Neuroanatomy of the human hypothalamic kisspeptin system

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Abstract

Hypothalamic kisspeptin (KP) neurons are key players of the neuronal network that regulates the onset of puberty and the pulsatile secretion of gonadotropin-releasing hormone (GnRH). In various mammalian species, the majority of kisspeptin synthesizing neurons are concentrated into two distinct cell populations in the preoptic region and the arcuate nucleus (ARC). While studies of female rodents provide evidence that preoptic KP neurons play a critical sex-specific role in positive estrogen feedback, KP neurons of the ARC have been implicated in negative sex steroid feedback and also hypothesized to contribute to the pulse generator network which regulates episodic GnRH secretion in both females and males. Except for relatively few morphological studies available from monkeys and humans, our neuroanatomical knowledge in the hypothalamic KP systems is dominantly based on observations on laboratory species which are phylogenetically distant from the human. This review article discusses the currently available literature about the topographic distribution, network connectivity, neurochemistry, sexual dimorphism and aging-dependent morphological plasticity of the human hypothalamic kisspeptin neuronal system.

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1. Introduction

Members of the kisspeptin (KP) neuropeptide family encoded by the *KISS1* gene are potent stimulators of luteinizing hormone (LH) secretion in various mammalian species, including rodents [1], sheep [2], monkeys [3] and humans [4]. The hypothalamic KP neuronal system is critically involved in the central regulation of puberty and reproduction. KP acts mainly via stimulating gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus. Accordingly, the KP-induced release of LH can be prevented by GnRH antagonists in mice [1] and monkeys [5]. The actions of KP on GnRH neurons are mostly direct. GnRH neurons receive KP-immunoreactive (IR) afferent inputs [6-10], express the KP receptor (*Kiss1r*) transcripts [2, 11, 12] and respond with cFos expression [11, 13] and depolarization [12, 14, 15] to KP.

Inactivating mutations of the *KISS1* [16] or the *KISS1R* [17, 18] genes produce hypogonadotropic hypogonadism in humans and similar reproductive deficits also characterize the *Kiss1*- [19, 20] or the *Kiss1r* [18, 21] knockout mice. While resembling fertility problems observed in mutants of the two species suggest that the reproductive significance of KP/KISS1R signaling is conserved in different mammals, potentially significant species differences have remained mostly unexplored in the absence of sufficient neuroanatomical information from the human. About 150 KP review articles have been published over the past 8 years to address various aspects of KP/KISS1R signaling. The aim of the present article is to provide an overview of the currently available anatomical literature on the human hypothalamic KP system. The topographic distribution, network connectivity, neurochemistry, sexual dimorphism and aging-dependent morphological plasticity of human hypothalamic KP neurons are discussed in the light of anatomical and functional information mostly available from animal experiments.

2. Major groups of human hypothalamic kisspeptin neurons

KP synthesizing neurons in various mammalian species have been localized to two major anatomical sites, the preoptic area and the arcuate nucleus (ARC) [22]. Both cell populations have also been

identified in the human hypothalamus [10]. The distribution of human hypothalamic KP neurons is illustrated schematically in **Figure 1**.

2.1. Kisspeptin neurons in the rostral periventricular area of the third ventricle

In several species, a major KP cell population has been identified in the preoptic region [22]. In laboratory rodents, the somata of these neurons form a compact cell mass in the anteroventral periventricular nucleus (AVPV) and the preoptic periventricular nucleus [1, 7, 23], defined together as the rostral periventricular area of the third ventricle (RP3V) [24]. Importantly, this KP cell group comprises many more neurons in females than in males; this conspicuous sexual dimorphism (see also section 4.1) develops in response to the organizational effects of neonatal testosterone exposure in males [7, 25, 26]. A KP synthesizing cell population is also present in the preoptic region of the sheep, although preoptic KP neurons in this species appear to be more scattered and less numerous [27, 28] than in rodents. The KP cell group of the ovine preoptic area also exhibits higher cells numbers in females compared with males; this sexual dimorphism develops prenatally in response to testosterone exposure of the male [29]. Some neurochemical properties of preoptic (RP3V) KP neurons have already been investigated and revealed in rodents. *In situ* hybridization and immunohistochemical studies identified galanin mRNA and immunoreactivity, respectively, in varying subsets of RP3V KP neurons in mice [30, 31]. In addition, subpopulations of the RP3V, but not of the ARC, KP neurons exhibited immunoreactivities to met-enkephalin [31] and to the dopaminergic marker tyrosine hydroxylase [32]; in this species KP/tyrosine hydroxylase neurons were proposed to represent the major source of dopamine in the afferent regulation of GnRH neurons [32]. *In situ* hybridization studies on mice also identified GABA-ergic and glutamatergic marker mRNAs in subsets of the RP3V KP neurons [33], indicating that these cells also use classic amino acid neurotransmitters for synaptic communication.

The first systematic study to localize KP expressing neurons in *post-mortem* human hypothalami used *in situ* hybridization with radiolabeled cDNA oligonucleotide probes on sagittal sections [34]. In addition to visualizing the bulk of KP neurons in the hypothalamic infundibular (arcuate) nucleus (Inf), this study

only identified rare, sparsely labeled neurons scattered within the hypothalamic sections including the medial preoptic area; notably, these preoptic neurons were not grouped in discrete foci in a distribution reminiscent to the AVPV (or RP3V) of the rodent [34]. Similarly, the immunohistochemical mapping of KP neurons in neonatally gonadectomized male monkeys only identified KP-IR neurons in the posterior two-thirds of the ARC but not in the preoptic area [8]. In contrast with the results of the above two studies, the *in situ* hybridization analysis of *KISS1* mRNA in cycling female monkeys detected quite significant numbers of KP neurons in the preoptic area [35]. Preoptic KP neurons of the monkey formed a compact cell group and exhibited the highest levels of expression in the late follicular phase [35], suggesting the positive estrogenic regulation of their *KISS1* mRNA expression which also characterizes KP neurons in the rodent RP3V [23, 25, 26]. The different results of this [35] and the previous two [8, 34] studies may have technical explanations. The choice of the sagittal human tissue sections [34] and the use of the neonatally gonadectomized male monkey model [8] could be suboptimal for visualizing preoptic KP neurons. To map the human hypothalamic KP system in our laboratory, we performed immunohistochemical studies on free-floating sections that were prepared from immersion-fixed *post-mortem* human hypothalamic tissue blocks [10]. Two different KP antisera were used in these studies. The first one (#566; gift from Dr. A. Caraty; Nouzilly, France) was directed against peptide YNWNSFGLRY-NH2 which is common to all forms of mouse kisspeptin [27] and 90% identical to the corresponding human sequence (YNWNSFGLRF). Although the single amino acid substitution at the C-terminal KP sequence of the human results in a relatively low cross-reactivity (1%) of the #566 rabbit antiserum with the human kisspeptin-10 peptide in radioimmunoassay [27], this antibody was still suitable for the immunohistochemical detection of human KP with the highly sensitive ABC technique and silver-gold-intensified nickel-diaminobenzidine chromogen [10]. A second polyclonal antiserum (GQ2; gift from Dr. S.R. Bloom; London, UK) we used was raised in sheep specifically against the full-length KP-54 sequence of the human. This antiserum reacts with human KP-54, KP-14 and KP-10 and shows virtually no cross-reactivity (<0.01%) with other related human RF amide peptides, including prolactin releasing

peptide, neuropeptide FF, neuropeptide AF and RF amide-related peptides (RFRP1, RFRP2, RFRP 3) [4]. In immunohistochemical assays, both KP antibodies visualized a group of relatively lightly labeled neurons in the rostral periventricular area, overlapping with the ventral periventricular nucleus, the anterior parvicellular paraventricular nucleus and the parvicellular and magnocellular subdivisions of the paraventricular nucleus, according to the anatomical atlas of Mai et al. [36] **(Figures 1A, B)**. This relatively compact cell group showed sexual dimorphism and was most obvious in tissue samples obtained from young women [10]. Information regarding the presence of tyrosine hydroxylase, enkephalins, galanin or amino acid neurotransmitters in the rostral periventricular KP neurons of the human is currently unavailable. Moreover, an AVPV/RP3V-like anatomical entity and other sexually dimorphic systems at a similar location of the primate hypothalamus have not been reported yet.

From a functional point-of-view, there is a strong case that in rodents, the KP cell population of the RP3V is critically involved in positive estrogen feedback to GnRH neurons [24]. The higher number of KP neurons in the female *vs.* the male rodent RP3V [7, 25, 26] correlates with the ability of female, but not male, rodents to respond to the positive feedback action of estradiol with a GnRH/LH surge (see also section 4.1). Preoptic KP neurons are activated before the preovulatory GnRH/LH surge not only in rodents [26, 37-39] but also in the sheep [40, 41].

The presence of a sexually dimorphic KP cell population in the rostral periventricular area of the human [10] and monkey [35] hypothalami raises a challenge to the prevailing view that the positive estrogen feedback in primates takes place exclusively in the infundibular region [42]. Spontaneous menstrual cyclicity and LH/FSH responses to estrogen in non-human primates remain well preserved after mediobasal hypothalamic deafferentation [43, 44] and estradiol can elicit gonadotropin surges after acute complete removal of the neural tissue dorsal and anterior to the optic chiasm [45]. Although the above data seem to suggest that the preoptic/anterior hypothalamic region is not essential for the GnRH/LH surge, multiple feedback centers and some redundancy in the mechanism of the preovulatory GnRH/LH surge remain possible, with important modulatory roles of an anterior preoptic KP cell population.

Notably, Cogen and colleagues reported that monkeys with bilateral anterior hypothalamic disconnection ceased to have cyclic gonadotropin release and ovulation after surgery, and these animals also failed to release FSH and LH in response to estrogen [46]. However, 4-7 months after surgery, the animals showed spontaneous resumption of cyclic gonadotropin release in response to endogenous or exogenous estrogen [46]. These data make it likely that although the cycles can be maintained by an anatomically isolated medial basal hypothalamic-hypophyseal unit, the preoptic/anterior hypothalamic region plays important modulatory roles in normal menstrual cyclicity. The preoptic region also contains a considerable population of hypophysiotropic GnRH neurons in the monkey [47], indicating further that the reproductive significance of this anatomical site should not be overlooked in primates. Future studies of cFos expression in the rostral preoptic KP neurons of monkeys will be critically important to clarify whether these neurons are activated at the time of the positive estrogen feedback and the mid-cycle GnRH/LH surge.

2.2. Kisspeptin neurons in the infundibular area

In a variety of mammalian species including non-human primates [8, 35], the largest KP cell population has been localized to the mediobasal hypothalamus. Unlike the preoptic KP cell population, KP neurons in the ARC co-synthesize the tachykinin peptide neurokinin B (NKB) in the sheep [28, 29], the goat [48], the mouse [49] and the monkey [50]. NKB plays a crucial role in reproduction and inactivating mutations of the genes encoding for NKB (*TAC3*) and the NKB receptor NK3 (*TACR3*) cause hypogonadotropic hypogonadism in the human [51, 52]. The *Tacr3* knockout mice are also subfertile [53], suggesting that NKB/NK3 signaling also plays important roles in the reproduction of this species. The recently introduced ‘KNDy neuron’ terminology [54] to refer to the KP cell population of the ARC is based on the synthesis of the opioid peptide dynorphin by the majority of KP/NKB cells, at least in the sheep [28, 29, 55], the goat [48], the mouse [49] and the rat [56, 57]. In the sheep, dynorphin neurons of the ARC are critically involved in progesterone negative feedback to GnRH neurons. The majority of these cells contain progesterone receptor [58] and progesterone treatment increases preprodynorphin

mRNA expression in the ARC and dynorphin levels in the cerebrospinal fluid [59]. Endogenous opioid peptides exert inhibitory effect on the episodic secretion of LH in this species [60]. In mice, varying subsets of KNDy neurons, similarly to RP3V KP cells, contain galanin mRNA and immunoreactivity [30, 31] and also express glutamatergic [33, 56] and GABAergic [33] phenotype markers.

In humans, the largest KP cell population has been detected in the Inf (analogous to the ARC) both with *in situ* hybridization [34] and with immunohistochemistry [10, 61] (**Figures 1D, E, 2A**). The majority of these KP neurons appear to be multipolar, although dendritic labeling is often insufficient to safely assess cell morphology (**Figure 2B**). KP-IR cell bodies in the Inf, which often intermingle with scattered GnRH neurons (**Figure 2B**), form a continuum with labeled KP perikarya in the infundibular stalk (InfS) (**Figure 1E, 2A**).

Previous colocalization experiments in our laboratory addressed the presence of NKB [10, 61, 62] and dynorphin [63] immunoreactivities in KP neurons of the human Inf. These immunohistochemical studies revealed that the majority of KP and NKB neurons in the Inf of postmenopausal women express both neuropeptides [10]. In recent studies of a large cohort of postmenopausal women ($\geq$55 years; N=19), we have found that 71.3±5.9% of KP-IR somata contain NKB immunoreactivity and 83.7±3.7% of NKB-IR somata contain KP immunoreactivity (**Figure 4**). These specimens were processed in parallel with samples from male individuals [61], allowing quantitative comparisons with the young male (<50 years) and aged male ($\geq$50 years) human models. Combined results of these dual-immunofluorescent experiments indicate that the extent of KP colocalization in NKB neurons of young men [61, 63] is much lower (35.8±5.1%) than observed in postmenopausal women (83.7±3.7%), whereas in aged male individuals (>50 years) it increases to a similarly high percentage (68.1±6.8%) [61]. On the other hand, the percentages of NKB-immunopositive KP perikarya in the Inf are similarly high in postmenopausal women (71.3±5.9%), young men (72.7±6.0%) [61] and aged men (77.9±5.9%) [61]. Colocalization results from the three available models are combined in **Figure 4**. Unfortunately, similar coexpression data are currently unavailable from premenopausal women. In addition to identifying many single-labeled

perikarya in the human Inf (in particular, NKB neurons without KP labeling in the young male model), in previous immunofluorescent studies [10, 61-64] we also observed a remarkable segregation of KP and NKB immunoreactivities in neuronal fibers and identified many single-labeled KP and NKB axons in and around the Inf. It is interesting to note that the majority of KP-IR and NKB-IR axons forming contacts with GnRH neurons of the Inf were also single-labeled [61, 62], although sex-specific subsets (~ 8-10% in young and aged males and ~ 25-30% in postmenopausal females) co-contained KP and NKB signals [61, 62]. The differential coexpression of KP and NKB immunoreactivities in the distinct human models may have important functional implications which will require clarification.

We have also carried out colocalization studies in an attempt to detect dynorphin A and dynorphin B immunoreactivities in KP (putative 'KNDy') neurons of the Inf [63]. These experiments revealed unexpectedly low levels (if any) of dynorphin signal in neuronal cell bodies of the Inf from young human male subjects [63]. Dynorphin signal was absent from most KP neurons and fibers, in contrast with the extensive coexpression reported previously in rodents [49, 56, 57, 65], sheep [28, 29, 55] or goat [48]. Given that opioid peptides play important roles in the negative regulation of pulsatile prolactin and LH release in humans [66, 67] and similarly to the ARC of laboratory animals, the human Inf [68] and the monkey ARC [69] also express preprodynorphin mRNA, the absence of dynorphin immunoreactivity in the majority of KP-IR neurons of the human Inf (at least in young men) was somewhat unexpected [63]. It will require clarification if the negative colocalization data represent an important species difference of the human from laboratories species or caused by *post-mortem* degradation of dynorphin in KP-IR neuronal elements.

Information regarding the putative expression of galanin and glutamatergic/GABAergic markers in KP neurons of the human Inf is currently unavailable.

In previous *in situ* hybridization studies by Rance and Young [70], NKB neurons in the Inf showed a similar distribution pattern as did Substance P (SP) neurons. This observation raised the possibility that the two tachykinin peptides derived from different genes might be co-expressed in a subset of KP

neurons. Indeed, results of our recent triple-immunofluorescent studies indicate that 25.1% of NKB-IR and 30.6% of KP-IR perikarya contain SP in the Inf of postmenopausal women; furthermore, 16.5% of all immunolabeled cell bodies are triple-labeled (KP/NKB/SP-positive) in this human model [64]. The quantitative analysis of SP cell numbers in the Inf of postmenopausal women also revealed significantly more SP-immunoreactive neurons in the Inf of postmenopausal women than in either age-matched or young men [64].

From a functional point-of-view, KP (KNDy) neurons of the ARC/Inf in different species have been strongly implicated in negative sex steroid feedback to GnRH neurons [28, 71, 72]. Accordingly, the selective ablation of these cells in rats with the locally injected neurotoxin NK3-saporin prevented the rise in serum LH and attenuated the rise in serum follicle stimulating hormone (FSH) following ovariectomy [73]. It is worthy of note that the suppressive effects of estradiol on gonadotropin secretion were not entirely blocked in this lesioned animals, indicating some redundancy in the neuronal pathways that mediate estrogen negative feedback [73]. The hypothalamic Inf of humans has also been known for a long time to represent an important site of sex steroid negative feedback to the reproductive axis. *In situ* hybridization studies revealed a robust postmenopausal hypertrophy of neurons that express estrogen receptor alpha mRNA at this site [74] and later *in situ* hybridization experiments determined that neuronal hypertrophy in the absence of estrogens occurs selectively in SP [70], NKB [70], KP [34] and dynorphin [68] neurons (see also section 4.2).

In some species, KP neurons of the ARC may also play a role in positive estrogen feedback to GnRH neurons. In sheep, estradiol treatment to induce a GnRH/LH surge results in cFos expression in ARC KP neurons [41]. In monkeys, menstrual cyclicity is preserved after deafferentation of the mediobasal hypothalamus [43, 44].

As discussed further in section 3.1, KP (KNDy) neurons of the ARC establish frequent contacts among one another [28, 55, 57]; this intranuclear communication was proposed to play a critical role in the regulation of GnRH/LH pulses [29, 48, 49, 54, 65].

2.3. Additional kisspeptin neurons

In addition to the two major KP cell populations, relatively darkly stained KP neurons are scattered throughout the rostro-caudal extent of the human periventricular nucleus [10]. The neurochemical characterization of these neurons will help to determine if they are functionally analogous with KP neurons of the rostral periventricular region or rather, the Inf. KP neurons at similar periventricular locations have not been reported in rodents [22].

The small population of KP mRNA-expressing cells identified with *in situ* hybridization in the bed nucleus of the stria terminalis of monkeys [35] has not been revealed yet in the human [10, 34], although a relatively dense KP-IR fiber network occurs at this site [10]. KP-IR fibers in the human bed nucleus of the stria terminalis are devoid of NKB immunoreactivity, indicating their origin outside the Inf [10]. The possibility of KP expression in other extrahypothalamic areas of the human has not been addressed using morphological tools. Anatomical studies will thus need to confirm the presence of KP neurons in the caudate nucleus, globus pallidus, nucleus accumbens, putamen, and striatum, sites where the *KISS1* transcript has been detected with RT-PCR [75].

3. Connections of kisspeptin neurons

The major anatomical projections of rodent KP neurons have been mapped using lesioning [76] and classical neuroanatomical tract tracing studies [76, 77] as well as with the application of site-specific topographic markers [30, 32, 78] colocalized with the two distinct subsets of KP neurons and their projections. Similar neuroanatomical information from the human is less complete and restricted to the NKB-containing fiber projections that arise from the Inf [10].

3.1. Intranuclear network connectivity of kisspeptin neurons in the infundibular region

ARC KNDy neurons provide abundant axo-somatic and axo-dendritic inputs to one another [28, 55, 57]. It occurs that this intranuclear communication primarily uses excitatory neurotransmission by NKB via NK3 autoreceptors and inhibitory dynorphin signaling through κ-opioid autoreceptors. Accordingly, NK3 immunoreactivity [49, 57, 79, 80] as well as *Tac2* and κ-opioid receptor mRNA expression [49, 81]

have been revealed in mouse KNDy neurons. These cells respond with cFos expression [82] and depolarization [82] to the NK3 agonist senktide. NKB increases [65, 81, 83], whereas dynorphin or a selective kappa-opioid receptor agonist decreases [81, 83] the activity of mouse KNDy neurons. While KP does not seem to influence the electric activity of KNDy neurons [83], it is the likely protagonist in the communication between KNDy cells and GnRH neurons, which, indeed, express Kissr1 [2, 11, 12]. The pulsatile KP output and GnRH secretory pulses are temporally correlated in the median eminence of the female rhesus monkey [84].

Information on the major neuropeptides and receptors in the above communication network was incorporated into new models of the GnRH/LH pulse generator [29, 48, 49, 54, 65]. Evidence from ovariectomized goats indicates that central NKB increases whereas dynorphin A decreases the frequencies of multiunit activity volleys and LH secretory pulses [48]. The pulse generator model is very likely to change substantially in the future. For example the role of some players including dynorphin [63] might not be universal in all species, whereas others can have more complex actions than thought initially. KP can also act in the ARC to modulate LH pulse frequency, in addition to providing the output signal of KNDy neurons toward the GnRH neuronal system. Accordingly, administration of a KP antagonist into the ARC could suppress LH pulse frequency [85]. In addition, in male humans chronic KP infusion could stimulate LH pulsatility [86] and a single injection of KP could reset the hypothalamic GnRH clock [87]. The role of new neurotransmitters/neuromodulators and receptors influencing and/or fine-tuning the GnRH/LH pulse generator may also emerge in the future, including SP that has been colocalized in human KP and NKB neurons [64]. Recent evidence from male mice indicates that multiple tachykinin receptors (NK1-3) account together for the excitatory effects of NKB on ARC KP neurons [83]. Interestingly, while the NK3 agonist senktide did not elicit a discernible electrophysiological response from GnRH neurons in earlier studies [65], recent evidence indicates that it can elicit GnRH release from the median eminence via a KP-independent mechanism [88].

The presence of the classic amino acid neurotransmitters GABA and glutamate in KP neurons [33] increases further the complexity of signaling mechanisms in ARC KP neurons.

In our studies of human hypothalami, we also found numerous axo-somatic and axo-dendritic appositions among NKB neurons of the Inf [63] which are partly identical with KP neurons [10]. High-power light microscopic images reveal that KP-immunoreactive neurons form a compact cell group in the Inf (especially in aged human individuals) and establish frequent contacts with one another (**Figures 2B, 3A**).

3.2. Axo-somatic and axo-dendritic efferent connections to GnRH neurons

Previous studies analyzing the efferent targets of KP cells focused on GnRH neurons in view of convincing evidence that the KP-induced release of LH can be prevented by GnRH antagonists in mice [1] and monkeys [5]. KP-IR neuronal contacts onto GnRH cell bodies and dendrites exist in all species examined so far [6-9, 35], although several authors noted the surprising paucity and restricted occurrence of these contacts on a subpopulation of GnRH neurons [7, 8]. While immunohistochemical data are still unavailable to visualize the putative distribution of the KISS1R protein on the somatic and dendritic compartments of GnRH neurons, the findings that KP induces cFos expression [11, 13] and depolarization [12, 14, 15] in GnRH neurons provide functional support to the concept that KP can excite GnRH neurons via these axo-somatic and axo-dendritic inputs. The major source of the KP input to GnRH neurons of the rodent preoptic area appears to be the RP3V, in view that these KP inputs rarely contain the ARC-specific neuropeptide marker NKB [30].

Light microscopic immunohistochemical studies from our laboratory established that axo-somatic (**Figure 3A**) and axo-dendritic (**Figure 3B**) appositions also occur on human GnRH neurons [10]. The quantitative analysis of this innervation was carried out in the Inf which contains relatively high numbers of GnRH neurons in the human. Comparison of the innervation patterns between aged male and female individuals provided evidence for a robust sexual dimorphism in the incidence of these KP-IR axo-somatic and axo-dendritic contacts, being several times higher in postmenopausal women compared with

age-matched men [62]. For further sexually dimorphic features of the human KP and NKB systems, see section 4.1. Comparison of hypothalamic tissue samples from men below and above 50 years of age also revealed a significant aging-related enhancement in the density of this innervation [61] (See also section 4.2). Unlike in ovariectomized and estrogen treated mice where only 5.6% of the KP-IR appositions to GnRH neurons contained NKB as an index of their ARC origin [30], about 26% and 10% of KP-IR afferent contacts on GnRH neurons in postmenopausal women and age-matched men, respectively, contained also NKB. Together with the frequent occurrence of single-labeled KP-IR and NKB-IR axons in the Inf which indicates a considerable degree of segregation of the two neuropeptides in the human [10], the Inf is likely a major source of the KP-IR input to human GnRH neurons. Topographic markers that would help identify putative KP projections to GnRH neurons from the human rostral periventricular region need to be identified.

3.3. Axo-axonal connections between kisspeptin and GnRH neurons

In addition to influencing the somatic and dendritic compartments of GnRH neurons, there is accumulating evidence from different species that KP also regulates GnRH secretion via acting in the median eminence where GnRH axon terminals are apposed to KP-IR [8, 10, 89] fibers. A large subset of the participating KP fibers arises from the ARC KP neuron population; these fibers are partly identical with NKB-IR fibers of ARC origin [76, 78] that are immediately apposed to GnRH-IR axons [56, 79]. Such direct axo-axonal contacts lack classical synaptic specializations at the ultrastructural level in goats [89] and rats [56]. While immunohistochemical evidence is still missing to indicate KISS1R expression on GnRH axons, NK3 receptors have already been detected on hypophysiotropic GnRH axons of the rat [79]. These receptors may account for the KP-independent induction of GnRH release from the mouse median eminence by senktide [88].

Dual-label immunohistochemical studies of the human hypothalami established that KP-IR axons in the mediobasal hypothalamus form sporadic appositions to the hypophysiotropic GnRH-IR fibers in the InfS (**Figures 3C, D**) and around the portal capillary vessels of the postinfundibular eminence [10].

Unlike in rats where most GnRH axons entering the median eminence terminate in the external zone, many GnRH axons in the human and the monkey travel large distances in the InfS and descend all the way down to the neurohypophysis [90]; GnRH fibers in this descending GnRH fiber tract are also accompanied and occasionally contacted by KP-IR axons.

There is abundant functional evidence from different species that KP has an important site of action on the axonal compartment of GnRH neurons. First, GnRH release from the mediobasal hypothalamic explants of mice (which contain the hypophysiotropic GnRH axons but only few, if any, GnRH cell bodies) can be stimulated by KP in a Kiss1r-dependent and action potential-independent manner [91] and KP can similarly stimulate GnRH release from cultured ovine ME explants [92]. Furthermore, systemic KP injection induces *in vivo* LH secretion in a variety of species [5, 13, 93] including humans [4, 94], in accordance with putative site/s of KP action outside the blood-brain barrier. It has to be recognized that GnRH neurons send fiber projections to multiple circumventricular organs that can be reached by KP from the systemic blood. Such brain sites include the organum vasculosum of the lamina terminalis. It has been shown recently that mouse GnRH neurons in the immediate vicinity of the organum vasculosum of the lamina terminalis have a highly branched dendritic tree which is accessible to molecules circulating in the systemic blood [95]; KP puffed onto these dendrites could excite GnRH neurons [95]. Of note, the relevance of this site and mechanism of action of KP in the human is uncertain, considering that human GnRH neurons are widely distributed in the hypothalamus and most of them do not seem to send projections to the lamina terminalis [90].

In different species the GnRH/LH pulse generator is thought to be located in the mediobasal hypothalamus. Accordingly, mediobasal hypothalamic explants from fetal and adult human brains release GnRH in a pulsatile manner [96]. Similarly, GnRH is released episodically from mediobasal hypothalamic explants of the rat which are devoid of GnRH cell bodies and only contain the hypophysiotropic GnRH axon projections [97]. This observation makes it likely that the proposed pacemaker KP cells of the ARC/Inf generate GnRH pulses via influencing the secretory output of GnRH axons, instead of acting on

386 the somato-dendritic compartment. This assumption gains support from the observation that pulsatile KP
387 output and GnRH secretory pulses are temporally correlated in the median eminence of the monkey [84].

388 **3.4. KP fiber projections to the hypophysial portal vasculature**

389 KP-IR fibers in the mouse [98] and the rat [99] median eminence preferentially target the internal zone,
390 suggesting little if any communication between KP neurons and the hypophysial portal capillaries of the
391 external zone. This view is strengthened by the lack of Fluorogold uptake from the systemic circulation
392 by mouse KP neurons [77]. KP fibers were also observed mostly in the internal zone of the monkey
393 median eminence [8]. The major source of KP fibers in the rodent median eminence appears to be the
394 ARC [76, 78], although KP fibers of RP3V origin also reach the mediobasal hypothalamus [78].

395 Previous immunohistochemical studies from our laboratory [10, 63] showed a highly abundant
396 network of KP-IR axons around the portal vasculature of the human postinfundibular eminence which
397 contains a superficial and a deep portal capillary plexus [100]. These observations raise the possibility
398 that, unlike in rodents, KP is secreted into the hypophysial portal circulation of the human as a
399 hypophysiotropic factor. It occurs that species may vary considerably regarding the presence/absence of
400 hypophysiotropic KP axon projections. While there is evidence from ewes to indicate KP secretion into
401 the portal circulation [101], similarly low portal blood KP levels observed in ovariectomized ewes that
402 were untreated or given estrogen to elicit an LH surge, suggest that the anterior pituitary is not a major
403 site of action of KP on LH release. This view is supported by the lack of effect of *iv.* KP on LH release in
404 hypothalamo-pituitary-disconnected ewes [101]. Somewhat conflictingly, some [101-103], albeit not all
405 [13, 93], *in vitro* studies did identify mild stimulatory KP effects on LH release. Furthermore, *Kiss1r*
406 mRNA expression [75, 101, 102, 104] and Kiss1r immunoreactivity [104] have been detected in the
407 adenohypophysis.

408 **3.5. Other efferent projections**

409 Further important KP fiber tracts arising from the ARC as well as the RP3V were localized
410 periventricularly and found to carry fibers to several important preoptic, hypothalamic and septal nuclei

and to the bed nucleus of the stria terminalis [76, 77]. A few hypothalamic target neurons to KP fiber projections have already been been identified, Anatomical information exists from rats that the tuberoinfundibular dopaminergic system of the dorsomedial ARC receives sexually dimorphic KP-IR and NKB-IR innervation from KNDy neurons [105] whereby KP and NKB may regulate the secretion of prolactin [106]. Neuronal NO synthase cells in the preoptic region also receive KP-IR innervation and express Kiss1r [107]. The KP-induced phosphorylation of neuronal NO synthase in this circuitry has been strongly implicated in the KP-dependent preovulatory activation of GnRH neurons, whereas basal NO synthase activity maintains the tonic inhibition on the GnRH system during negative estrogen feedback [107].

The bulk of KP fiber projections in the human hypothalamus also occurs periventricularly in the medial hypothalamus [10]. Beyond GnRH cells innervated by the KP axon projections [10], further target cells of KP fibers in the human remain to be explored. Preliminary immunohistochemical data from our laboratory suggest that a similar connectivity between KP cells and the dopaminergic systems also exists in the human periventricular nucleus. The distinction between axon projections arising from KP neurons in the rostral periventricular area of the third ventricle and from those in the infundibular area, respectively, will be greatly facilitated once site-specific immunofluorescent markers for the two subsets of KP neurons are identified.

3.6. Afferent inputs to kisspeptin neurons

Specific inputs to the KP cells may play important roles in mediating stress-, metabolic-, and hormonal signals to the putative GnRH pulse generator in adults. Relatively little information has been published about these neuronal afferents. For example, KP neurons in the RP3V of mice receive vasopressinergic innervation from the suprachiasmatic nucleus which is thought to play a critical role in the circadian signaling to GnRH neurons for the timing of the proestrous afternoon GnRH/LH surge [108]. Recent evidence indicates that GnRH-immunoreactive axons also provide synaptic input to both the RP3V and ARC populations of KP neurons [109]. Further neurotransmitters acting upstream from KP

cells, possibly include glutamate which can induce the bursting activity of KP neurons [110]. The glutamatergic regulation of KP neurons may also be critically involved in the onset of puberty [111].

The innervation of human KP neurons is currectly unexplored.

4. Sexual dimorphism and aging-dependent changes of the human kisspeptin system

4.1. Sexual dimorphism of human kisspeptin and neurokinin B neurons

Both the preoptic (RP3V) and the ARC subsets of KP neurons contain receptors for estradiol, testosterone and progesterone [23, 26, 27, 71]. In rodents, androgens as well as estrogens can upregulate KP expression in the RP3V [23, 26, 71] at the putative site of positive estrogen feedback [24]. In contrast, KP expression in the ARC/Inf is regulated negatively by sex steroid hormones in rodents and other mammals [23, 26, 69, 71] and so is NKB expression at this site [69, 70, 112]. Sex differences of the KP and NKB neuronal systems are partly caused by the activational effects of the gonadal steroid hormone milieu which changes depending on the reproductive status and differs in the male and the female. Steroid hormones also exert robust organizational effects on the expression of KP and NKB in various species during development. Organizational effects have been studied most extensively in case of the sexually dimorphic KP neuron population of the rodent RP3V which is imprinted neonatally and results in higher KP cell numbers in adult females compared with males [25] (see also section 2). Other studies identified organizational effects in the formation of sex-specific projection fields by NKB neurons in the rat ARC [56] and in KP immunoreactive labeling of the mouse ARC [113]. Unlike in rodents where the sexual dimorphism of the ARC KP and NKB systems seems to be relatively mild, the ARC of the female sheep contains much higher NKB [114] and KP [29] cell numbers, compared with males. A recent study identified estrogen-dependent and -independent components of the sexual dimorphism developing in the mouse RP3V and ARC [113].

Putative anatomical sex differences of the human hypothalamic KP and NKB systems are likely to develop under combined organizational and activational gonadal steroids effects. Recent

immunohistochemical work provides evidence that human KP and NKB neurons are highly sexually dimorphic [10, 62, 115].

First, the rostral periventricular region of the third ventricle was found to contain a compact KP cell population in premenopausal women but not in men [10]. The full characterization of this cell population will require the further investigation of samples from male and female individuals of different age groups. In this study we also noticed a conspicuous sex difference in the regional density of KP-IR cell bodies and fibers in the Inf [10]; specimens from male subjects (especially those derived from young men) often exhibited extremely low numbers of KP-IR perikarya and fibers at this site.

A second quantitative immunohistochemical study from our laboratory analyzed sexually dimorphic features in hypothalamic samples from ‘aged male’ (>50 years) and postmenopausal female (>55 years) subjects [62]. The density of KP-IR cell bodies, the density of KP-IR fibers and the incidence of contacts these fibers established on the cell bodies and dendrites of GnRH neurons were significantly higher in aged women compared with men [62]. A milder sex difference of the NKB system was reflected in a somewhat higher regional density of NKB-IR somata in women compared with men [62]. In addition, larger KP-IR and NKB-IR cell bodies (mean immunolabeled profile area) were observed in females than in males. Somewhat unexpectedly, immunofluorescent studies only identified a partial overlap between KP-IR and NKB-IR axons. The colocalization in fibers showed a significant sex-dependence, with KP being colocalized in a higher percentage of NKB-IR afferents to GnRH neurons in women (31%) compared with men (9%). The percentage of KP-IR contacts co-containing NKB was also higher in females (26%) than in males (10%) [62]. These sex differences might be mostly attributable to the lack of estrogen negative feedback in aged women, whereas testosterone can continue to suppress KP, and to a lesser extent, NKB synthesis in men. Accordingly, comparative *in situ* hybridization studies of *KISS1* [34] and *TAC3* [70] mRNA expressing neurons in pre- *vs.* postmenopausal women provided evidence that these cells exhibit hypertrophy and higher cell numbers and cellular mRNA levels in the postmenopausal compared with the premenopausal period. The negative regulation of the KP- and NKB-encoding genes

by sex steroids is in accordance with similar observations from other species [23, 25, 26, 69, 71, 116, 117]. Because samples from young individuals were not available for immunohistochemical comparisons to samples from young males, based on these studies it was impossible to determine whether or not the quantified neuroanatomical features would also be sexually dimorphic when sex steroid levels are high and negative feedback is in place in both sexes.

The sexual dimorphism of the human hypothalamic NKB system has also been addressed by other investigators [115]. In this study the NKB-IR innervation of the Inf was found to be higher in adult human females compared with males, whereas the pars tuberalis received dense NKB-IR innervation in adult males but not females [115]. Furthermore, the Inf volume occupied by NKB immunoreactivity was significantly lower in adult men than in adult women and in adult male-to-female transsexuals [115]. These anatomical differences were present in young adults under the influence of negative sex steroid feedback, raising the possibiliy that they are partly due to organization sex steroid effects earlier in development.

4.2. Menopausal changes of kisspeptin and neurokinin B neurons in the infundibular nucleus

With the onset of menopause, the depletion of ovarian follicles leads to the loss of circulating estrogens. This causes the absence of negative estrogen feedback [118]. Comparison of histological samples from pre- and postmenopausal women revealed profound anatomical changes in the Inf where negative feedback is thought to take place [118]. *In situ* hybridization studies identified postmenopausal hypertrophy in neurons that express the transcripts encoding for estrogen receptor alpha [74], SP [70], NKB [70], KP [34] and dynorphin [68]. These morphometric alterations were also associated with increased *TAC1* [70], *TAC3* [70] and *KISS1* [34] and decreased prodynorphin [68] mRNA expression in this region.

The increased synthesis of *TAC3* [70] and *KISS1* [34] mRNAs in postmenopausal women also results in very high levels of KP and NKB immunoreactivities [62]. It is interesting to note that our laboratory has processed a large number of samples for KP and NKB immunohistochemistry from women above 80

510 years of age; KP and NKB immunoreactivities (including KP and NKB cell and fiber densities, and
511 incidences of contacts on GnRH cell bodies and dendrites) remained very high in these aged individuals,
512 indicating that these neurons do not have an intrinsic mechanism to halt the enhanced neuropeptide
513 synthesis in the absence of circulating estrogens. The dysregulation of NKB (or another KNDy peptide)
514 synthesis during menopausal transition was proposed to contribute to hot flushes via an altered NKB
515 input to thermoregulatory centers [119]. In addition, KNDy neuron ablation prevented the dramatic effects
516 of ovariectomy and estradiol replacement on body weight and abdominal girth. This finding indicates that
517 KP and/or NKB also play an important role in the estrogenic regulation of body weight homeostasis [73].

518 **4.3. Aging-dependent changes of the kisspeptin and neurokinin B systems in men**

519 Aging-related decline in reproductive functions is less dramatic in human males than in females
520 because of the sustained testosterone production by the testes [120]. Although the gonadal functions of
521 men can be well preserved throughout life, the negative feedback response of the reproductive axis to
522 testosterone shows a declining trend in aging men [121]. Clinical symptoms of hypogonadism, including
523 decreased morning erections, erectile dysfunction and decreased frequency of sexual thoughts, become
524 more common in men with aging [122]. Midlife transition is often characterized by decreased serum
525 levels of free testosterone and dihydrotestosterone and increased levels of LH, FSH and sex hormone
526 binding globulin [123, 124]. In addition, aging is associated with decreased pulsatile and increased
527 basal LH secretion, and a decline in the LH secretory burst mode [121]. Elderly men also secrete LH
528 and testosterone more irregularly and more asynchronously than do young men [125, 126]. Some of
529 these endocrine alterations result from a reduced androgen receptor-mediated negative feedback to
530 the hypothalamus [121]. In view of animal experiments indicating that KP and NKB neurons also play
531 an important role in testosterone negative feedback to the male hypothalamus [65, 71], we anticipated
532 enhanced central KP- and NKB-signaling in the Inf of aged *vs.* young men. To address the predicted
533 age-dependent enhancements of central KP- and NKB-signaling, we carried out quantitative

534 immunohistochemical studies on a relatively large number (N=20) of hypothalamic samples from men
535 [61].

536 Indeed, the comparative analysis of KP and NKB immunoreactivities of the Inf between
537 arbitrarily defined 'young' (<50 years) and 'aged' (≥50 years) men revealed conspicuous aging-
538 related anatomical changes [61]. Robust aging-dependent enhancements were identified in the
539 regional densities of KP-IR perikarya and fibers, and in the incidence of contacts they established
540 with the cell bodies and dendrites of GnRH neurons [61]. NKB-IR perikarya, fibers and axonal
541 appositions to GnRH neurons also increased with age, but to lesser extents [61]. In addition, in dual-
542 immunofluorescent studies, the incidence of NKB-IR perikarya that co-contained KP increased from
543 36% in young to 68% in aged men, indicating that more NKB neurons started to express detectable
544 levels of KP in aged individuals (**Figure 4**) [61]. Finally, we identified a mild but significant
545 hypertrophy of KP-IR and NKB-IR neurons which was reminiscent in magnitude to the previously
546 reported hypertrophy of unidentified neurons in the Inf of aged men [127].

547 It seems likely that the aging-related enhancements of the immunohistochemical signals are the
548 consequences of the reduced negative sex steroid feedback to KP and NKB neurons in aged, compared
549 with young, men. The heavier KP and NKB inputs to GnRH neurons may cause the enhanced stimulation
550 of the reproductive axis in aged men. It is worthy of note that the KP system showed an overall higher
551 response (fold-change of quantified immunohistochemical measures) to aging than the NKB system [61].
552 This finding might be explained by a higher sex steroid responsiveness of the *KISS1 vs.* the *TAC3* gene.
553 This putative regulatory difference is also reflected in the higher degree of sexual dimorphism of the KP
554 *vs.* the NKB system that we observed in aged subjects [62]. Of note, the mouse *Kiss1* gene also shows a
555 higher responsiveness to estrogen in comparison with the NKB-encoding *Tac2* gene [128]. It requires
556 clarification to what extent the enhanced KP and NKB signaling upstream from the human GnRH neurons
557 represents an adaptive response to reduced androgen levels or alternatively, the consequence of an aging-
558 related decline in the androgen sensitivity of the hypothalamus.

5. Remaining important issues

In situ hybridization and immunohistochemical studies of *post-mortem* human hypothalami will remain valuable tools to study several questions unanswered so far. The aims of future studies will include:

5.1. Further anatomical characterization of the KP cell population in the human rostral periventricular area

5.2. Localization of steroid hormone receptors in human KP neurons

5.3. Identification and subcellular localization of neuropeptide receptors (KISS1R; NK1-3; κ-opioid receptor, etc.) in KP and GnRH neurons

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

5.5. Characterization of the afferent connectivity of KP neurons

5.6. Neurochemical characterization of KP neuron populations

5.7. Identification of pubertal changes in KP neurons

5.8. Clarification of organizational and activational effects contributing to the sexual dimorphism of the human KP neuronal system

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Legends

Figure 1. Topographic distribution of kisspeptin-immunoreactive cell bodies in the human hypothalamus.

Schematic diagrams of coronal sections were generated with CorelDRAW from representative Nissl-stained sections of human hemihypothalami. Green dots correspond to the distribution of kisspeptin-immunoreactive cell bodies at the different rostro-caudal 1evels (**A-F**). Anatomical information is combined from male and female individuals of various ages. Most rostrally (**A**), a prominent group of faintly-stained kisspeptin neurons occurs in the VPe and in the PaAP. Behind this level (**B**), labeled somata are accumulated in the PaMc. These two cell groups are most numerous in young female individuals and appear to be analogous, at least anatomically, to kisspeptin neurons in the rostral periventricular area of the third ventricle (RP3V) in rodents [24]. Kisspeptin neurons are most numerous in the caudal Inf (**E**). This cell group is likely to correspond to kisspeptin neurons of the arcuate nucleus in laboratory animals and extends into the proximal portion of the InfS. Kisspeptin neurons in this region are most numerous in samples from postmenopausal women [62]. A third population of relatively darkly labeled kisspeptin neurons is scattered in the periventricular region through the rostro-caudal extent of the human hypothalamus. Abbreviations: **3V**, third cerebral ventricle**; Ac**, anterior commissure; **BST**, bed nucleus of the stria terminalis; **DHA**, dorsal hypothalamic area; **DMH**, dorsomedial hypothalamic nucleus; **Fx**, fomix; **HDB**, horizontal limb of the diagonal band of Broca; **Inf**, infundibular nucleus; **InfS**, infundibular stalk; **LHA**, lateral hypothalamic area; **LSV**, ventrolateral septal nucleus; **Ltu**, lateral tuberal nucleus; **Mfb**, medial forebrain bundle; **MMC**, rnagnocellular part of the mammillary nucleus; **Opt**, optic tract; **OX**, optic chiasm; **Pa**, paraventricular hypothalamic nucleus; **PaAP**, anterior parvocellular subdivision of the paraventricular nucleus; **PaMc**, magnocellular part of the paraventricular hypothalamic nucleus; **Sch**, suprachiasmatic nucleus; **SO**, supraoptic nucleus; **VMH**, ventromedial hypothalamic nucleus; **VPe**, ventral periventricular hypothalamic nucleus. Scale bar=2.5 mm.

606

607

608 **Figure 2. Immunohistochemical detection of kisspeptin neurons in the mediobasal hypothalamus of**
609 **the human. A:** The largest kisspeptin cell population of the human is located in the infundibular area.
610 Immunoreactive neurons in the infundibular nucleus (Inf), detected with black silver-gold-intensified
611 diaminobenzidine, are most numerous in samples from postmenopausal women. This cell population
612 extends to the infundibular stalk (InfS). **B:** High-power image illustrates that scattered gonadotropin-
613 releasing hormone-immunoreactive neurons (brown diaminobenzidine chromogen) often intermingle with
614 kisspeptin-immunoreactive perikarya in the Inf. Scale bar=285µm in **A** and 20µm in **B**.

615

616

Figure 3. Efferent targets of kisspeptin neurons. In the infundibular nucleus (Inf), kisspeptin-immunoreactive axons (black silver-gold-intensified diaminobenzidine) establish frequent contacts with the cell bodies and dendrites of other kisspeptin neurons (**A**) and innervate (arrows) the somatic **(A)** and dendritic **(B)** compartments of gonadotropin-releasing hormone-immunoreactive neurons (brown diaminobenzidine). Hypophysiotropic gonadotropin-releasing hormone axon projections in the infundibular stalk (InfS; **C**) intermingle with a dense kisspeptin-immunoreactive fiber network. At high-power **(D)**, the two types of axon form occasional contacts (arrows). Scale bar=20μm in **A-C** and 10μm in **D**.

Figure 4. Overlap between neurokinin B-immunoreactive and kisspeptin-immunoreactive perikarya in three different human models. The ratios of double-labeled neurokinin B (NKB) and kisspeptin (KP) perikarya were determined quantitatively from dual-immunofluorescent specimens in which tyramide signal amplification approaches were applied to maximize both types of labeling [61]. In young male (<50 years), aged male (≥50 years) and aged (postmenopausal) female (≥55 years) models available for these quantitative studies, the majority of KP-IR perikarya (72.7±6.0% in young men, 77.9±5.9% in aged men and 83.7±3.7% in postmenopausal women) also contained NKB immunoreactivity. Similarly, the majority of NKB-IR neurons in aged human subjects (68.1±6.8% in aged men and 71.3±5.9% in postmenopausal women) contained KP immunoreactivity. However, in young human males, most of the NKB-IR perikarya were single-labeled and only 35.8±5.1% contained KP immunoreactivity. *P<0.05. For details of methods, analysis and the colocalization results from males, see Molnár et al., 2012 [61].

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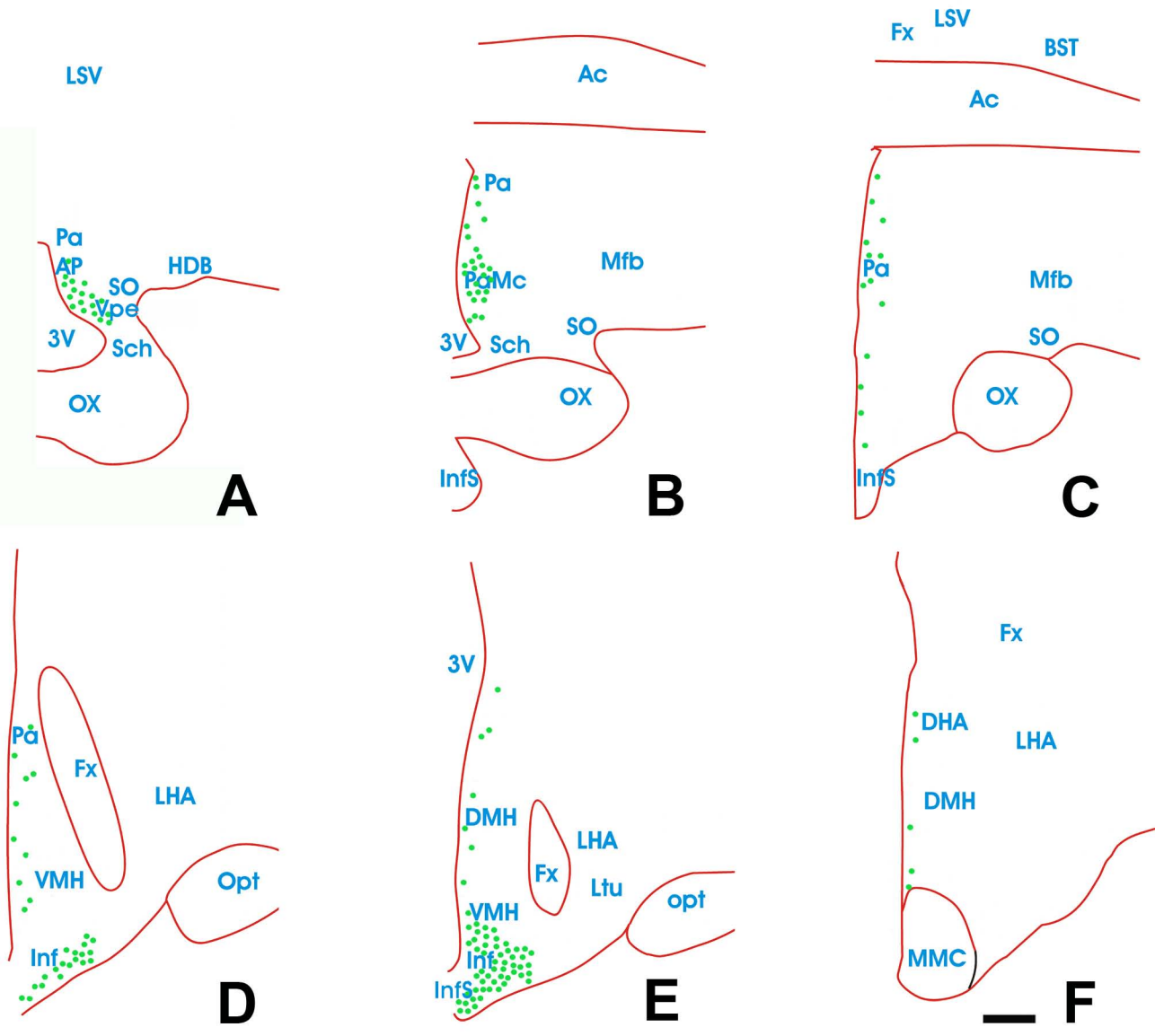
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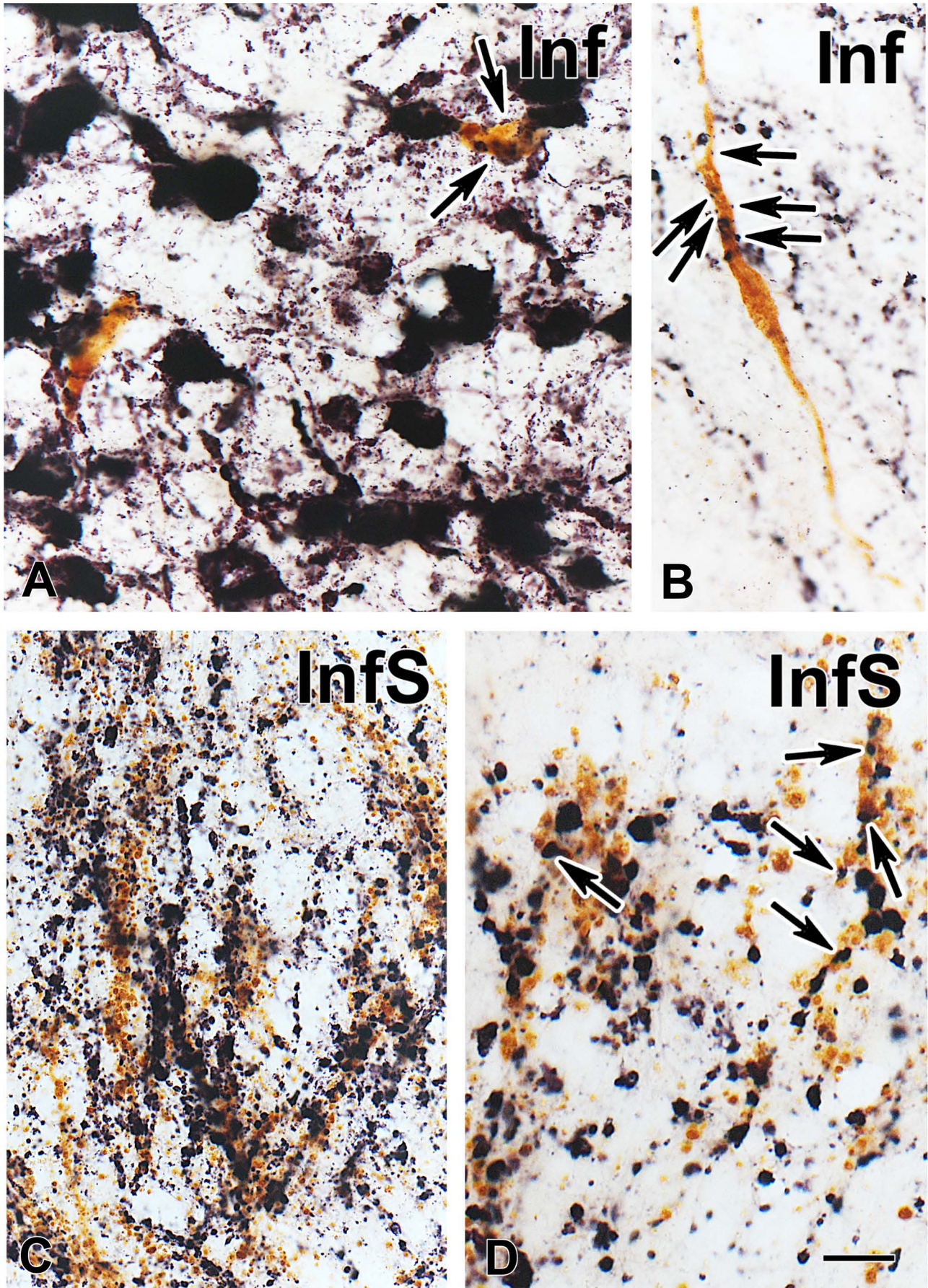
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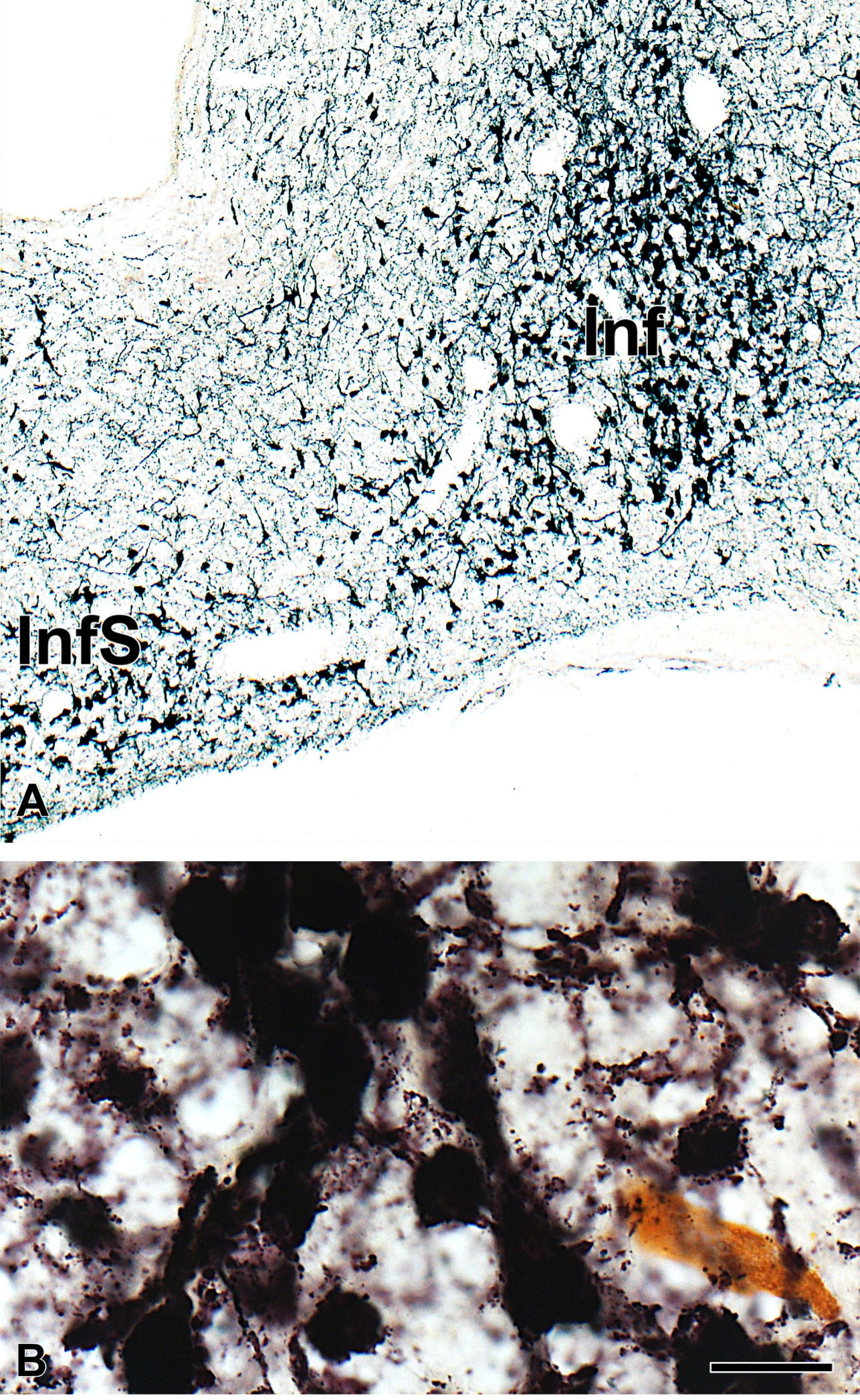
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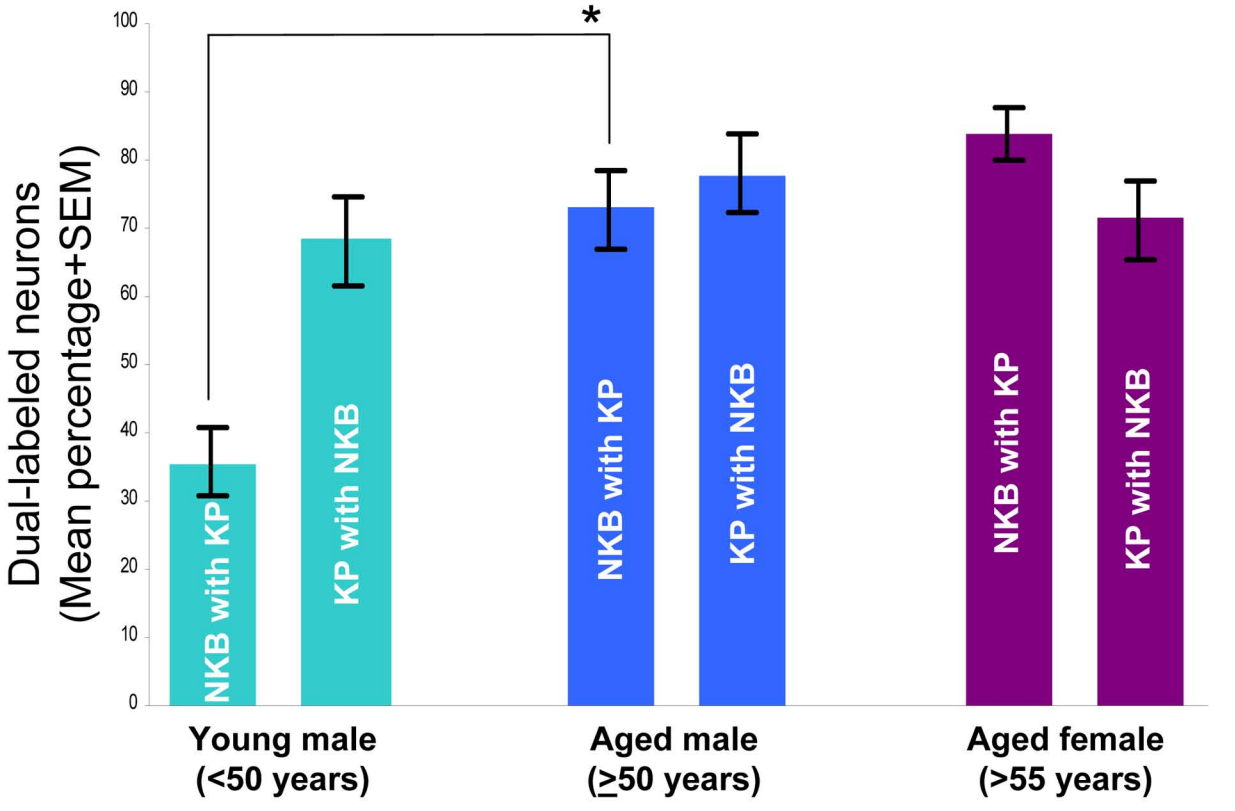
Dual-labeled neurons
(Mean percentage+SEM)
100
90
80
70
60
50
40
30
20
10
0
*
NKB with KP
KP with NKB
NKB with KP
KP with NKB
NKB with KP
KP with NKB
Young male
(<50 years)
Aged male
(≥50 years)
Aged female
(>55 years)