New perspectives for anatomical and molecular studies of kisspeptin neurons in the aging human brain

Running head: Human kisspeptin neurons and aging

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Abstract

The human infundibular nucleus (corresponding to the rodent arcuate nucleus) serves as an important integration center for neuronal signals and hormones released by peripheral endocrine organs. Kisspeptin (KP) producing neurons of this anatomical site many of which also synthesize neurokinin B (NKB) are critically involved in sex hormone signaling to gonadotropin-releasing homone (GnRH) neurons. In recent years, the basic topography, morphology, neuropeptide content and connectivity of human KP neurons have been investigated with *in situ* hybridization and immunohistochemistry on *post mortem* tissues. These studies revealed that human KP neurons differ neurochemically from their rodent counterparts and show robust aging-related plasticity. Earlier immunohistochemical experiments also provided evidence for temporal changes in the hypothalamus of aging men whose NKB and KP neurons undergo hypertrophy, increase in number, exhibit increased neuropeptide mRNA expression and immunoreactivity and give rise to higher numbers of immunoreactive fibers and afferent contacts onto GnRH neurons. Increasing percentages of KP-expressing NKB perikarya, NKB axons and NKB inputs to GnRH neurons raise the intriguing possibility that a significant subset of NKB neurons begins to co-synthesize KP as aging proceeds. Although use of *post mortem* tissues is technically challenging, recently-available single-cell anatomical and molecular approaches discussed in this review article provide promising new tools to investigate the aging-related anatomical and functional plasticity of the human KP neuronal system.

Keywords: GnRH, hypothalamus, immunohistochemistry, kisspeptin, LHRH, neurokinin B, reproduction

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1. Introduction

Hypothalamic kisspeptin (KP)/kisspeptin receptor signaling is critical for mammalian puberty and fertility [1, 2]. The topography, morphology, connectivity and plasticity of human KP neurons was reviewed in this journal five years ago [3]. In the present minireview we briefly summarize the current state-of-the-art, recent developments and future perspectives of single-cell anatomical and molecular research on *post mortem* human hypothalamic tissues, with a focus on plastic changes of the KP system during reproductive aging.

2. Topography and structure of human kisspeptin neurons

The regional distribution of KP neurons in the human hypothalamus has been studied and clarified with *in situ* hybridization [4] and immunohistochemistry [3, 5]. The results of these anatomical studies agreed in that the bulk of KP cells is located in the caudal infundibular nucleus (INF). In addition, immunohistochemical mapping experiments revealed a relatively lightly-labeled second neuronal population in the rostral periventricular area of the third ventricle in female subjects [3]. Given that positive estrogen feedback might be regulated by a similarly located sexually dimorphic (more abundant in females) preoptic cell group in laboratory rodents [6], the observation of this second KP cell population in humans is conceptually interesting. Currently, positive estrogen feedback in primates is thought to take place primarily in the mediobasal hypothalamus [7, 8]. In humans the pituitary also seems to play a considerable role in the preovulatory LH surge [9, 10], whereas no solid evidence exists to support the reproductive significance of the preoptic area. The human hypothalamus also contains a third KP-immunoreactive (IR) cell population which consists of scattered periventricular neurons that can be immunostained relatively heavily [3, 5].

It is interesting to note that the rodent brain contains extrahypothalamic KP neurons as well within the medial amygdala, the bed nucleus of the stria terminalis and the lateral septum [11]. The issue of whether or not equivalent cell groups exist in the human brain will require clarification. Earlier we observed a dense

KP-IR axon plexus in the human bed nucleus of the stria terminalis. The absence of neurokinin B in these fibers [5] raises the possibility of their local origin.

The basic shape of individual KP-IR neurons has been established in our laboratory using 100-µm-thick sections [12]. The majority of human KP neurons (79.3%) are bipolar, with two primary dendrites, as also reported in mice [13]. In addition, we have observed tripolar (three primary dendrites; 14.1%) and unipolar (a single emerging dendrite; 6.6%) neuronal phenotypes which have not been reported in earlier studies of rodents[13] (**Fig. 1**).

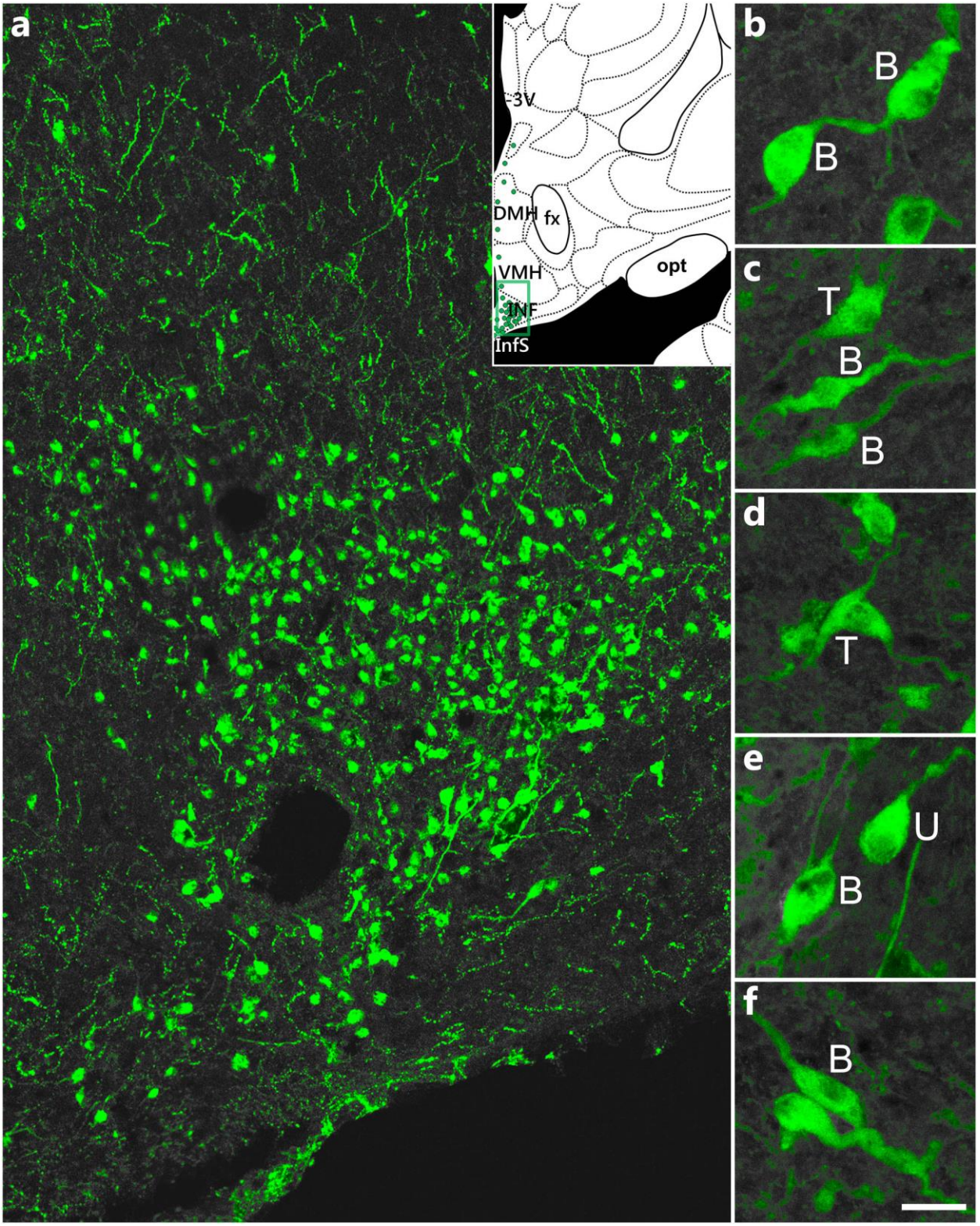

Fig.1. Distribution and morphology of kisspeptin-immunoreactive neurons in the caudal infundibular nucleus of postmenopausal women. (a) The majority of labeled neurons are present in the caudal infundibular nucleus (INF) and the infundibular stalk (InfS), as shown in a representative 30-µm-thick section of a 57-year-old woman. **(b-f)** The analysis of non-truncated neurons in 100-µm-thick sections

of a 72-year-old female reveals that KP neurons have two (B=bipolar neuron; 79.3%), three (T=tripolar neuron; 14.1%) and occasionally, only one (U=unipolar neuron; 6.6%) primary dendrite. DMH, dorsomedial nucleus of the hypothalamus; fx, fornix; opt, optic tract; VMH, ventromedial nucleus of the hypothalamus; 3V, third ventricle. Scale bars= 100 μm in **a** and 25 μm in **b-f.** Image has been reproduced with permission from [12].

3. Species-specific neurochemistry of the human kisspeptin cell

Important species differences exist between the neurochemistry of KP neurons in humans *vs.* rodents, as reviewed recently [14]. Accordingly, the colocalization between KP and neurokinin B (NKB) is only partial in humans. While independently of sex and age, the majority of human KP neurons express NKB, the colocalization in the opposite direction is more limited and depends significantly on the age and the sex of the subjects [15, 16]; the highest percentage of KP-expressing NKB neurons (84%) has been detected in postmenopausal women and the lowest (36%) in young men (**Fig. 2a**) [14, 17]. Dynorphin which is detectable in the majority of KP cells in the sheep [18] and in rodents [19] can be visualized very rarely with immunohistochemistry in human KP cells [14, 17]. It is worth to note that tissue samples from premenopausal women having higher prodynorphin expressing cell numbers in the INF than postmenopausal women [20] have not been tested yet in this context. Another technical consideration is that alternative splicing [21] and/processing of prodynorphin by human KP cells may result in protein fragments unrecognized by the dynorphin A and dynorphin B antibodies used in previous colocalization experiments [17].

Similarly to dynorphin, galanin is also present in murine [22, 23], but not in human, KP neurons. Conversely, neuropeptides showing species-specific colocalization with KP in humans, but not in laboratory rodents, include substance P [24] and cocaine- and amphetamine-regulated transcript [25] (**Fig. 2b**), as we reviewed recently [14].

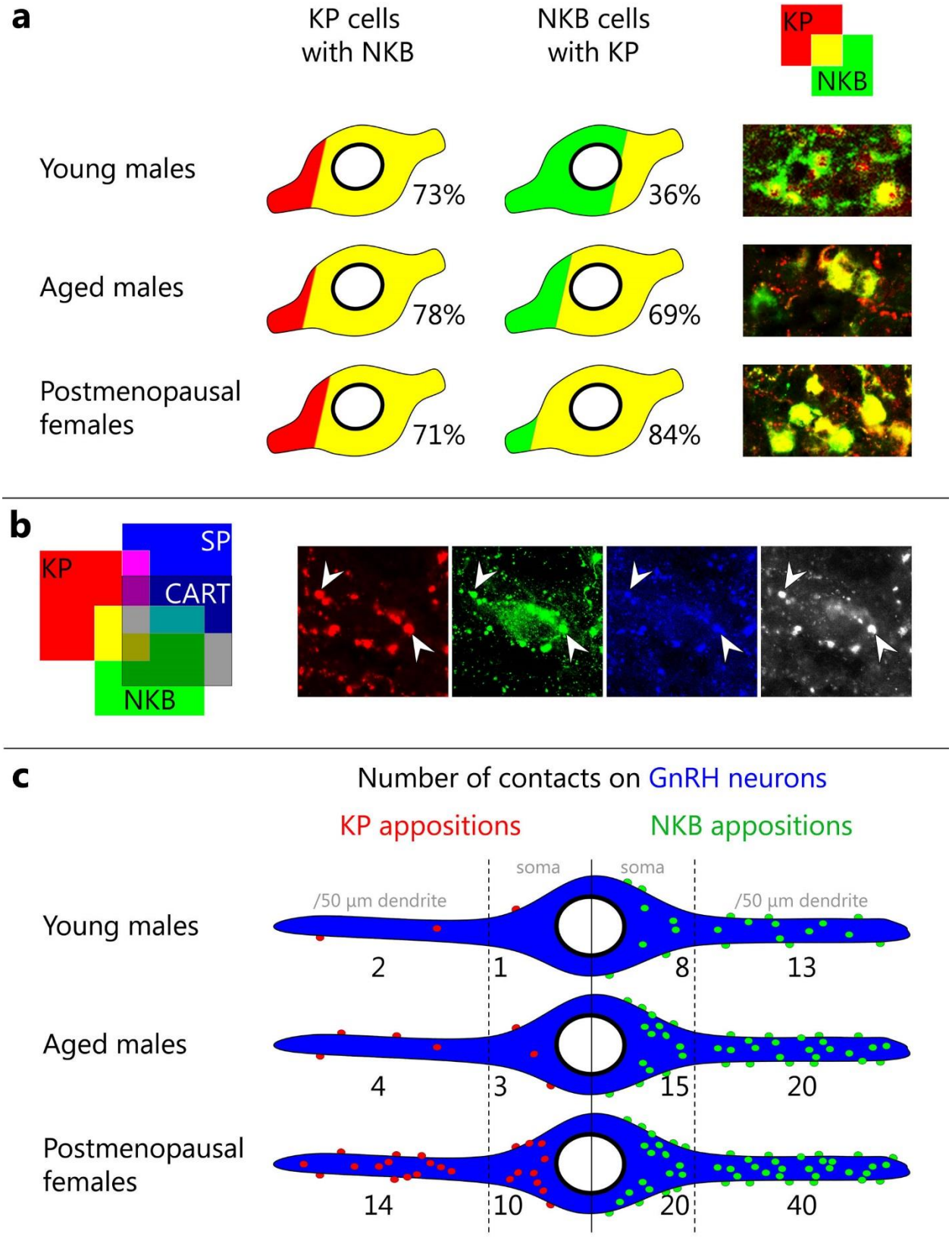

Fig.2. Age- and sex effects on the co-synthesis of kisspeptin in neurokinin B neurons and on the incidences of kisspeptin and neurokinin B afferents to gonadotropin-releasing hormone neurons. (a)

KP and NKB signals and the extent of their colocalization show robust sex- and age-dependence in the human. Independently of the sex and the age of the subject, most KP neurons express NKB, whereas colocalization in the opposite direction is limited and highly sex- and age-dependent. Accordingly, the percentage of NKB neurons that also contain KP increases from 36% in young (<50 years) to 69% in middle-aged/aged (≥50 years) adult male individuals. An even higher percentage (84%) can be found in samples form postmenopausal women. **(b)** The neuropeptide complement of human KP neurons differs considerably from that of laboratory rodents and the sheep. Unlike rodent KP neurons, human KP cells do not contain galanin and rarely seem to contain dynorphin, whereas they express substance P (SP) and cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivities. Arrowheads in immunofluorescent insets point to quadruple-IR axon varicosities co-expressing the KP, NKB, SP and CART immunofluorescent signals. **(c)** The incidences of KP-IR and NKB-IR afferent contacts onto the cell bodies and dendrites of GnRH neurons also vary with age and sex. Both axo-somatic and axo-dendritic inputs increase with age in males and the highest numbers can be oberved in postmenopausal women.
Note that similar quantitative immunohistochemical data from premenopausal women are currently unavailable. For more thorough description of the above data, see original reports [14-17, 24, 25].

4. Menopausal alterations

In 1966 Sheehan and Kovacs reported hypertrophied neurons with enlarged nuclei and nucleoli and a prominent Nissl substance in the INF of postmenopausal women and of women with post-partum hypopituitarism. They attributed these anatomical changes to ovarian failure [26]. Later, *in situ* hybridization studies from Rance and co-workers demonstrated the expression of the mRNAs encoding estrogen receptor-α [27], substance P [28], NKB [28], kisspeptin [4] and prodynorphin [20] in these hypertrophied cells. Subsequent studies from our laboratory used quantitative immunohistochemical analyses to compare sex differences between KP and NKB neurons in postmenopausal women (>55years) and middle-aged/aged (≥50years) men [15]. These studies confirmed the postmenopausal neuronal hypertrophy and showed twice as large profile areas for KP neurons in females than in age-matched males. NKB labeling was generally more abundant than KP labeling in both sexes, whereas quantifiable parameters of KP immunoreactivity differed more between the two groups. The number of KP cell bodies, the density of KP fibers, and the incidence of their contacts on GnRH neurons (**Fig. 2c**) were much higher in middle aged/aged women compared with men [15]. The immunohistochemical signal for NKB was also more abundant in females, but fold differences between the two sexes were less pronounced. The dimorphic patterns/sex differences could be attributed mostly to the lack of estrogen negative feedback in aged women, as opposed to males in which testosterone negative feedback remains functional. However, we have to note

that some sex differences may also reflect the organizational effects of a developmental sex steroid exposure. An important health consequence of the altered NKB signaling in postmenopausal women is the dysregulation of the heat dissipation center which seems to play a critical role in the pathogenesis of hot flushes [29]. In accordance with this concept, recent studies of mice have shown that the artificial activation of arcuate nucleus KP neurons evokes a heat-dissipation response which can be sensitized by ovariectomy [30]. Within this volume, Modi and Dhillo provide a review of the growing evidence supporting antagonism of the NKB receptor (NK3R) as a potential new treatment for menopausal hot flushes [31].

5. Aging of kisspeptin neurons in males.

Our laboratory carried out a series of quantitative immunohistochemical experiments in an attempt to address the putative aging-related anatomical alterations of the KP and NKB systems in the human male [16]. The samples were arbitrarily subdivided into 'young' (<50 years) and 'aged' (≥50 years) groups. We assessed and compared between these two age 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. Overall, the abundance and labeling of NKB-IR neuronal elements exceeded those of the KP-IR structures. On the other hand, aging-related changes of the KP system were more pronounced than those of the NKB system. We identified robust aging-dependent enhancements in the regional densities of KP-IR perikarya and fibers and the incidence of contacts they established onto GnRH neurons (**Fig. 2c**). The abundance of NKB-IR perikarya and fibers and the number of inputs they provided for GnRH neurons also increased with age, albeit to lower extents than did these parameters for KP. 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. In dual-immunofluorescent studies, the incidence of KP-IR NKB perikarya increased from 36% in young to 68% in aged men (**Fig. 2a**). Collectively, these immunohistochemical observations on human males suggest an aging-related robust enhancement in central KP signaling and moderate enhancement in central NKB

signaling. Overall, these alterations may be compatible with a reduced negative sex steroid feedback to KP and NKB neurons. Middle aged/aged male subjects showed a mild 22% age-dependent increase in the mean profile area of NKB neurons which was reminiscent to a previously reported mild (12%) increase in the size of unidentified neurons in the INF of the aging men [32]. This phenomenon may be analogous to the much more robust hypertrophy of KP [4] and NKB [28] cells in postmenopausal women. As reviewed recently [33], serum testosterone and free testosterone levels decline with advancing age from the third decade onward with an average rate of 1 % and 3%, respectively, per year. Low levels of circulating sex steroids in middle aged/aged men may thus serve as the endocrine background for anatomical changes of KP and NKB neurons. It can be debated that the decreased levels of male sex hormones necessarily result from normal aging. Confounders include the increasing incidences of obesity and chronic health issues [33]. The most interesting aging-related change in our studies were the increasing percentages of KP-expressing NKB perikaryal (**Fig. 2a**), NKB axons and NKB inputs to GnRH neurons. The increased colocalization rates raise the intriguing possibility that a significant subset of NKB neurons only begins to co-synthesize KP as aging proceeds. This may be due to epigenetic derepression of the KISS1 gene in these cells.

6. Recent technical advancement and perspectives

Recent technical advancements will allow us to ask previously unanswered questions about the hypothalamic neuronal network of human fertility and its changes during reproductive aging. These include:

6.1. Generation of new preprokisspeptin antibodies for immunohistochemical experiments

KP antibodies used previously to study human KP neurons [5] were directed against the receptor ligands mouse KP-10 and human KP-54 [5]. The targeted sequences included the conserved C-terminal amidated RF or RY motif which is common to members of the RF-amide peptide family, potentially causing erroneous antibody binding to cells like RF amide-related peptide neurons [34]. To eliminate this problem, new polyclonal antibodies have been designed and raised against different human preproKP peptide

fragments in ways to exclude the C-terminal RF-amide motif of the receptor ligand KP-54 (aa 68-121 of NP_002247.3). This approach makes cross-reactions with unwanted members of the RF-amide peptide family very unlikely. Two products sold recently by Antibody Verify were generated in rabbits against aa 21-80 (AAS26420C) and aa 47-106 (AAS27420C) of NP_002247.3. Results of dual-immunofluorescent experiments in our laboratory with the combined use of these and the sheep GQ2 reference KP-54 antibodies [35] confirmed that the new products only recognize KP cells in immersion-fixed human hypothalamic tissues [36]. Recently, our laboratory has also designed an antigen in which an N-terminal cysteine was added to a synthetic peptide corresponding to aa 70-93. The peptide was conjugated to keyhole limpet hemocyanin using the Sulfo-SMCC crosslinker and five mice were immunized intraperitoneally to generate antibodies in ascites fluid, as reported for other antigens [37]. Antibody production was carried out in accordance with the Council Directive of 24 November 1986 of the European Communities (86/609/EEC) and approved by the Animal Welfare Committee of the Institute of Experimental Medicine (No. PE/EA/1510-7/2018). One mouse provided excellent antibodies (BG#01) which was collected by aspiring ascites fluid 8 days after booster injections. Positive control experiments used the triple-immunofluorescent labeling of hypothalamic sections from the INF of a postmenopausal woman (**Fig. 3**). Three different primary antibodies raised in different species (sheep, mouse and rabbit, respectively) recognized essentially identical neurons and fibers. Triple-immunoreactivity of nearly all labeled structures indicates that preproKP antibodies will be applicable not only to label KP cell bodies but also to trace KP fiber projections. We occasionally observed single-labeling of a few scattered axons with the GQ2 antiserum against KP-54. This may reflect either the higher sensitivity or a negligible non-specific binding of this antiserum, although *in vitro* studies show virtually no cross-reactivity of this antiserum with several tested RF-amide peptides [35]. These specificity control experiments were carried out with permission from the Regional and Institutional Committee of Science and Research Ethics of Semmelweis University (SE-TUKEB 251/2016), in accordance with the Hungarian Law (1997 CLIV and 18/1998/XII.27. EÜM Decree/).

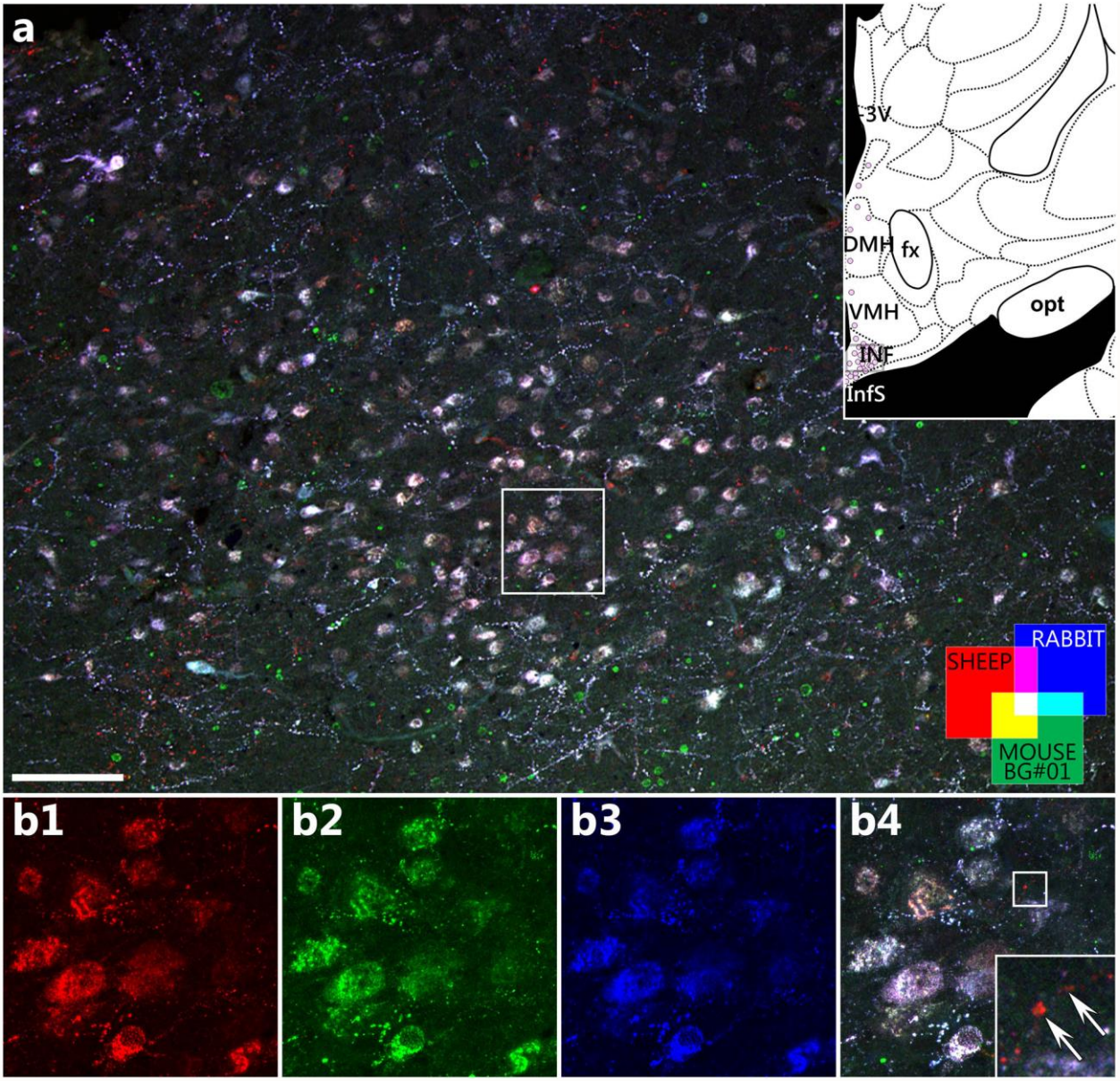

Fig.3. Results of positive control experiments to confirm specificity of immunohistochemical labeling with newly available prepokisspeptin antibodies. (a) Low-power confocal image of a triple-immunolabeled section from a postmenopausal woman illustrates that the distribution pattern of the KP signal is essentially identical using three primary antibodies from different host species. White color corresponds to triple-labeled neuronal elements in the merged red, green and blue channels. (**b1-b4**) High-power images of the framed region in **a** are shown in separate (**b1-3**) and merged (**b4**) color channels. The reference KP-54 antiserum (GQ; **b1**) has been raised in sheep against aa 68-121 of NP_002247.3 [35]. Our polyclonal mouse KP antibodies (BG#01) used in **b2** has been generated in ascites fluid after immunizing a mouse intraperitoneally with an antigen comprising aa 70-93 of NP_002247.3. The commercially available rabbit antiserum used in **b3** (AAS26420C; Antibody Verify) has been directed against aa 21-80 of NP_002247.3. The secondary antibodies from Jackson ImmunoResearch Laboratories were conjugated to Cy3, FITC and Cy5, respectively, in **b1-3**. Note that the vast majority of cell bodies and processes are triple-labeled, although very few fibers occasionally exhibit KP-54 immunoreactivity only. This extra labeling may reflect either the higher sensitivity or a negligible non-specific binding of this antiserum. The two preproKP antibodies provide excellent options to label KP neurons as well as fibers in future

immunohistochemical studies. DMH, dorsomedial nucleus of the hypothalamus; fx, fornix; INF, infundibular nucleus; InfS, infundibular stalk; opt, optic tract; VMH, ventromedial nucleus of the hypothalamus; 3V, third ventricle. Scale bar= 140 μm in **a**, 50 μm **b1-b4**, and 15 μm in **b4** inset.

6.2 Use of perfusion-fixed human brains to analyze KP neuron synaptology

In an attempt to study for the first time human KP neuron synaptology, recent studies from the Human Hypothalamus Research Unit of our laboratory (http://hhru.koki.hu/) used brain samples that were perfusion-fixed through the Willis circle 3-4 hours *post mortem* with a glutaraldehyde-containing fixative. The well-preserved ultrastructure of such samples allowed us to study the synaptic connectivity of human KP neurons with electron microscopy **(Fig. 4a, b1, b2)**. Immunoreactive axons formed axo-axonal contacts and established asymmetric axo-dendritic and axo-somatic synapses with each other. KP terminals many of which synapsed on dendritic spines, contained small-clear vesicles, in addition to dense-core granules. This finding, together with the asymmetric morphology of synapses, raised the possibility that the amino acid co-transmitter of KP neurons is glutamate. Indeed, high frequency optogenetic stimulation of KNDy neurons in rodents evokes glutamatergic signaling onto rostral periventricular KP neurons [38] and KNDy neurons express vesicular glutamate transporter-2 (VGLUT2) mRNA [39] and immunoreactivity [40]. Moreover, VGLUT2 has also been detected in axon terminals of ovine KNDy neurons [41]. Although ultrastructural features of human KP terminals highly indicates the use of glutamatergic cotransmission, we note that the direct demonstration of vesicular glutamate transporters in human KP terminals has not been successful in our recent dual-immunofluorescent experiments [12].

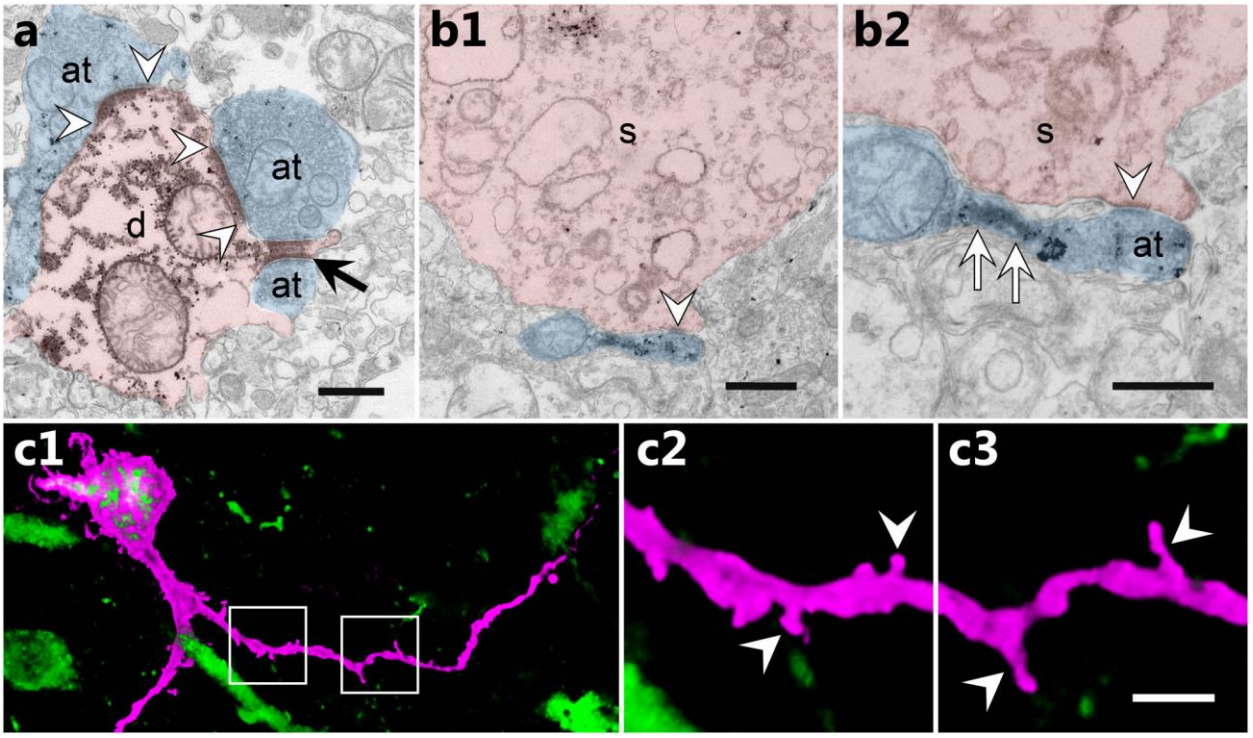

Fig.4. Fine structure of human kisspeptin neurons studied using immuno-electron microscopy and random diolistic labeling with DiI

(a, b1, b2) KP input to KP neurons has been studied with preembedding immunoelectron microscopy using perfusion-fixed brain samples from a 55-year-old male subject. KP-IR axon terminals (at; silver-gold intensified nickel-diaminobenzidine particles) form exclusively asymmetric synapses (arrowheads) on the dendrites (d; **a**) and somata (s; **b1**, **b2**) of other KP neurons. The IR terminals contain both large dense-core (∅ 80-100 nm; white arrows in **b2**) and round small clear (∅ 20-30 nm) vesicles, which, together with the asymmetric synaptic morphology, suggest the use of glutamatergic co-transmission. Black arrow in **a** points to a KP/KP synaptic contact on a spine neck. (**c1-c3)** Diolistic labeling of KP neurons with a Helios gene gun allows the visualization of the fine structure of the somato-dendritic neuronal compartment. The KP-IR (green) neuron in **c1** has been hit randomly by a tungsten bead preabsorbed with the lipophilic dye, DiI (magenta color). The uneven somato-dendritic surfaces are caused by fungiform (**c2**) and filiform (**c3**) spines shown by arrowheads in high-power images which correspond to the framed areas in **c1**. Scale bars= 500 nm in **a** and **b2** and 1 μm in **b1.** Scale bar in **c3**= 20 μm in **c1** and 5 μm in **c2** and **c3**. Images were reproduced with permission from [12].

6.3. Use of diolistic labeling with DiI to study the KP dendritic arbor and spines

A frequently encountered limitation of the immunohistochemical technique is the poor visualization of the distal dendrites and cell surface appendages. Accordingly, earlier immunohistochemical studies in our laboratory could only provide limited insight into the dendritic organization of the human KP system, leaving important fine structural details unexplored [5]. To overcome this limitation, in our recent study [12] we have random-labeled the KP cell membrane with a Helios Gene Gun using bullets loaded with

tungsten beads to which the lipophilic dye, DiI was preabsorbed. Use of light tissue fixation and mild tissue permeabilization before the immunofluorescent detection of KP were important to achieve successful random-labeling of KP neurons in 100-µm-thick vibratome slices (**Fig. 4c1-c3**). The dendritic tree of KP neurons was found to branch sparsely. The mean length of non-truncated dendrites was 290 µm. The labeled axons emerged from the proximal dendrite or the cell body. The DiI labeling also visualized a large number of multiform spines on the KP somata and dendrites; these appendages remained entire invisible using immunohistochemistry only. *Post mortem* labeling with DiI of KP neurons from different reproductive statuses is a promising approach for studying the aging related morphological plasticity of the human KP system. In recent experiments on mice, KNDy neurons filled *in vitro* with biocytin exhibited an interesting steroid-dependent structural plasticity in that they responded to estradiol treatment with reduced cell size and dendritic spine density [13]. Assuming a similar regulation in the human, we predict higher spine densities on KP neurons of postmenopausal *vs.* premenopausal women. DiI labeling will also offer an excellent approach to study pubertal changes of the dendritic tree via the comparison of prepubertal to adult samples.

6.4. Use of short *post-mortem* time tissues for *in situ* hybridization experiments

Several laboratories including our own (http://hhru.koki.hu/) have access to human tissues in which appropriate RNA preservation allows *in situ* hybridization experiments. Important early publications with a focus on estrogen-responsive neurons of the INF used isotopically labeled oligoDNA probes on *post mortem* tissues which provided sufficient sensitivity to detect the mRNAs encoding estrogen receptor-α [27], substance P [28], NKB [28], KP [4] and prodynorphin [20]. A recent technical advancement was the development of the revolutionary RNAScope *in situ* hybridization technology. This technique may provide extremely high specificity and sensitivity for future multiple-labeling *in situ* hybridization experiments. It is worth to note that the use of fluorescent signal detection to study human KP cells may be challenging due to the high tissue autofluorescence caused by spotty lipofuscin deposits especially in samples from aged subjects. Prior to immunofluorescent experiments, we routinely quench this autofluorescence using tissue

delipidation with acetone, followed by a 0.3% Sudan black treatment of the sections in 70% ethanol for 30 min. For detailed protocol, see [42]. In *in situ* hybridization experiments, quenching of lipofuscin autofluorescence with Sudan Black is better placed after the fluorescent signal detection steps, keeping also in mind that fluorochromes have to be chosen to withstand use the above organics. In recent years, several alternatives to Sudan Black became available commercially, including TrueBlack from Biotium.

6.5. Newly available techniques for single-cell transcriptomics

Modern single-cell microarray and RNA-Sequencing techniques [43] with high-throughput approaches enable the interrogation of RNA sequences on a large scale. The majority of single-cell techniques like Drop-Sequencing [44] start with living tissues and cells with well-preserved RNA which could not be accessed easily from the human hypothalamus. Second, using dissection material, *post mortem* delay before optimal tissue processing may already compromise cellular RNA integrity and freshly-dissected surgical samples are not readily available from this deep brain site. In mice, transgenic expression of cell type-specific fluorescent markers can be achieved and used to collect cell type-specific RNA following the isolation of the labeled cell population with FACS, LCM or a patch pipette. An additional technical challenge in human tissues will be to preserve RNA integrity while introducing cell-type specific labels to KP neurons. Because of these technical difficulties, RNA-Seq methods could not so far be carried out on human KP neurons. Laboratories are currently working on the development of pulse-immunolabeling approaches which can preserve RNA integrity while visualizing individual neurons in unfixed or only lightly fixed *post mortem* brains. Once this task is achieved, laser capture microdissection (LCM) can be used to dissect and pool individual KP-IR neurons for subsequent analysis on the Illumina platform. Promising alternative approaches compatible with the use of frozen *post mortem* brain tissues include the recently developed DroNc-seq technology, a high-throughput single nucleus RNA-seq method [45].

7. Unresolved tasks

7.1. Single-cell transcriptomics of KP cells

As mentioned above, multiple technical requirements will need to be met to study the transcriptome profile of human KP cells. The immunohistochemical identification of KP neurons appears to require at least a short fixation with formalin. In itself, this fixation step can somewhat compromise RNA integrity and quality. Then, the technical parameters of immunohistochemical pulse-labeling have to be optimized. Brief use of RNase-free antibody and buffer solutions containing RNase inhibitors will be key to maintain RNA integrity during the immunohistochemical visualization of KP cells. Laser capture microdissection (LCM) can be used to collect RNA from the immunolabeled cells, followed by RNA-Seq. The identification of steroid and neuropeptide receptors in these neurons and aging-related changes in the transcriptome profile of the KP cell will be particularly interesting.

7.2. Identification of new hypothalamic and extrahypothalamic target cells to KP neurons

From the putative target cells of human KP projections, only GnRH [5] and KP [12] neurons have been studied and identified so far. As KP fibers are quite widespread especially in the medial hypothalamus [5], many additional target neurons are likely to exist. Several KP target neurons have already been identified in rodents. These include POMC [46], AgRP [46] and oxytocin cells [47]. The issue of whether or not these cells are also innervated by KP fibers in the human, as well as the location and neurochemistry of additional KP target neurons, will require clarification. It will also remain an interesting challenge to visualize the thermoregulatory pathway proposed to account for hot flushes in postmenopausal women [29].

7.3. Characterization of the afferent connectivity of KP neurons

In a recent study we demonstrated glutamatergic and GABAergic inputs to human KP neurons [12]. The phenotypes and sources of other specific inputs require immunohistochemical clarification. A particularly interesting task will be to identify the putative pathways that mediate metabolic effects to the reproductive axis.

7.4. Characterization of the sexually dimorphic KP cell population in the rostral periventricular area

We now possess multiple optional preproKP antibodies including our own (**Fig. 3**), to study the neurochemical characteristics, projections, target cells and the age- and hormone-dependent plasticity of the sexually dimorphic KP cell population observed originally in the rostral periventricular area of young human females [5]. Sexual dimorphism of the equivalent rodent cell group develops in response to the perinatal sex steroid exposure of males [48]. This makes it very likely that early-life organizational events also contribute to sex differences of this cell group in humans.

7.5. Determining the sex steroid-, puberty and age-dependent anatomical and molecular plasticity of KP neurons

Quantitation of immunohistochemical labeling patterns [15, 16] and currently unavailable routine approaches of single-cell transcriptomics will need to determine how sex steroids, puberty and age influence the transcriptome and proteome profiles of human KP neurons.

8. Conclusion

Although critical information has accumulated in recent years from animal experiments, laboratory rodents sometimes have limited translational value as models for the hypothalamic regulation of the human reproductive cycle and fertility. Therefore, studies of the *post mortem* human hypothalamus will remain indispensable in the future. Development and use of high resolution and high throughput molecular and anatomical techniques on human tissues will be critically important to clarify the basic mechanisms of GnRH/LH pulsatility, sex steroid feedback, puberty and reproductive aging.

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