunohistochemistry were used to study the relationship of fibers with the superficial and deep capillary plexuses of the human postinfundibular eminence (25). Then, the extents of overlap between NKB and KP immunoreactivities and between DYN and KP immunoreactivities were assessed from dual-immunofluorescent specimens.

Experiment 4. Studies of the incidences of KP-IR and NKB-IR appositions onto GnRH-IR neurons of the Inf

Dual-immunoperoxidase labeled sections were selected (1-2 from each individual) to determine the number of axonal contacts along the outlines of GnRH-IR cell bodies and dendrites. Counting of the

appositions was carried out using a 63X oil-immersion objective and contacts defined using stringent criteria (18, 26, 27). For each subject, the mean number of contacts per GnRH soma and 100µm GnRH dendrite were calculated (18).

Results

The comparative analysis of NKB, KP and DYN immunoreactivities in immunoperoxidase-labeled sections of the Inf (Fig. 1) and the InfS (Fig. 2) revealed strikingly different labeling intensities for KNDy neuropeptides. In general, NKB-IR elements showed much higher abundance than KP-IR elements. DYN immunoreactivity, both in perikarya and fibers, was relatively sparse and weak.

Experiment 1. Incidence of IR perikarya in the Inf

In peroxidase-based immunohistochemistry, many NKB-IR perikarya were identified in the Inf (Figs. 1A, B). KP-IR cell bodies occurred in much lower numbers in neighboring sections (Figs. 1C, D). Quantitative analysis showed that the density of KP neurons was about 5 times lower than that of NKB-IR perikarya (Graph 1; P=0.01 by ANOVA). DYN-IR perikarya were either entirely absent in some subjects (Figs. 1E, F) or extremely rare in the Inf of others, preventing quantitative studies. In contrast, the supraoptic nucleus contained many labeled perikarya (Fig. 1G), 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 (Figs. 3A, B). Tyramide signal amplification was crucial for sensitive detection of NKB/KP dual-labeled cell bodies which represented only 32.9±4.7% of the

NKB-IR and 75.2±6.6% of the KP-IR perikarya (Figs. 3A, B). Tyramide signal amplification was capable of visualizing only a few DYN-IR perikarya (not shown).

Experiment 2. Abundance of IR fibers in the Inf

The incidence of immunolabeled fibers in the Inf followed a similar trend as labeled perikarya. The most frequently encountered phenotype was, again, IR for NKB. These axons established many appositions (Fig. 1B) 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 as high as that of KP-IR fibers (Graph 2; P=0.0001 by ANOVA). DYN-IR fibers were also detectable in the Inf, although less frequently than either NKB-IR or KP-IR axons (Figs. 1E, F).

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 (Figs. 3A, B). DYN-IR fibers showed a high intensity of labeling only if the tyramide signal amplification approach was also used. Most of them were distinct from KP-IR axons, although dual-labeled KP/DYN-IR fibers occasionally occurred (Figs. 3F, G). Similarly, the majority of KP-IR fibers were also devoid of dynorphin B immunoreactivity in the Inf and the InfS (Figs. 3I, J), whereas this second antiserum also performed well in regions rich in DYN fibers, including the ventromedial nucleus (Fig. 3K).

Experiment 3. Abundance of immunoreactive fibers in the InfS

The InfS was associated with the superficial and the deep capillary plexuses of the postinfundibular eminence (25). Both were abundantly innervated by GnRH-IR axons (brown color in Figs. 2A-F), suggesting they contribute to the GnRH supply of

adenohypophysial gonadotropes. The relative abundance of the different types of labeled fibers around the two capillary plexuses matched what was seen in the Inf. Accordingly, portal blood vessels were surrounded by dense networks of NKB-IR fibers (Figs. 2A-C) and innervated only moderately by KP-IR fibers (Figs. 2D-F). Very few DYN-IR fibers occurred in the proximity of the portal capillaries (Figs. 2G, I, J). This low level of DYN signal did not reflect a technical limitation, considering that the magnocellular neurosecretory tract was immunolabeled heavily in the same sections (Fig. 2H).

The analysis of immunofluorescent specimens confirmed that NKB dominates over KP around the portal vasculature and NKB-IR fibers often lack KP labeling (Figs. 3C-E). Similarly to the Inf, the InfS contained both single-labeled and double-labeled KP-IR fibers (Figs. 3C-E). In sections dual-labeled for KP and DYN, labeled fibers were mostly distinct, although rare colocalization cases were also detectable (Fig. 3H).

Experiment 4. Frequency of kisspeptin-IR and NKB-IR appositions onto GnRH-IR neurons

Sections double-labeled with the silver-gold-intensified nickel-diaminobenzidine and diaminobenzidine chromogens were used to obtain quantitative estimates about NKB-IR and KP-IR inputs to GnRH-IR neurons. Microscopic analysis confirmed that NKB-IR (18) and KP-IR (17, 18) axons provide axo-somatic and axo-dendritic inputs to GnRH neurons in the Inf (Fig. 4). Quantitative analysis (Graph 3) established that GnRH-IR perikarya and dendrites, respectively, received 6 and 5 times heavier NKB-IR (Fig. 4A) than KP-IR (Fig. 4B) innervation (GnRH perikarya: $P=0.004$; GnRH dendrites: $P=0.005$, by ANOVA).

Discussion

Immunohistochemical results of this study provide evidence for limitations of the ‘KNDy neuron’ terminology and concept. Specifically, our observations indicate that in young male humans

the majority of NKB-IR neurons in the Inf, their processes and contacts onto GnRH neurons do not contain detectable amounts of KP immunoreactivity. Furthermore, KP-IR neuronal elements without NKB labeling also occur frequently, in addition to NKB/KP dual-phenotype structures. Finally, we observed that most KP-IR neurons and fibers are devoid of DYN immunoreactivity in this human model.

Species differences in the colocalization of KNDy peptides

We propose that the different colocalization patterns in the present human study and in previous animal experiments partly reflect species differences in reproductive mechanisms (28). These may include the absence of DYN signal from most KP neurons and fibers in humans, which is in contrast with the extensive coexpression in the rodent (3, 4, 14), sheep (5) and goat (8) ARC. Another putative species difference is the large excess of NKB-IR over KP-IR perikarya in the present human study, as opposed to the two-fold excess in the ARC of male mice (4).

Sex-dependent variations in the absolute and relative abundances of KP and NKB and their colocalization pattern

In a previous study of KP-IR and NKB-IR neurons we identified a series of sex-dependent morphological differences between aged human males and females (18). In particular, KP immunoreactivity was highly sexually dimorphic; the number of KP-IR perikarya, the density of KP-IR fibers and the incidence of KP-IR afferents on GnRH neurons were much higher in aged women compared with men (18). It is worth of note that the NKB/KP neuron density ratio also differed, being 2.18 in aged males (>50 years) and 1.55 in postmenopausal females (>55 years) (18). In further support of the idea that the KP/NKB colocalization pattern is sexually dimorphic, the ratio of dual-labeled NKB-IR as well as KP-IR afferent contacts onto GnRH-IR neurons was significantly higher in postmenopausal women (≈25-30%) than in aged men (≈8-10%) (18). We proposed that sex differences are either due to

organizational effects of sex steroids during critical period(s) of sexual differentiation or alternatively, to the loss of negative sex steroid feedback in postmenopausal women, unlike in aged men who maintain relative high testosterone levels. In our previous dual-immunofluorescent study we demonstrated a high degree of overlap between the KP and NKB systems of postmenopausal women; above 80% of KP-IR perikarya and NKB-IR perikarya contained also the other neuropeptide (17). The degree of this colocalization is likely to be sexually dimorphic and much lower in aged males whose Inf contained over twice as many NKB-IR as KP-IR perikarya (18).

It is worthy of note that the sexual dimorphism of KP and NKB neurons in the ARC/Inf region is not unique to humans. The ARC of the sheep contains higher NKB (29) and KP (1) cell numbers in females than in males. In rats, sex differences were reported in the projection fields of NKB-IR axons within the infundibular area (23).

Aging-dependent variations in the absolute and relative abundances of KP and NKB

The human NKB and KP systems, at least in the male, also exhibit robust aging-dependent changes. Our preliminary data indicate that the enhancements of the absolute NKB and KP cell numbers coincide with a significantly enhanced percentage of KP-expressing NKB neurons in aged men (Molnár et al., in preparation).

Role of NKB in the regulation of the human reproductive axis

The tachykinin peptide NKB plays a crucial role in human reproduction and inactivating mutations of the NKB and NK3 encoding genes cause normosmic hypogonadotropic hypogonadism (30, 31). Although the first reports did not indicate fertility deficits in the NK3 mutant mice (32), later analysis focusing on the reproductive phenotype noticed subfertility (33), suggesting functional similarities in NKB/NK3 signaling between the human and mouse species.

Out of the three KNDy peptides, NKB provided the heaviest immunohistochemical signal in the Inf and the InfS of young men (present report), aged men (18) and aged women (18). In immunoperoxidase-based studies, the dominance of NKB over KP was highest in young men (present study) where the density of NKB-IR perikarya and fibers were about 5 times as high as those of the KP-IR elements and NKB-IR axons established about 6-times as many axo-somatic and 5-times as many axo-dendritic contacts onto GnRH neurons as did KP-IR axons.

Conflicting results of previous experiments suggest that the net effect of NKB on LH secretion depends on animal species and endocrine paradigms. Intraperitoneal or intracerebroventricular NKB administration to male mice had no effect on serum LH (34), whereas the intracerebroventricular injection of the selective NK3 agonist senktide reduced LH secretion in ovariectomized rats treated with a low dose of estradiol (35). Reduced LH secretion in response to senktide was also observed in ovariectomized and in ovariectomized and estradiol treated rats (36) and in ovariectomized mice (3), whereas another study on rats found stimulatory effect on LH secretion in the presence of physiological levels of estradiol (37). Senktide also stimulated LH secretion in castrated male monkeys (16), and in the follicular, but not in the luteal, phase in ewes (38), whereas reduced net LH secretion was observed in ovariectomized goats (8).

Multiunit activity recorded in the ARC is considered to be an electrophysiological correlate of the GnRH pulse generator activity. The coordinated bursts of neuronal firing occur in synchrony with the LH secretory pulses in various animal species (8, 39), including primates (40). Senktide dose-dependently suppressed the frequency of pulsatile LH secretion and inhibited hypothalamic multiunit activity volleys in ovariectomized rats, independently of gonadal steroid levels (36). In contrast, a robust increase in the frequency of multiunit activity volleys was observed in ovariectomized goats (8).

NKB mainly acts upstream from GnRH neurons. For example, the stimulatory effect of intravenous NKB and senktide on LH secretion could be abolished by the GnRH receptor antagonist acyline (16).

One major target site for NKB actions appears to be on NKB (KNDy) neurons of the ARC/Inf. Accordingly, i) we found numerous NKB-IR afferent contacts on human NKB neurons, ii) similar contacts were reported previously in rats (14) and sheep (5, 41), iii) NK3 receptors are present on these cells in rodents and sheep (3, 14, 42) and iv) KP (KNDy) neurons in the ARC of male mice respond with c-Fos expression (4) and depolarization (4) to senktide.

There is little consensus regarding the possibility that NKB also influences GnRH neurons directly. In sheep, GnRH neurons do not express NK3 immunoreactivity (42). In mice, while single-cell microarray and RT-PCR studies provided proof for NK3 mRNA expression in GnRH neurons (43), *in situ* hybridization studies were unable to confirm this finding (4). Also, senktide did not activate mouse KNDy neurons *in vitro* (4). In rats, while immunohistochemical studies found evidence for NK3 immunoreactivity in only 16% of GnRH-IR cell bodies (44), the receptor was more abundant on GnRH-IR axon terminals in the median eminence (44) where frequent appositions between GnRH-IR and NKB-IR axons occurred (23, 44). Although NKB in itself did not alter GnRH release from hypothalamic explants of male rats, it abrogated the KP-induced release of GnRH, suggesting a complex mode of action which is likely parallel with, and not upstream from, the KP action (45). Recent functional evidence from KISS1R-KO mice indicates that intact KP/KISS1R signaling is required for the suppression of LH secretion by senktide. This finding provides support for the concept that the dominant action of NKB is upstream from KP neurons, instead of being exerted directly on GnRH cells (45).

Results of the present and a previous (18) human study indicate that NKB-IR axons abundantly innervate human GnRH neurons and the incidence of these contacts is several times as high as those of KP-IR axons. It will require clarification whether this anatomical pathway uses NKB/NK3 signaling. Alternatively, neurotransmitter (s) other than NKB may act in this communication, which is not likely to be KP or DYN, in view of their relative paucity in young male individuals. In addition to innervating GnRH-IR cell bodies and dendrites, NKB-IR axons also represented the most abundant KNDy peptide

around the portal capillary plexuses of the human postinfundibular eminence (25). This hypothalamic site that lies outside the blood-brain barrier may represent an important site of interaction between NKB-IR and GnRH-IR axons. It also remains possible that NKB is released into the hypophysial portal circulation to influence adenohypophysial functions. *In vitro* evidence from rats, indeed, indicates that NKB can induce prolactin secretion from perifused pituitary cells (46).

Role of KP in the regulation of the human reproductive axis

In humans, KP/KISSR1 signaling plays a pivotal role in reproduction. Loss of function mutations of the genes encoding KISSR1 (47-49) and KP (50) result in hypogonadotropic hypogonadism. In recent models of the GnRH pulse generator, KP was proposed to provide the main output signal of the pulse generator neuronal network toward GnRH neurons, whereas NKB and DYN seem to primarily account for the intranuclear communication of KNDy neurons via acting on NK3 and KOR, respectively (3, 8). It is generally believed that independently from the species, KP acts directly on GnRH neurons that express KISS1R mRNA (10-12) and in mice, respond with depolarization to KP (11, 51, 52).

As in laboratory and domestic animals, KP increases LH secretion in men (20, 53, 54) and women (55, 56), most potently during the preovulatory phase of the menstrual cycle in the latter. It is interesting to note that the continuous intravenous infusion of KP enhanced the LH pulse frequency in men (53), indicating that KP not only acts on GnRH neurons, but also upstream from the pulse generator network. This finding suggests a species difference from the mouse in which KNDy neurons do not appear to synthesize KISS1R (57) and express only NK3 (3) and KOR (3) mRNAs.

In humans, axo-somatic, axo-dendritic and axo-axonal contacts may serve as communication pathways between KP and GnRH neurons (17). In previous studies we showed that the KP system of aged human individuals exhibits a robust sexual dimorphism (18) with postmenopausal women having several times higher densities of KP-IR cell bodies and fibers in the Inf and higher incidences of KP-IR

afferent contacts onto GnRH neurons, than aged men above 50 years. Preliminary data that KP immunoreactivity is even much lower in the hypothalamus of young men (Molnár et al., in preparation) led us to carry out the present study to challenge the validity of the KNDy neuron terminology and concept for the young male human model. Our present immunohistochemical data indicate that the density of KP-IR perikarya and fibers in the Inf were about 5 times lower than those of the corresponding NKB-IR elements and the number of KP-IR appositions to GnRH-IR cell bodies and dendrites only reached about one-fifth and one-sixth, respectively, of those established by NKB-IR axons. The functional consequences of the surprisingly low level of KP immunoreactivity in young men (in somata as well as fibers) requires clarification. In view that KP is thought to represent the main neurotransmitter output of the pulse generator system (3, 8), its low level in the mediobasal hypothalamus of young men is compatible with only a moderate stimulation of GnRH/LH secretion. Much higher raw numbers of the KP-IR cell bodies, fibers and contacts onto GnRH neurons in aged male human subjects (18) and their highest incidence in postmenopausal women (18) are in accordance with the idea that KP immunoreactivity and serum LH levels are linked. A future challenge will be to correlate the immunohistochemical images of KP and the other KNDy peptides with the GnRH neurosecretory output at the different human age and sex groups.

Absence of DYN immunoreactivity from most KP neurons and their fiber projections

The opioid peptide DYN is rather ubiquitous and may have multiple sites of action upon the reproductive axis. These sites are likely upstream from the GnRH neuron that does not appear to express KOR in rats (58). DYN is critically involved in progesterone negative feedback to GnRH neurons in ewes; the majority of DYN cells in the ARC of ovariectomized ewes contain progesterone receptor (59) and progesterone treatment increases preprodynorphin mRNA expression in the ARC and DYN levels in the cerebrospinal fluid (60).

DYN is an important regulator of the pulse generator system. In sheep, KOR antagonists stimulate the episodic secretion of LH during the luteal phase (61). In ovariectomized goats, central administration of DYN decreases and KOR antagonist increases the frequencies of the multiunit activity volleys and of the LH secretory pulses (8). Opioid peptides also regulate negatively the pulsatile release of prolactin and LH in humans; this inhibitory tone can be suspended by the blockade of opioid receptors with naloxone (62, 63).

The concept and terminology of the 'KNDy neurons' rely on the similar results of colocalization experiments from several animal species. DYN has been detected in NKB (and/or KP) neurons in the ARC of sheep (5, 41), mice (3, 4), rats (14, 23) and goats (8). Moreover, the DYN receptor KOR is present in subsets of KNDy neurons in the ARC of mice (3, 4). Our present immunohistochemical study to address the presence of DYN immunoreactivity in human KP neurons was also informed by previous reports in which preprodynorphin mRNA expression was detected in the human Inf (64) and the monkey ARC (65).

The somewhat unexpected absence of DYN immunoreactivity in most KP-IR somata and fibers of young male humans questions the universal importance of DYN peptides within NKB and KP neurons of the Inf and reveals an important difference from the rodent, sheep and goat species (1-5, 8, 23). It is worthy to note that species also vary considerably regarding the sex steroid regulation of DYN in the ARC/Inf. Preprodynorphin expressing neurons showed reduced numbers in postmenopausal women (64) and in ovariectomized ewes (60), whereas there was no change in mRNA expression in postmenopausal monkeys (65), whereas preprodynorphin mRNA was increased in the absence of sex steroids in mice (3).

The absence of DYN immunoreactivity from most KP-IR neurons and their fibers we report in this study is unlikely to be entirely caused by the limited sensitivity of the applied immunohistochemical method because i) DYN-IR cell bodies (e.g. magnocellular perikarya in the supraoptic nucleus) and fibers (e.g. a dense fiber plexus in the ventromedial nucleus) were readily detectable elsewhere in the

hypothalamus, ii) substantial colocalization with KP was also undetectable using the highly sensitive tyramide signal amplification method to visualize DYN or iii) using an antiserum against a different prodynorphin cleavage product, dynorphin B.

Summary of neuroanatomical findings

i) The regional density of NKB-IR cell bodies, fibers and contacts onto GnRH neurons exceed about five-fold those of KP-IR neuronal elements in the Inf.

ii) In addition to NKB-IR cell bodies and processes (in both the Inf and the InfS) that are devoid of KP labeling, KP-IR elements lacking NKB immunoreactivity are also highly abundant, as established in dual-immunofluorescent studies. In this study only 32.9±4.7% of the NKB-IR and 75.2±6.6% of the KP-IR perikarya were dual-labeled.

iii) DYN-IR cell bodies and fibers occur much less frequently than either NKB-IR or KP-IR elements; KP-IR axons in the Inf and the InfS contain DYN immunoreactivity only occasionally.

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

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468

Legends

Figure 1. Relative abundances of NKB-, KP- and DYN-IR cell bodies and fibers in the Inf of young male humans. The silver-gold intensified nickel-diaminobenzidine chromogen was used to visualize NKB (**A, B**), KP (**C, D**) and DYN (**E-G**) immunoreactivities in adjacent sections of the Inf from a 31-year-old male subject. NKB-IR perikarya as well as nerve fibers (**A, B**) occur in much higher numbers than do KP-IR elements (**C, D**). For results of the quantitative analyses which reveal about five-fold differences for both perikaryon and fiber densities, see Graphs 1 and 2, respectively. Arrowheads point to NKB-IR cell bodies in **B** and a KP-IR cell body in **D.** Note that NKB-IR neurons receive numerous afferent contacts (arrows in **B**) from NKB-IR varicose axons; analogous juxtapositions in other species were proposed to underlie the main peptidergic signaling mechanism among the putative pulse-generating KNDy neurons. Few if any DYN-IR cell bodies are detectable in the Inf (none visible in this specific case) and only scattered DYN-IR fibers occur (**E, F**). This low level of the DYN signal does not appear to reflect a technical limitation of the immunohistochemical approach, given that IR perikarya and fibers are abundant elsewhere in the hypothalamus, including the supraoptic nucleus (SO; **G**). Scale bar=100μm for **A, C, E** and 9μm elsewhere.

Figure 2. Results of immuno-peroxidase studies illustrating the differential innervation of the portal capillary plexus by NKB-, KP- and DYN-IR fibers in a 31 year old men. A-I: The black silver-gold intensified nickel-diaminobenzidine chromogen was used to detect NKB (**A-C**), KP (**D-F**) and DYN (**G-J**) immunoreactivities in adjacent sections of the InfS. Note that GnRH has also been visualized in **A-F** with brown diaminobenzidine. The postinfundibular eminence with its deep (**B, E, I**) and superficial (**C, F, J**) plexuses of portal blood vessels (BV) is surrounded by GnRH-IR hypophysiotropic axons (brown color in **A-F**). Out of the axons immunoreactive for the three KNDy peptides (black color), those with NKB immunoreactivity represent the most frequently encountered phenotype (**A**) and densely innervate both the deep (**B**) and the superficial (**C**) portal capillaries; this innervation raises the possibility of NKB release into the hypophysial portal circulation. The KP-IR innervation of the portal BVs is of much lower density (**D-F**). Although DYN-IR axons are readily detectable in the InfS (**G**) and contribute to the magnocellular axon tract (**H**), they only occur rarely around the portal BVs (**H-I**). Scale bar=100μm for **A, D, G** and 10μm elsewhere.

Figure 3. Results of immunofluorescent studies revealing a significant degree of mismatch between KP and NKB immunoreactivities (A-E) and KP and DYN (or dynorphin B) immunoreactivities (F-K) in the Inf (A, B, F, G) and the InfS (C-E, H). The dual-immunofluorescent visualization of NKB (green) and KP (red) immunoreactivities in the Inf (**A, B**) not only confirms the dominance of NKB-IR (green) over KP-IR (red) cell bodies (arrowheads) and axons (arrows) in the Inf, but also reveals a considerable degree of segregation between the two different perikaryon (arrowheads) and fiber (arrows) populations. Yellow double-arrows and arrowheads point to dual-labeled fibers and cell bodies, respectively. Single-labeled NKB-IR axons are also typical around the portal blood vessels (BV) of the postinfundibular eminence in the InfS (**C-E**), in addition to single-labeled KP-IR fibers (red) and NKB/KP-dual-labeled (yellow) axons. The dual-immunofluorescent visualization of KP and DYN (**F-H**) illustrates the absence of DYN immunoreactivity from KP-IR cell bodies in the Inf (red arrowheads in **F**). With the exceptions of a few scattered dual-labeled fibers (yellow double-arrows) in the Inf (**F, G**) and the InfS (**H**), the majority of KP-IR (red) and DYN-IR (green) axons are separate. Negative colocalization results are reproducible in the Inf (**I**) and the InfS (**J**) using an antiserum against a different prodynorphin cleavage product, dynorphin B. Note the high density of dynorphin B-IR fibers in the neighboring ventromedial nucleus (VMH) in **K**. High-power micrographs (**A, B, D-K**) represent single optical slices (0.7µm). Scale bar=100µm for **C**, 5µm for **D** and 10µm for **A**, **B**, **E-K**.

Figure 4. Different incidences of NKB-IR and KP-IR afferent contacts onto GnRH-IR neurons. NKB-IR axons (black color in **A**) show a much higher abundance in the Inf and establish considerably more axo-somatic and axo-dendritic juxtapositions (arrows) onto GnRH-IR neurons (brown color) than do KP-IR axons (black color in **B**). For quantification of these results, see Graph 3. Scale bar=20μm.

Graph 1. Regional abundance of NKB-IR and KP-IR neuronal perikarya in the Inf of young male humans. The maximal number of immunoreactive cell bodies per 0.25mm^2 counting frame (1-6 per subject) was determined with the aid of an ocular frame and used as the index of the density of labeled perikarya. Results show that in young male human individuals, the mean incidence of NKB-IR cell bodies is about 5-times as high as the incidence of KP-IR cell bodies. *P<0.05 by ANOVA.

Graph 2. Density of NKB-IR and KP-IR fibers in the Inf of young men. The area covered by immunoreactive fibers (divided by the total area analyzed) was determined with the ImageJ software in digital photographs of the Inf and used as a fiber density measure. Areas that were occupied by labeled cell bodies and their thick proximal dendrites were deleted from the photographs with the eraser tool of the Adobe Photoshop software and thus, excluded from the analysis. The density of NKB-IR fibers (expressed in arbitrary units) is 5.4 times as high as the density of KP-IR fibers. *P<0.0005 by ANOVA.

Graph 3. Incidences of NKB-IR and KP-IR contacts onto GnRH-IR neurons in the Inf in young men

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

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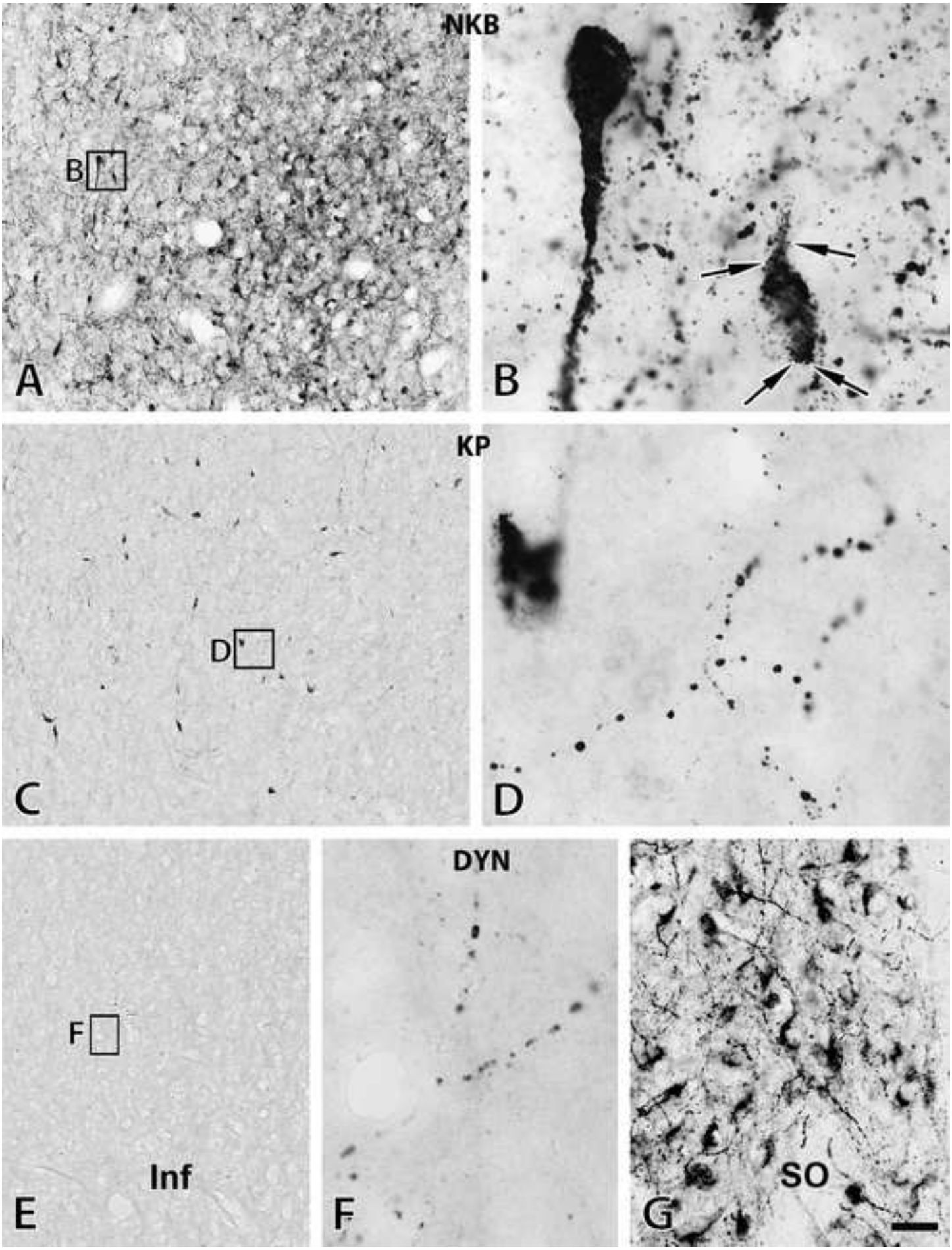

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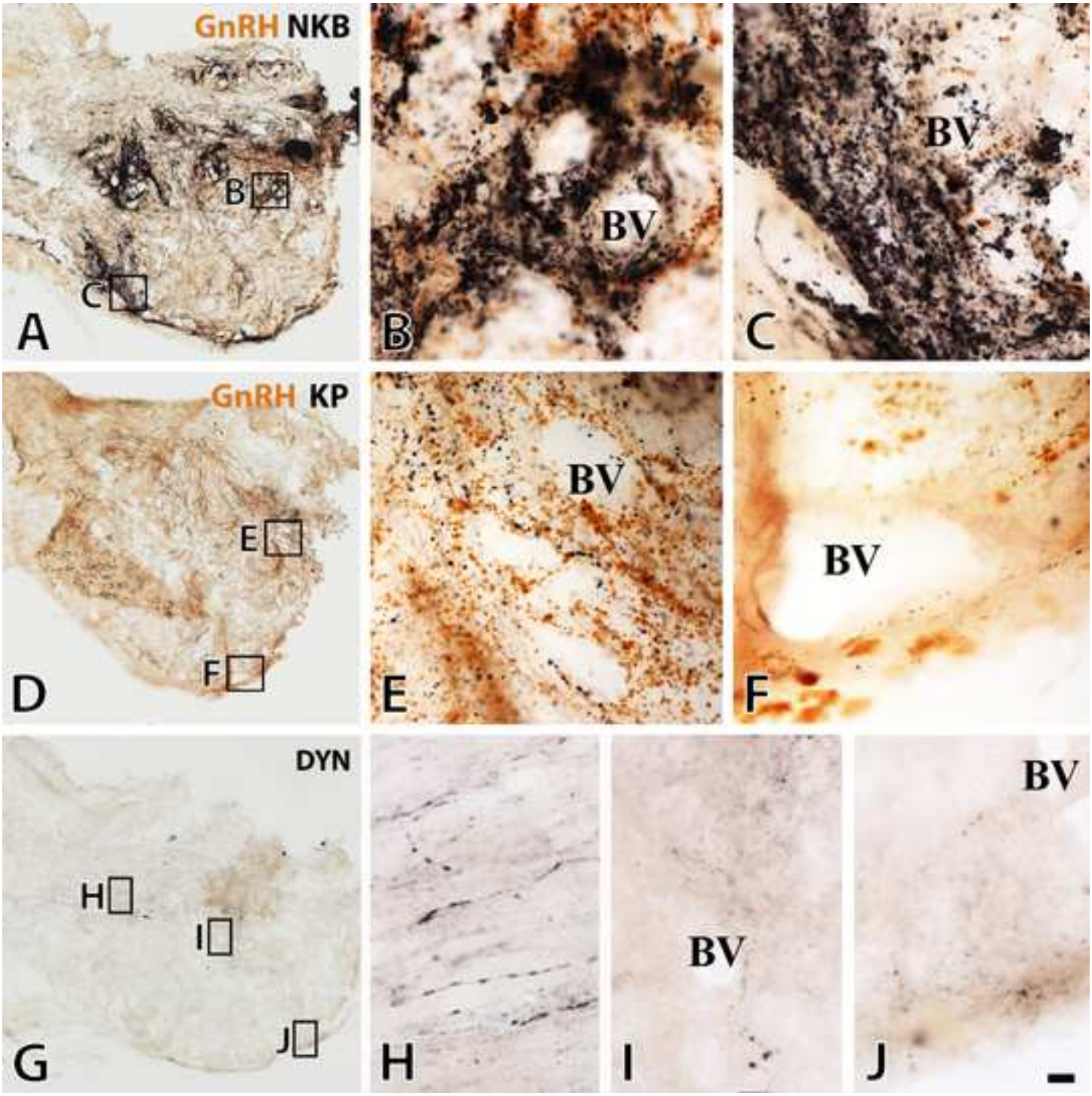

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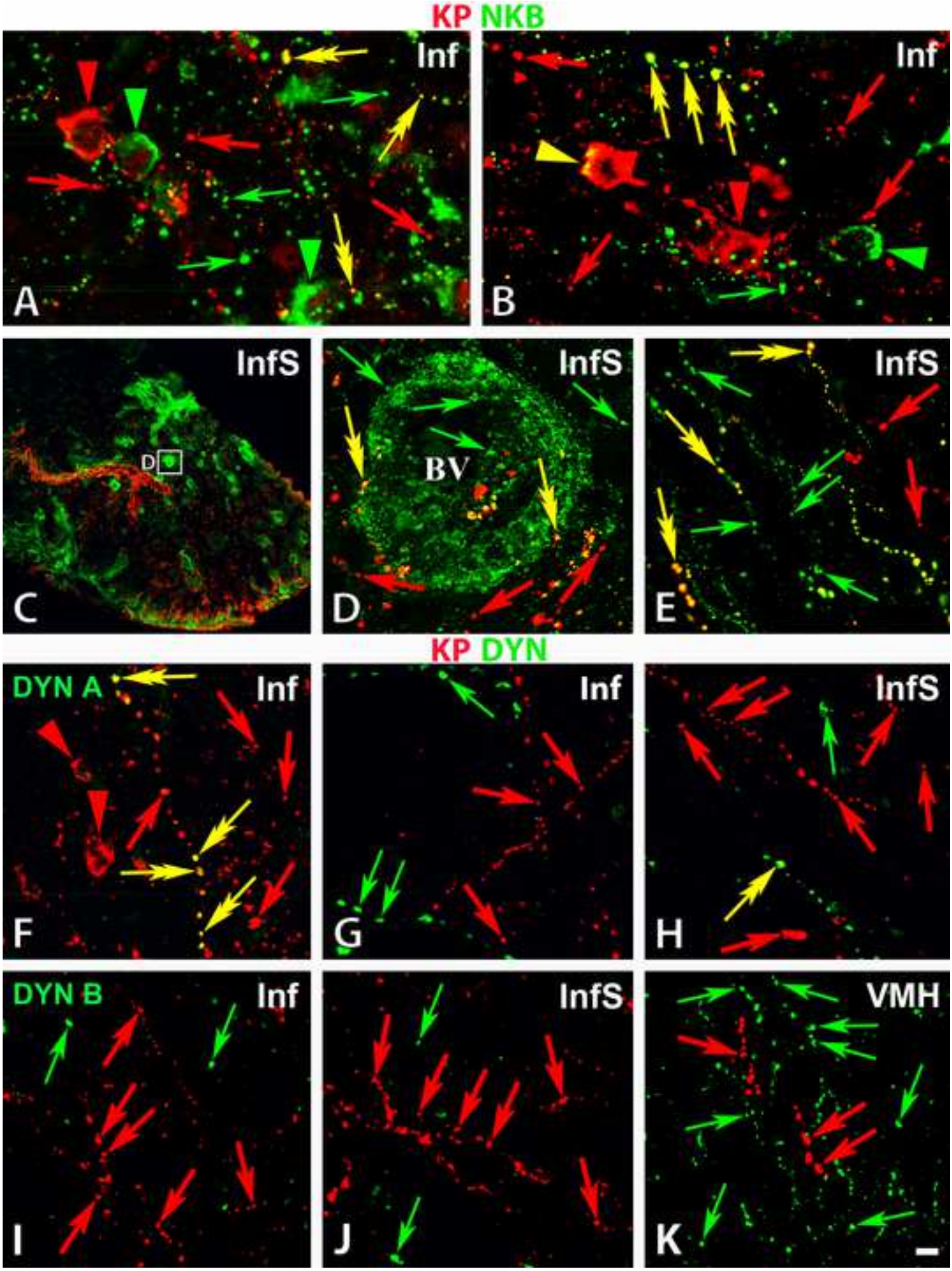

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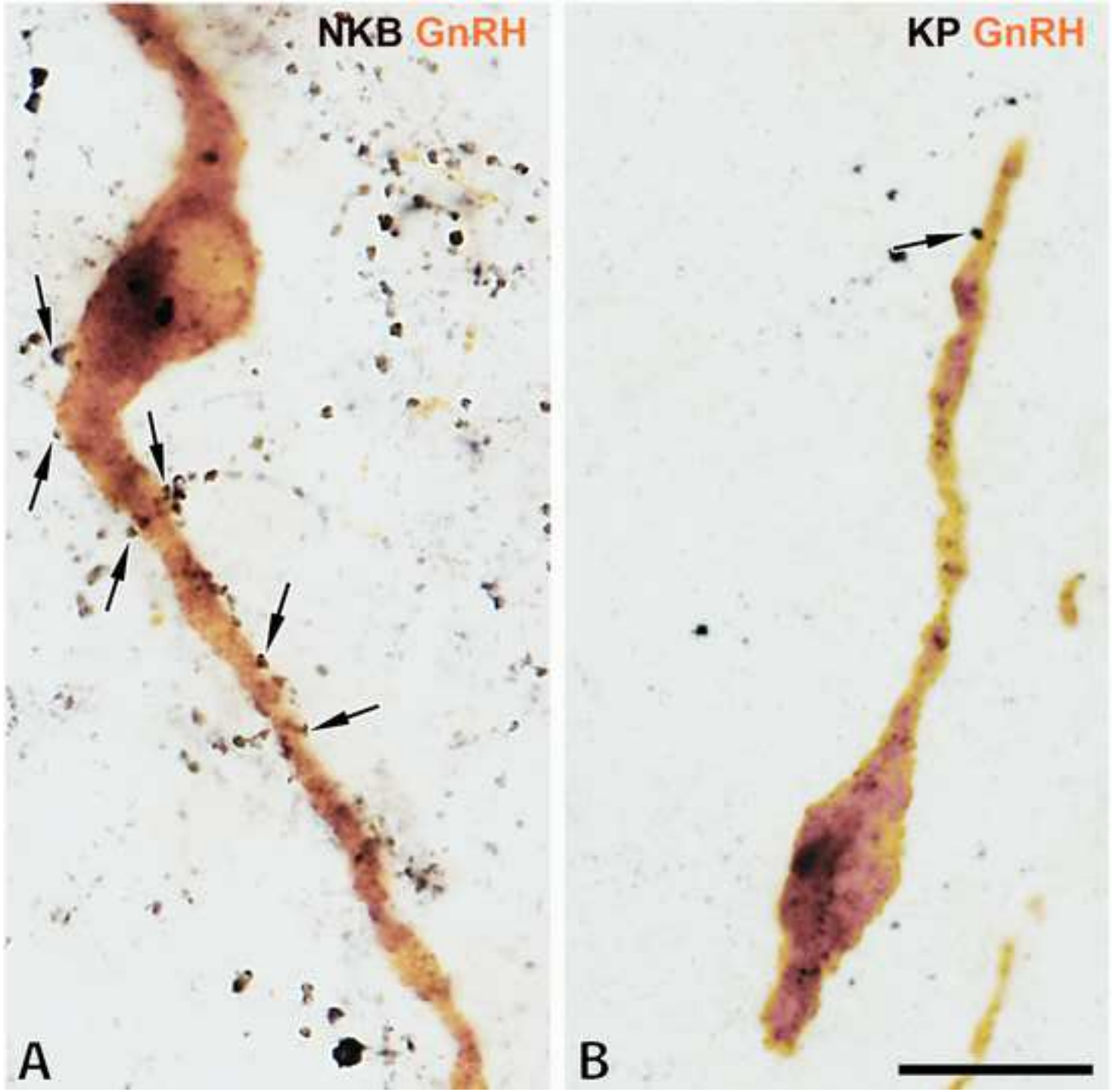

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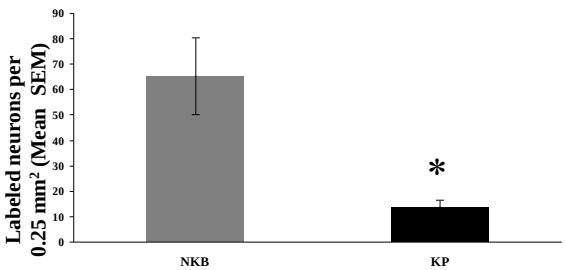

Graph 1

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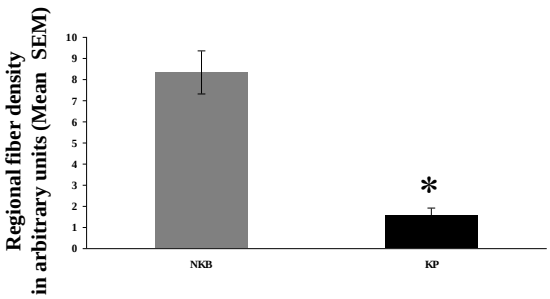

Graph 2

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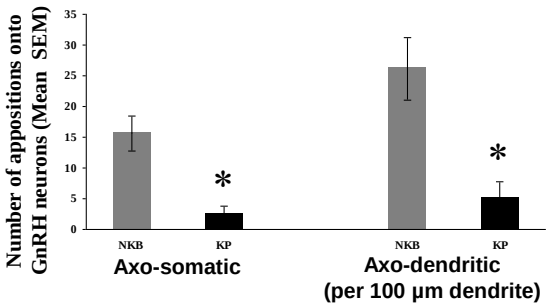

Graph 3