

Hypophysiotropic gonadotropin-releasing hormone projections are exposed to dense plexuses of kisspeptin, neurokinin B and substance P immunoreactive fibers in the human: a study on tissues from postmenopausal women

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Abstract

Neuronal populations that synthesize kisspeptin (KP), neurokinin B (NKB) and substance P (SP) in the hypothalamic infundibular nucleus of humans are partly overlapping. These cells are important upstream regulators of gonadotropin-releasing hormone (GnRH) neurosecretion. Homologous neurons in laboratory animals are thought to modulate episodic GnRH secretion

primarily via influencing KP receptors on the hypophysiotropic fiber projections of GnRH neurons. To explore the structural basis of this putative axo-axonal communication in humans, we analyzed the anatomical relationship of KP-immunoreactive (IR), NKB-IR and SP-IR axon plexuses with hypophysiotropic GnRH fiber projections. Immunohistochemical studies were carried out on histological samples from postmenopausal women. The neuropeptide-IR axons innervated densely the portal capillary network in the postinfundibular eminence. Subsets of the fibers formed descending tracts in the infundibular stalk, some reaching the neurohypophysis. KP-IR, NKB-IR and SP-IR plexuses intermingled, and established occasional contacts, with hypophysiotropic GnRH fibers in the postinfundibular eminence and through their lengthy course while descending within the infundibular stalk. Triple-immunofluorescent studies also revealed considerable overlap between the KP, NKB and SP signals in individual fibers, providing evidence that these peptidergic projections arise from neurons of the mediobasal hypothalamus.

These neuroanatomical observations indicate that the hypophysiotropic projections of human GnRH neurons in the postinfundibular eminence and the descending GnRH tract coursing through the infundibular stalk to the neurohypophysis are exposed to neurotransmitters/neuropeptides released by dense KP-IR, NKB-IR and SP-IR fiber plexuses. Localization and characterization of axonal neuropeptide receptors will be required to clarify the putative autocrine and paracrine interactions in these anatomical regions.

Introduction

A peptidergic neuron population identified in the hypothalamic arcuate nucleus (ARC) co-synthesizes kisspeptin (KP), neurokinin B (NKB) and dynorphin [1-3] in a variety of mammalian species; these 'KNDy neurons' [4] were proposed to constitute an important regulatory component of the pulse generator which shapes the episodic secretion of gonadotropin-releasing hormone (GnRH) into the hypophysial portal circulation [2, 3, 5]. While homologous KP and NKB neurons are also present in the human infundibular nucleus [6, 7], species differences are also likely to exist in the neurotransmitter complement that these neurons use for communication [8]. Notably, morphological studies have found only poor evidence for dynorphin expression in KP-IR neurons in the infundibular nucleus of young men [9]. On the other hand, substance P (SP) has been revealed in considerable subsets of KP and NKB neurons in the human [10], but not in the rodent or sheep, KNDy neurons.

The regulation of GnRH release by overlapping populations of KP and NKB neurons is thought to take place primarily via KP signaling through the kisspeptin receptor (KISS1R) which is expressed in GnRH cells [11-13]. While additional immunohistochemical evidence from rats [14] and single-cell microarray evidence from mice [12] indicate that a subset of GnRH neurons also contain the NK3 receptor for NKB, NK3 immunoreactivity has not been revealed in GnRH neurons of the sheep [15]. In addition, the effect of NKB on LH release seems to require KP signaling in the monkey [16], raising further the possibility of species difference. The issue of whether GnRH neurons possess the NK1 receptor for SP, has not been addressed.

Hypothalamic KP neurons are capable of regulating reproduction via acting on the somato-dendritic compartment of the GnRH cell. Accordingly, GnRH neurons receive KP-IR afferent contacts [7, 17-20] and show depolarization [12, 21, 22] and cFos expression [11, 23] in response to KP. Similarly to KP fibers, NKB-IR axons also establish axo-somatic and axo-dendritic contacts with GnRH neurons in rats [24], mice [25] and humans [9, 26, 27] and 16% of the GnRH-IR perikarya in the preoptic area of the rat exhibit NK3 immunoreactivity [14]. Somewhat conflictingly, mouse GnRH

neurons do not give electrophysiological responses to the NK3 receptor agonist senktide in slice preparations [28] and senktide does not induce GnRH release from the preoptic area which contains the GnRH cell bodies [29]. In the human mediobasal hypothalamus, many SP-IR neurons are identical with KP and NKB neurons [10]. SP neurons provide axo-somatic and axo-dendritic inputs to GnRH neurons, as indicated by light microscopic immunohistochemical observations on humans [30] and immuno-electronmicroscopic data from rats [31].

In addition to influencing the somatic and dendritic compartments of GnRH neurons, there is accumulating evidence from a variety of species that KP and NKB can also regulate GnRH secretion in the median eminence where GnRH axon terminals are juxtaposed to KP-IR [7, 19, 32] and NKB-IR [14, 33] processes. Such direct axo-axonal contacts are devoid of classical synaptic specializations at the ultrastructural level [32, 33]. Although immunohistochemical studies are still unavailable to demonstrate the presence of the KP receptor (KiSS1R) at this putative axo-axonal communication site, a previous immunofluorescent study on rats identified NK3 immunoreactivity on GnRH-IR fibers in the median eminence [14]. Abundant functional evidence exists to support the concept that both KP and NKB act on the axonal compartment of GnRH neurons. Accordingly, GnRH secretion from mediobasal hypothalamic explants of mice (which contain the hypophysiotropic GnRH axons but few if any GnRH cell bodies) can be stimulated by KP in a KiSS1R-dependent and action potential-independent manner [34]. A site for KP action outside the blood-brain barrier gains additional support from the observations that systemic KP injection induces LH secretion in rats [23, 35], monkeys [36] and humans [37, 38], although it remains possible that KP causes these effects in other circumventricular organs, including the organum vasculosum of the lamina terminalis [39]. Similarly to KP, the NK3 agonist senktide is capable of eliciting GnRH release from the median eminence of mice and this action does not require KP signaling [29].

In different species, the GnRH pulse generator is thought to be located in the mediobasal hypothalamus which also contains the KNDy neurons. Accordingly, mediobasal hypothalamic explants from foetal and adult human brains release GnRH in a pulsatile manner [40]. Similarly, GnRH

secretion occurs episodically from mediobasal hypothalamic explants of the rat. In this rodent species the mediobasal hypothalamus contains the hypophysiotropic GnRH axon projections but no GnRH cell bodies [41], suggesting that upstream elements of the GnRH pulse generator act via influencing the neurosecretory output of hypophysiotropic GnRH fibers. Information accumulated about KNDy neurons in the last few years has been incorporated into new models of the GnRH/LH pulse generator. According to these models, the peptidergic communication of KNDy neurons with each other and with GnRH neurons is critically involved in the regulation of pulsatile GnRH secretion [3, 4, 28, 42].

Here, we have used immunohistochemistry to study the putative axo-axonal interaction sites where KP-IR and NKB-IR fibers might influence GnRH neurosecretion in the human. The analysis was extended to SP-IR axons many of which are identical with KP-IR and NKB-IR axons in the human [10]. The anatomical relationship of KP-IR [7], NKB-IR [7] and SP-IR [43] axons with GnRH-IR [44] axons was first studied around the postinfundibular eminence which contains the majority of the portal capillaries in the human [45]. GnRH axons in the human and the monkey enter the infundibular stalk (InfS) and many fibers descend all the way down to the neurohypophysis [44]. Therefore, we have also examined the anatomical relationship of these long descending GnRH fiber projections through the InfS to the neurohypophysis with the descending KP-IR, NKB-IR and SP-IR axon tracts.

Materials and methods

Tissue collection

Human hypothalamic and pituitary tissues from five postmenopausal female subjects (53-83 years) were obtained at autopsies [7, 26] 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). The subjects were not known to suffer from neurological or endocrine disorders and the *post mortem* intervals before dissection were below 36h.

Tissue preparation for immunohistochemistry

Brain removal from the skull was carried out in a way to maintain a long pituitary stalk. Dissection guidelines from the hypothalamic blocks were the optic chiasm rostrally, the mammillary bodies caudally and the anterior commissure dorsally [7, 26]. The hypophysis was also taken out from the sella following the removal of the brain. The tissue blocks were rinsed briefly with running tap water, and then, immersion-fixed in 4% formaldehyde in 0.1M phosphate buffer saline (PBS; pH 7.4) for 7-21 days. The fixed hypothalami were cut in half before section preparation. The pituitaries and the hemihypothalami were infiltrated with 20% sucrose for 5 days at 4°C. The tissues were placed in freezing molds, 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 then, sectioned at 30µm with a Leica SM 2000R freezing microtome (Leica Microsystems). The hypothalami were sectioned in the coronal plane and the pituitaries either in the horizontal or the coronal plane. 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

Every 72nd section of the hypothalamic infundibular nucleus (2-3 sections per subject) and the hypophysis was used for each immunohistochemical experiment. The relationship of KP-IR, NKB-IR and SP-IR axons to the portal capillary plexuses and to GnRH-IR fibers was studied on adjacent sections. 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, epitope retrieval was carried out using a 30-min treatment in 0.1M citrate buffer (pH=6.0) at 80°C. In immunofluorescent experiments, similar series of sections were used and treated additionally with Sudan black as described previously [46], to reduce tissue autofluorescence [47].

Dual-labeling immunohistochemistry

Incubation of neighboring sections in KP, NKB or SP antibodies for 48 h at 4°C 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

signal was visualized with nickel-diaminobenzidine chromogen. Then, the chromogen was silver-gold-intensified as detailed elsewhere [48], except that the thioglycolic acid pretreatment step was left out. KP immunoreactivity was detected with a sheep polyclonal antiserum (GQ2; 1:150,000) against human kisspeptin-54. This antiserum recognizes human kisspeptin-54, kisspeptin-14 and kisspeptin-10 and shows no cross-reactivity (<0.01%) with other human RF-amide peptides, including prolactin releasing peptide, neuropeptide FF, neuropeptide AF and RF-amide related peptides (RFRP1, RFRP2, RFRP3) [37]. The GQ2 antibodies were used successfully in previous immunohistochemical experiments on hypothalamic sections from the rhesus monkey [19, 49] and the human [7, 9, 26, 27]. NKB synthesizing neuronal fibers were visualized with a previously characterized [7, 9, 26, 27, 49] rabbit polyclonal antiserum (IS-682; P. Ciofi; 1:100,000) directed against the C-terminal 28 amino acids of human pro-NKB or alternatively, with a mouse monoclonal antibody against human NKB (Biosensis Pty. Ltd, Thebarton, SA Australia; M-871-100; 1:50,000). SP immunoreactivity was detected either with a rat monoclonal antibody (Serotec #8450-0505; Bio-Rad Laboratories, Inc., Hercules, CA; 1:30,000) [10] or a rabbit polyclonal antiserum (#505D3) raised against the carboxyl terminus of SP (kind gift from Dr. P. Petrusz, Department of Anatomy, University of North Carolina, Chapel Hill, NC; 1:150,000). This rabbit antiserum required an amidated carboxyl terminus group for recognition and showed less than 0.05% cross-reactivity with either neurokinin A or NKB [50].

Following the detection of KP, NKB or SP, the sections were processed further to detect GnRH-IR axons, using a previously characterized guinea pig antiserum against mammalian GnRH (#1018; 1:50,000) [26]. GnRH-IR elements were visualized with the biotinylated secondary antibody-ABC technique and brown diaminobenzidine chromogen, as in previous dual-label immunoperoxidase experiments [7, 9, 26, 27].

Triple-immunofluorescent labeling for the simultaneous visualization of KP, NKB and SP

A series of hypothalamic and hypophysial sections was used to study the putative colocalization between KP, NKB and SP immunoreactivities. First, the primary antibodies were applied to the sections in a cocktail consisting of the sheep KP (1:1000), the rabbit NKB (1:1000) and

the rat SP (1:1000) antibodies (4°C; 24h). Then, the sections were transferred for 12 h at 4°C into the following secondary antibody cocktail: anti-sheep-Cy3 (1:1000)+anti-rabbit-FITC (1:250)+anti-rat-Cy5 (1:500).

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.

Analysis

Peroxidase-labeled sections were analyzed and representative light microscopic images prepared with an AxioCam MRc 5 digital camera mounted on a Zeiss AxioImager M1 microscope, and using the AxioVision 4.6 software (Carl Zeiss, Göttingen, Germany).

Confocal images from the triple-immunofluorescent specimens were prepared with a Radiance 2100 (Bio-Rad Laboratories, Hemel Hempstead UK) confocal systems. Individual optical slices (<0.8µm) were collected for analysis and illustrations using the 'lambda strobing' function so that only one excitation laser and the corresponding emission detector were active during a line scan, to eliminate emission crosstalk between the fluorophores. The separately recorded red, green and far-red channels were merged and transferred into the red, blue and green channels of Adobe Photoshop (PSD) files, respectively.

Specificity controls

Various control approaches were used to confirm the specificity of immunohistochemical results. For peroxidase-based detection, specificity controls included the comparative analysis of the immunohistochemical staining obtained with two distinct antisera in neighboring sections. In case of KP, results obtained with the sheep GQ2 antiserum against the processed active peptide KP-54 (amino acids 68-121 of Q15726) and two commercially available affinity-purified rabbit polyclonal

antibodies (Antibody Verify, Inc., Las Vegas, NV, USA) were compared. These rabbit reference antibodies target amino acids 21-81 (AAS26420C) and 47-107 (AAS27420C), respectively, of the 138-amino acid human pro-KP sequence (Q15726). Of note, these peptide segments do not include the C-terminal RF-amide motif of KP which could account for unwanted cross-reactions with other members of the RF-amide peptide family. For NKB, results obtained with the rabbit antisera (IS-681 and IS-682) [7] and with the mouse monoclonal antibody (M-871-100) were compared. Similar positive control experiment for SP labeling were carried out by replicating the immunohistochemical labeling with the rabbit (#505D3) and the rat (#8450-0505) antibodies. Negative control experiments included the omission of the primary or secondary antibodies from the labeling procedure.

In triple-label immunofluorescent studies, the presence of many bright single-labeled, in addition to double- and triple-labeled structures served as an endogenous control for the absence of antibody cross-reactions and bleed-through.

Finally, series of test sections were dual-labeled with the combined use of the rabbit and the sheep KP antibodies, the rabbit and the mouse NKB antibodies or the rabbit and the rat SP antibodies. In these double-labeling experiments, the primary antibodies raised in the different species were detected with FITC-conjugated and Cy3-conjugated secondary antibodies.

Results

Demonstration of anatomical overlap between hypophysiotropic GnRH fibers and axons expressing KP-, NKB-, and SP immunoreactivities

The hypothalamic distribution of GnRH-IR [44, 51, 52], KP-IR [7, 8], NKB-IR [7] and SP-IR [30, 43] neuronal cell bodies and fibers was in agreement with previous reports. While the dual-labeling experiments confirmed that GnRH neurons in the medio-basal hypothalamus receive axo-somatic and axo-dendritic afferent contacts from KP-IR, NKB-IR and SP-IR neurons [7, 26, 30], the present study focused on the anatomical sites of major hypophysiotropic GnRH fiber projections. These GnRH projections were present in highest abundance in the postinfundibular eminence (Figs. 1A-I) which

contains a superficial and a deep plexus of hypophysial portal capillaries [45]. These capillary plexuses were surrounded by GnRH-IR axons and, at the same time, also by very dense KP-IR (Fig. 1A), NKB-IR (Fig. 1D) and SP-IR (Fig. 1G) fiber networks. High-power light microscopic analysis of double-labeled sections revealed occasional axo-axonal appositions between the GnRH-IR fibers and the other three phenotypes of peptidergic axons (Figs. 1B, C, E, F, H, I).

The analysis of immunostained histological sections from the neurohypophysis confirmed the previous observation [44] that a significant population of GnRH-IR fibers entering the human InfS descend to the neurohypophysis (Figs. 1J-R). In addition, we found that the neurohypophysis also contained abundant plexuses of KP-IR (Figs. 1J-L), NKB-IR (Figs. 1M-O) and SP-IR (Figs. 1P-R) fibers; the course and distribution of these fibers overlapped with the projection areas of the descending GnRH-IR processes (Figs. 1K, L, N, O, Q, R). In each dual-labeling experiment, occasional axo-axonal contacts were also detectable between the different types of peptidergic axons and the GnRH fibers (Figs. 1L, N, O, Q-R).

Visualization of the parallel descent of GnRH-IR and SP-IR fibers through the InfS

Some hypothalamic samples contained long InfS, allowing us to follow the parallel descent of different peptidergic projections (Fig. 2). The analysis of such specimens showed that peptidergic axons often intermingled with groups of immunoreactive cell bodies, in particular in the upper portions of the InfS (Figs. 2C, D, E). The descending KP-IR, NKB-IR and SP-IR axon tracts intermingled and established sporadic axo-axonal contacts with the GnRH-IR fibers (Figs. 2B, F).

Colocalization of KP, NKB and SP immunoreactivities in peptidergic axons

Triple-immunofluorescent studies revealed colocalization between KP (red), NKB (blue) and SP (green) immunoreactivities in individual axons in the InfS (Fig. 1S) and the neurohypophysis (Fig. 1T). Notably, the colocalization was only partial; single- and double-labeled (NKB/SP:turquoise; SP/KP:yellow; KP/NKB:purple) fibers occurred more frequently than the triple-labeled (white color) ones (Figs. 1S, T).

Dual-immunofluorescent control experiments to verify the specificity of KP, NKB and SP

immunolabeling

The combined use of two different primary antibodies (raised in different species) for immunofluorescent dual-labeling showed that the different KP, NKB and SP antibodies label essentially identical neuronal structures; the majority of immunoreactive neuronal elements appear to be dual-labeled (Figs. 3A-L), in strong support of labeling specificity.

Discussion

In this study, we present morphological evidence that hypophysiotropic GnRH axons in postmenopausal women form intermingling plexuses and establish occasional axo-axonal appositions with KP-IR, NKB-IR and SP-IR fibers. Such areas of regional overlap and axo-axonal contacts were detected throughout the lengthy course of the hypophysiotropic GnRH axons, including the postinfundibular eminence, the InfS and the neurohypophysis. We also show that many of the KP-IR, NKB-IR and SP-IR fibers in these regions are identical, indicating that their site of origin is the infundibular nucleus where a subsets of neurons co-synthesize two or all three of these neuropeptides [10].

Comparative analysis of the GnRH neuronal system in humans, monkeys, ferrets, bats and rats revealed conspicuous species differences regarding the course of hypophysiotropic GnRH axon projections. Unlike in rats where these fibers terminate in the palisade zone of the median eminence, considerable subsets of GnRH-IR axons in the human and the monkey also enter the internal zone of the InfS and even descend to the neurohypophysis [44]. A previous dual-label immunohistochemical study in the monkey revealed that the descending GnRH-IR axon projections and a descending KP-IR axon plexus intermingle in the posterior pituitary [53]. In our present study, we provide evidence that GnRH fibers in the human are accompanied by dense KP-IR, NKB-IR and SP-IR fiber plexuses in the postinfundibular eminence as well as throughout their lengthy course to the neurohypophysis. Occasional contacts could also be identified between these and

GnRH-IR fibers in all of these regions. The close anatomical relationship of these peptidergic plexuses may allow important paracrine interactions to occur at the level of the axonal compartments. The functional importance of the massive descending peptidergic fiber projections toward the neurohypophysis requires clarification. It is possible that neuropeptides released from the varicosities of these axon plexuses reach the adenohypophysis via the short portal veins to influence gonadotroph functions. In addition, the secreted neuropeptides might directly enter the systemic circulation from the portal capillary plexuses of the postinfundibular eminence [45] and through the tuberal veins of the InfS. It is important to recognize that many neuropeptides present in the external zone of the sheep median eminence are not necessarily secreted in significant amounts into the hypophysial portal blood of sheep [54] and this can also be the case in primates.

A recent study from our laboratory established that many of the KP-IR, NKB-IR and SP-IR cell bodies and lower subsets of axons in the human mediobasal hypothalamus are identical, with the largest degrees of neuropeptide coexpression observed in postmenopausal women [10]; in this endocrine status, 25.1% of the NKB-IR and 30.6% of the KP-IR perikarya contained SP and 16.5% of all immunolabeled cell bodies were triple-labeled [10]. In the present study we observed many single- and double-labeled KP-IR, NKB-IR and SP-IR fibers both in the infundibular nucleus and the InfS. The much lower degree of signal coexpression in axons vs. cell bodies implies that many fibers derived from KP/NKB/SP neurons only contain one or two neuropeptides in postmenopausal women. This observation raises the possibility that the axonal transport, processing and use for neurotransmission of the co-synthesized neuropeptides might be regulated depending on the functional status of these neurons. Earlier studies of the human KP and NKB systems in our laboratory already provided evidence that the extent of colocalization between KP and NKB is sex-dependent [8, 26] and also age-dependent, at least in men [8, 27]. In addition to this difference in the labeling of perikarya, we noticed that the neuronal contacts that these cells establish with GnRH neurons exhibit sex-specific patterns of KP/NKB co-labeling, with a significantly higher incidence of double-labeled fibers in postmenopausal women compared with age-matched men [26]. It will be important in the future to determine how the reproductive status

influences neuropeptide colocalization in the axon projections of these neurons, including the plexuses we propose here to interact with the hypophysiotropic GnRH fiber projections via autocrine/paracrine peptidergic mechanisms. At present, it is impossible to determine, whether or not, the occasionally observed axo-axonal contacts play a significant role in this putative axo-axonal interaction.

Axo-axonal interactions taking place in the median eminence/postinfundibular eminence region have long been thought to play important roles in neuroendocrine regulation [55]. However, it is difficult to appreciate the functional significance of this putative paracrine communication only on the basis of morphological studies, given that synaptic specializations are absent from such axo-axonal contacts at the ultrastructural level [33, 56]. While the immunohistochemical demonstration of specific receptors on neuroendocrine GnRH terminals would be indicative of such a paracrine communication, the evidence for axo-axonal interactions is mostly indirect and comes from functional studies. Because in rats, the mediobasal hypothalamus is devoid of GnRH-IR cell bodies and only contains hypophysiotropic projections [57], *in vitro* explants of this region can be used in pharmacological studies of receptor interactions that might influence GnRH secretion at the level of the GnRH axon. For example, GnRH terminals in the median eminence of the rat are juxtaposed to glutamatergic axons [56, 58] and express immunoreactivity for the KA2 and NR1 ionotropic glutamate receptor subunits [56]. There is *in vitro* evidence that glutamatergic drugs can induce Ca^{2+} -dependent GnRH release from median eminence fragments in a Ca-dependent manner [59]. It is interesting to note that the source of glutamate in this interaction may be, at least partly, intrinsic, since GnRH neurons of the adult male rat exhibit glutamatergic properties and express type-2 vesicular glutamate transporter mRNA and immunoreactivity [60]. Neuropeptide Y (NPY) also appears to act similarly on the GnRH axon terminals, in addition to occurring in axons that form axo-somatic and axo-dendritic contacts onto GnRH neurons of rats and humans [51, 61]. In rats, i) direct appositions exist between NPY-IR and GnRH-IR axons in the median eminence [62], ii) GnRH axon terminals

express Y1 receptor immunoreactivity [62] and iii) NPY agonists stimulate *in vitro* GnRH release from median eminence fragments [63].

While the putative presence of KiSS1R on GnRH terminals has not been detected formally with immunohistochemistry, there is abundant indirect evidence to support the concept that GnRH terminals represent important physiological sites of KP action in the regulation of pulsatile GnRH secretion. Accordingly, KP-IR axons are intimately juxtaposed to GnRH-IR axons in the goat [32], monkey [19] and human [7] median eminence and KP can stimulate GnRH release from median eminence fragments of wild type, but not KiSS1R mutant, mice [34]. This *in vitro* action of KP is independent of action potential generation and thus, persists in the presence of tetrodotoxin [34]. Axo-axonal communication may represent the primary mechanism whereby KNDy neurons influence the pulsatile secretion of GnRH. Accordingly, KP release into the monkey median eminence was found to be pulsatile, with secretory peaks that coincide with the LH pulses [64]. Furthermore, the proposed axonal site of action for KP is in accordance with the observation that the peripheral injection of KP results in rapid increases of LH release [23, 35, 36, 65] which can be prevented with the GnRH antagonist acylin [65]. KP is likely to influence GnRH neurons outside the blood-brain barrier because it does not induce c-Fos expression in GnRH cell nuclei of the preoptic area. We have to recognize that systemic KP may also act in other circumventricular organs lacking the blood-brain barrier, including the organum vasculosum of the lamina terminalis. In this context, it is important to mention that recent neuroanatomical studies of the mouse organum vasculosum of the lamina terminalis identified GnRH-IR processes with dendritic characteristics and extensive branching. Direct application of KP onto these processes caused electric activity and c-Fos expression in GnRH neurons [39]. We note that the GnRH-IR innervation of the human OVLT is much less abundant in comparison with the rodent [44]; therefore, it seems more likely that the LH release induced by systemic KP injection in humans [37, 38] is caused by the excitation of hypophysiotropic GnRH axons.

The present study also provided evidence for the regional overlap and axo-axonal contacts between NKB-IR and GnRH-IR axons. Similar appositions are also detectable in the rat median

eminence [14, 33] where the GnRH-IR axons express immunoreactivity for the NKB receptor NK3 in rats [14]. While recent models of the GnRH pulse generator agree in that KP provides the major output signal of the putative pacemaker KNDy neurons toward the GnRH network [2, 3], whether or not NKB can also regulate the release of GnRH directly, remains to be established. Given that NK3 is present in KNDy neurons and the NK3 agonist senktide excites KP neurons but not GnRH neurons [28], NKB/NK3 signaling might mainly act in the intranuclear communication of KNDy neurons within the network. It is possible that NKB can also regulate NKB axon terminals in the median eminence. In addition to exerting autocrine/paracrine actions on other KP and NKB neurons, recent studies on mice found evidence that senktide is capable of inducing GnRH release directly from the median eminence and this action does not require KP signaling [29]. Of note, species might considerably differ in this context and the role of KP seems to be essential for the NKB-mediated GnRH release in rhesus monkeys [16]. SP is a recently recognized neuropeptide player in the putative pulse generator KP and NKB neuronal systems. It will require clarification whether SP acts via influencing GnRH axon terminals directly or via regulating neuropeptide release from other types of fibers through autocrine/paracrine mechanisms.

In summary, in this study we provide evidence that the hypophysiotropic projections of human GnRH neurons are exposed to KP, NKB and SP in the postinfundibular eminence as well as throughout their lengthy descending projection pathway through the InfS to the neurohypophysis. Depending on the site/s of location of the SP receptor NK1, the NKB receptor NK3 and the KP receptor KiSSR1, the proposed axo-axonal interaction may involve important autocrine and paracrine components. To fully understand these mechanisms, information about the cellular and subcellular localization of the neuropeptide receptors will be critical.

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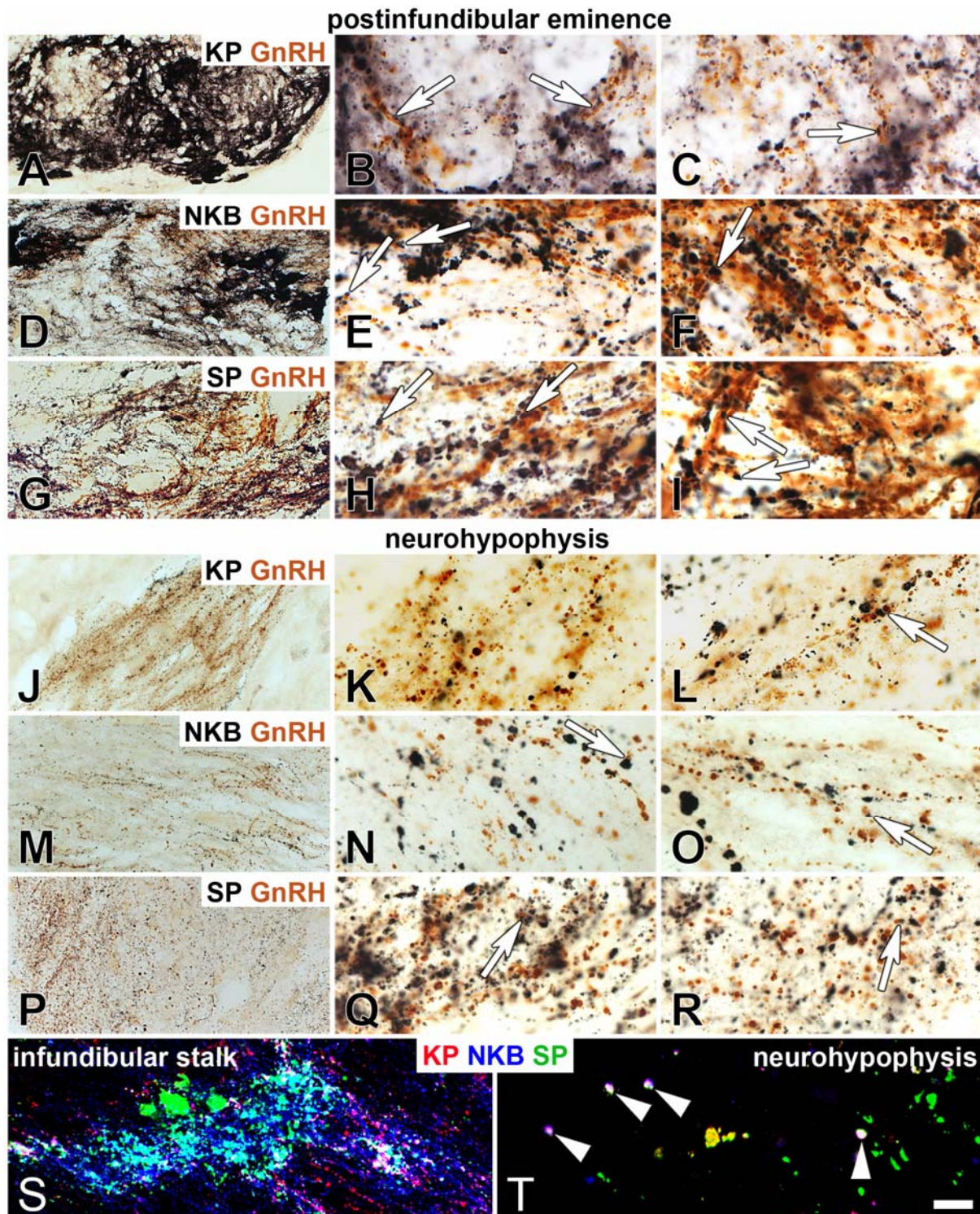


Figure 1. Overlapping distribution of GnRH-IR, KP-IR, NKB-IR and SP-IR axons in the postinfundibular eminence, the infundibular stalk and the neurohypophysis.

Dual-immunoperoxidase stained histological sections of the postinfundibular eminence from postmenopausal women illustrate very dense KP-IR (A-C), NKB-IR (D-F) and SP-IR (G-I) axon plexuses (black silver-gold-intensified nickel-diaminobenzidine chromogen) around the portal capillary vessels.

Brown diaminobenzidine labeling reveals the hypophysiotropic GnRH fiber plexuses that surround the portal vasculature similarly. Not all peptidergic axons terminate in the postinfundibular eminence and considerable subsets descend through the infundibular stalk to reach the neurohypophysis (**J-R**). Note that these descending fibers appear as parallel tracks in **J** and **M**, whereas they are transected in **P** due to the different section plane. Arrows in high-power panels point to the occasional axo-axonal contacts established by KP-IR, NKB-IR and SP-IR axons with GnRH-IR fibers in the postinfundibular eminence (**B, C, E, F, H, I**) and the neurohypophysis (**K, L, N, O, Q, R**). Results of triple-immunofluorescent studies (**S, T**) provide evidence that KP-IR (red), NKB-IR (blue) and SP-IR (green) fibers involved in the putative axo-axonal interactions with GnRH fibers are often identical. Colocalization of the neuropeptides also indicates that these fibers originate in the infundibular nucleus. White colour in the infundibular stalk (**S**) and in the neurohypophysis (**T**) corresponds to fibers that contain all three fluorochromes. Arrowheads in **T** point to triple-labeled (KP/NKB/SP-IR) axon varicosities in the neurohypophysis, whereas many single- and double-labeled (NKB/SP:turquoise; SP/KP:yellow; KP/NKB:purple) fibers also occur. Scale bar in **T** corresponds to 60 μm in **A, D, G**, 10 μm in **B, C, E, F, H, K, L, N, O, Q, R**, 30 μm in **S** and 6 μm in **T**.

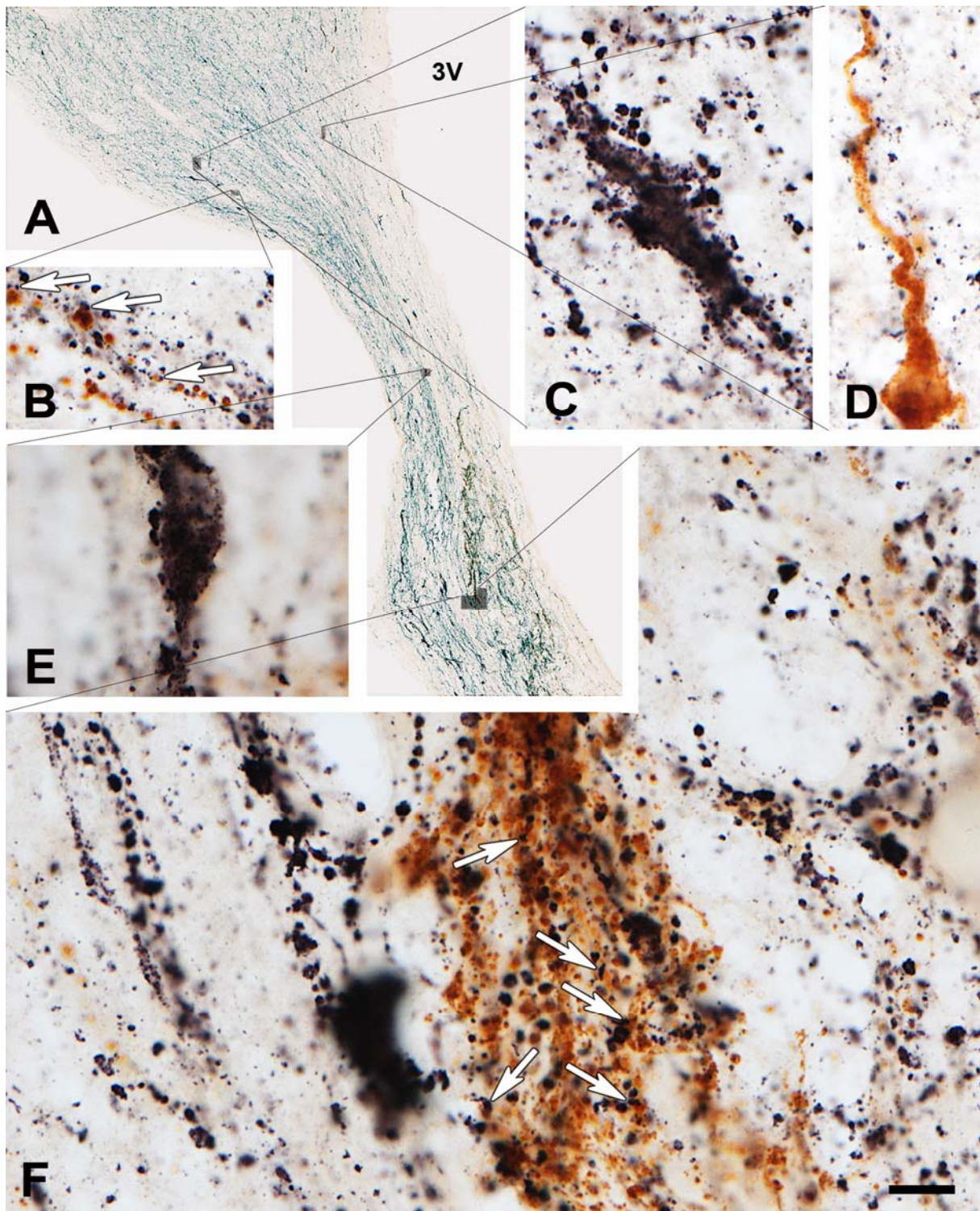


Figure 2. Simultaneous immunohistochemical visualization of SP-IR and GnRH-IR descending fibers in the infundibular stalk of a postmenopausal woman.

A coronal hemihypothalamic section of a postmenopausal woman (central figure A) through the long infundibular stalk has been dual-immunostained for SP (black silver-gold-intensified nickel-diaminobenzidine chromogen) and GnRH (brown diaminobenzidine). 3V, third ventricle. Note that

the dense longitudinal plexuses of SP-IR and GnRH-IR axons intermingle and descend deeply into the stalk, some reaching the neurohypophysis. As shown in high-power insets, the descending fibers often intermingle with groups of neuronal cell bodies (**C, D, E**). Arrows in **B** and **F** point to the sporadic axo-axonal contacts between SP-IR and GnRH-IR axons which may underlie putative paracrine interactions. Scale bar in **F** corresponds to 420 μm in **A** and 10 μm in **B-F**.

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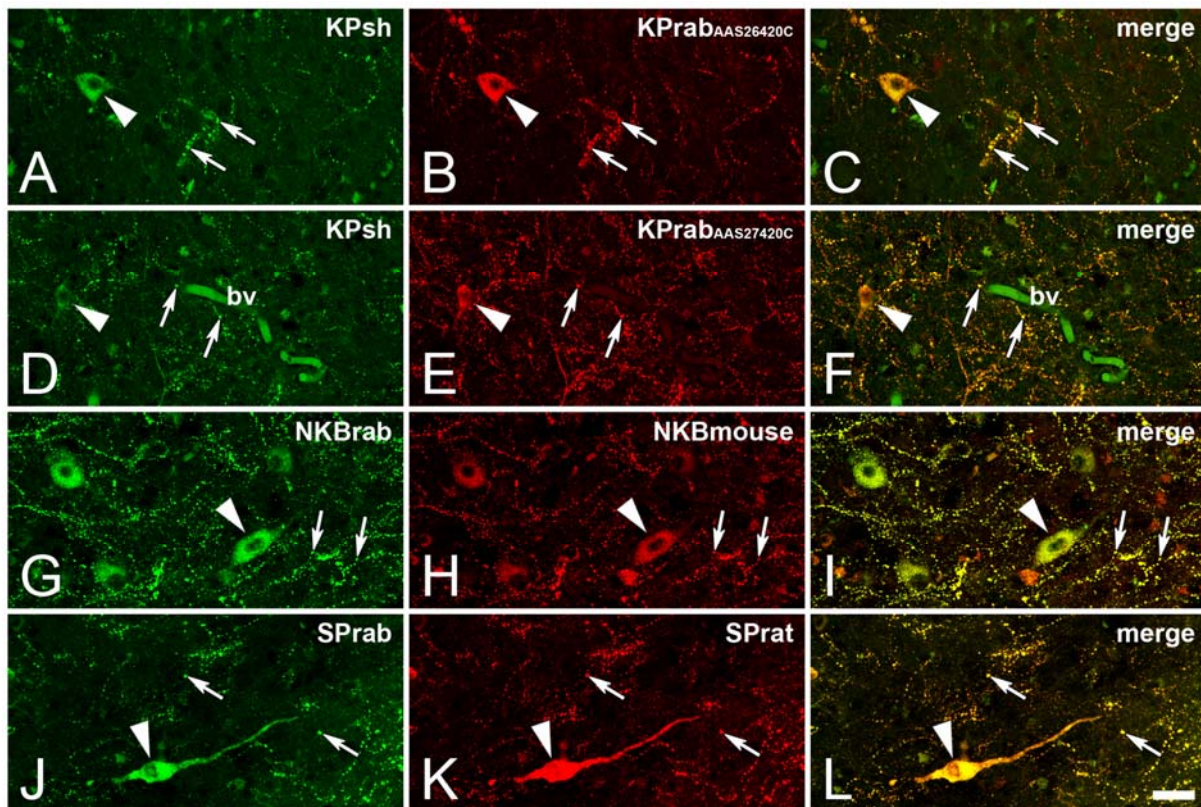


Figure 3. Results of positive control experiments using pairs of antibodies raised in two different animal species against the same neuropeptide target.

The specificity of labeling with the KP (GQ2; **A, D**), NKB (IS-682; **G**) and SP (#505D3; **J**) antisera has been challenged in dual-immunofluorescent experiments using other reference KP (AAS26420C; **B** and AAS27420C; **E**), NKB (M-871-100; **H**) and SP (#8450-0505; **K**) antibodies, respectively. The two primary antibodies used in combination were reacted with secondary antibodies conjugated with Cy3 and FITC, respectively. Results of these control experiments indicate that the vast majority of cell bodies and fibers in the infundibular nucleus can be dual-labeled (yellow color in the merged images **C, F, I** and **L**) with the red Cy3 and the green FITC chromogens. The specificity of labeling with the GQ2 antibodies against the processed active peptide KP-54 (amino acids 68-121 of Q15726; **A, D**) has been verified with two distinct commercial antibodies against the 138-amino acid human pro-KP protein (Q15726). These affinity-purified reference antibodies target amino acids 21-81 (AAS26420C; **B**) and 47-107 (AAS27420C; **E**), respectively. Dual-labeled cell bodies are indicated by arrowheads

and dual-labeled axon varicosities by arrows in 0.8 μm -thick optical slices. 'bv' labels a blood vessel exhibiting green autofluorescence. Scale bar in L corresponds to 35 μm in all panels.

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