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Chapter 18

Kisspeptin and neurokinin B expression in the human hypothalamus: Relation to reproduction and gender identity

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Abstract

Gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus are at the core of reproductive functioning. GnRH released into the median eminence regulates the secretion of the gonadotropins from the anterior pituitary, which in turn activates gametogenesis and steroid synthesis by the gonads. The GnRH system displays functional sex differences: GnRH is secreted in pulses at a constant frequency in men, whereas in women, pulse frequency varies over the menstrual cycle. In both sexes, GnRH release is regulated by sex steroid hormones, acting at the level of the hypothalamus and the anterior pituitary in a classic feedback loop. Because GnRH neurons do not express sex steroid receptors, hormone effects on GnRH release are presumed to be mediated indirectly through other steroid-sensitive neuronal systems, which then converge onto GnRH cell bodies and/or terminals. Human genetic studies demonstrated that kisspeptin (KP) as well as neurokinin B (NKB) signaling are both potent regulators of GNRH secretion. In humans, postmortem studies using immunohistochemistry have shown that women have higher KP and NKB expression in the infundibular nucleus than men. Sex differences in KP expression are present throughout life, which is from the infant/prepubertal into the elderly period, whereas sex differences in NKB expression do not emerge until adulthood. KP and NKB are often coexpressed together with dynorphin by the same population of neurons, also known as KDNy neurons in other species. Indeed, significant coexpression between KP and NKB but not with Dynorphin has been observed thereby challenging the KDNy concept in humans. Female-typical expression of both KP and NKB were observed in the infundibular nucleus of trans women (male sex assigned at birth and female gender identity). Taken together, sex differences in KP and NKB expression most likely reflect organizational actions of sex steroid hormones on the developing brain but they also remain sensitive to circulating sex steroids in adulthood. The female-dominant sex difference in infundibular KP and NKB expression suggests that this brain region is most likely involved in both the negative and positive feedback actions of estrogens on GnRH secretion. Finally, the sex-reversal observed in KP and NKB expression in trans women might reflect, at least partially, an atypical sexual differentiation of the brain.

INTRODUCTION

The human brain differentiates sexually under the influence of testosterone acting in the male fetus (Bao and Swaab, 2011). A large number of morphological and neurochemical sex differences induced by sex steroid hormones during the perinatal period are found predominantly in limbic–hypothalamic regions and are thought to be at the basis of sex differences in the control of reproductive functions, i.e., cyclic in women and tonic in men. Gonadotropin-releasing hormone (GnRH), which is a 10 amino-acid long peptide, highly

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conserved across species, is the master hormone within the hypothalamus that maintains normal reproductive functions. Pulsatile release of GnRH at the level of the median eminence (ME) regulates the secretion of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone, from specialized gonadotropic cells in the anterior pituitary. In turn, gonadotropins released into the blood activate gametogenesis and steroid synthesis by the gonads (Herbison, 2006).

There are only a few thousand GnRH neurons present in the hypothalamus, which is a remarkably low number in view of their critical function in reproduction. In addition, GnRH neurons do not arise from neurons within the developing central nervous system but are derived from progenitor cells in the epithelium of the olfactory placode. These nascent GnRH neurons migrate along the vomeronasal axons, across the cribriform plate and into the mediobasal hypothalamus. Patients with delayed puberty due to abnormal GnRH neuronal migration, such as Kallmann Syndrome, frequently have associated anosmia, i.e., the inability to smell, reflecting the fact that GnRH neurons indeed share common embryonic origins and migratory pathways with olfactory neurons (Stamou and Georgopoulos, 2018).

The GnRH system displays functional sex differences: GnRH is secreted in pulses at a constant frequency in men, whereas in women, pulse frequency varies over the menstrual cycle. In both sexes, GnRH release is regulated by sex steroid hormones, in particularly estradiol in women and testosterone in men, acting at the level of the hypothalamus and the anterior pituitary in a classic negative feedback loop inhibiting the secretion of GnRH and consequently of gonadotropins. In women, during the first part of the menstrual cycle, estrogens can also exert a positive feedback on the hypothalamus to produce an increase in GnRH release and ultimately the LH surge, which is necessary to induce ovulation. In men, this estrogen positive feedback does not normally occur but can be induced upon castration and prolonged estradiol treatment (Gooren, 1986; Goh and Ratnam, 1988). It should be noted that this study was conducted in trans women (male sex assigned at birth, female gender identity), who might have undergone a different developmental organization of the brain (see further under Section "KP and NKB expression in relation to gender identity and sexual orientation"). This suggests that LH responses to estrogens in humans might not be as perinatally fixed as is the case in rodents (Vreeburg et al., 1977) but might be dependent on the nature of circulating sex steroids with androgens generally being inhibitory.

Since immunohistochemical studies have failed to show colocalization of estrogen receptor α in GnRH neurons in different species (rat: Herbison and Theodosis, 1992; sheep: Lehman and Karsch, 1993; human and nonhuman primates: Rance et al., 1990; Sullivan et al., 1995), estradiol effects on GnRH release are presumed to be mediated indirectly via other steroid-sensitive neuronal systems, which then converge onto GnRH cell bodies or terminals. Furthermore, while GnRH release is clearly organized in a sexually dimorphic manner, no sex differences have been observed in the number of GnRH neurons for example.

Human genetic studies (de Roux et al., 2003; Seminara et al., 2003) have identified the neuropeptide kisspeptin (KP), the processed peptide product of the *KISS1* gene, and the endogenous agonist for the GPR54 receptor (also known as KISS1R) as a potent stimulator of GnRH neurons and by consequence as a major regulator of the hypothalamic–pituitary-gonadal axis. In rodents, KP-expressing neurons are mainly localized in two hypothalamic brain nuclei, i.e., the anteroventral periventricular nucleus/periventricular nucleus continuum (AVPv/PeN) and the arcuate nucleus (ARC), in humans called the infundibular nucleus (INF). KP neurons express sex steroid receptors (Smith et al., 2005a,b, 2006) that can be directly modulated by sex steroids (Smith et al., 2005a,b; Clarkson and Herbison, 2006; Kaufmann et al., 2007; Brock and Bakker, 2013). Over the last 15 years, the different roles of these two distinct KP neuronal populations in reproductive maturation and function in rodents were further refined by identifying their participation in the sexual differentiation of the brain (Clarkson and Herbison, 2006), puberty onset (Dungan et al., 2006), feedback regulation of gonadotropin secretion (Smith et al., 2006), neuroendocrine control of ovulation (Tena-Sempere, 2005; Clarkson et al., 2008), metabolic modulation of fertility (Kaufmann et al., 2007), environmental (photoperiod) control of reproduction in seasonal species (Simonneaux et al., 2009), in metabolism modulation and obesity (Tolson et al., 2014), and more recently in synchronizing female sexual behavior with ovulation (Hellier et al., 2018). The current view is that the AVPv/PeN KP population is predominantly involved in transmitting positive feedback actions of estradiol to GnRH neurons and thus in inducing the preovulatory LH surge, whereas the ARC KP population plays a major role in the negative feedback actions of sex steroids on GnRH neurons. This dichotomy is primarily based on the differential effects of estradiol and testosterone observed on KP neurons in the AVPv/PeN vs the ARC. Gonadal steroids dramatically inhibit ARC KP expression, both at the mRNA and protein level, and conversely, gonadectomy stimulates both ARC KP expression and LH secretion, with the postgonadectomy rise in LH secretion being blocked by a selective KP antagonist (Roseweir et al., 2009), confirming the important role of the ARC KP population in negative feedback actions of steroids on GnRH/LH secretion (Kauffman, 2010). By contrast, estrogens increase KP

mRNA expression in the AVPv/PeN and the overall expression of KP mRNA is increased at the time of ovulation (Smith et al., 2006; Robertson et al., 2009). Finally, AVPv/PeN KP neurons project to and stimulate GnRH cell bodies (which express GPR54), whereas ARC KP neurons project to GnRH fiber terminals in the ME (Wintermantel et al., 2006; D'Anglemont de Tassigny et al., 2008; Ramaswamy et al., 2008).

Interestingly, in a large number of species, sex differences have been reported in the number of KP neurons. For example, in mice, females show greater numbers of KP neurons in the AVPv/PeN (Clarkson and Herbison, 2006; Bakker et al., 2010), which is in line with a specific function for this population in transferring positive feedback actions of estradiol to GnRH neurons necessary to induce ovulation. Furthermore, this population appears to develop under the influence of estradiol during the peripubertal period, i.e., around the time the ovaries start to secrete estrogens (the ovaries are quiescent during prenatal development; Lamprecht et al., 1976). In a first study by Clarkson and Herbison (2006) in mice, it was shown that AVPv/PeN KP expression starts around postnatal day 15 (P15) and then rapidly increases to achieve adult-like levels by P30, the time of puberty onset. Ovariectomy of female pups at P15 resulted in a 70%–90% reduction in the number of KP neurons of mice sacrificed either at P30 or P60. Administration of estradiol to P15-ovariectomized mice until P30 completely restored the number of KP-expressing neurons. Likewise, female mice deficient in estradiol due to a targeted mutation in the *Cyp19* gene which encodes the enzyme aromatase that is crucial for the conversion of testosterone to estradiol (aromatase knockout mice or ArKO) showed significantly fewer numbers of KP neurons than wild-type females (Bakker et al., 2010). Prepubertal treatment (between P15 and P25) with estradiol significantly feminized the number of KP neurons in ArKO mice (Brock and Bakker, 2013). By contrast, the ARC KP population does not seem to show any sex differences in either the total number of KP mRNA expressing neurons or the amount of KP mRNA per cell (Kaufmann et al., 2007). The lack of a sex difference in ARC KP expression is not affected by circulating sex steroids: adult male and female mice and rats display similar high levels of KP expression in the ARC following gonadectomy and similar reductions in KP expression after sex steroid replacement (Kaufmann et al., 2007; Brock and Bakker, 2013).

In sheep (Hoffman et al., 2011), KP cells can also be found at similar rostral–caudal levels of the preoptic area (POA), but they are more scattered and less numerous than in rodents. The majority of KP cells are located in the ARC and, interestingly, this population shows sex differences with ewes having greater numbers of KP expressing neurons than rams. Furthermore, in sheep, both populations appear to contribute to the positive feedback actions of estradiol on GnRH and LH release, but this might be specific to this particular species. There is only fragmentary evidence on the role of rostral KP neurons in the preovulatory surge in other species than rodents. In nonhuman primates (Smith et al., 2010), KP neurons can be found in similar rostral areas as in sheep, but they are more periventricular and ventral in location, and like sheep, the vast majority of KP neurons can be found in the mediobasal hypothalamus. Therefore, the prevailing view is that the positive estrogen feedback in primates takes place exclusively in the ARC (or infundibular region, INF) and that there is no specific role for the more rostral KP population. It should be noted that the POA contains a considerable number of GnRH neurons in the monkey, as high as can be found in the INF, even though only 25% of these POA neurons actually send projections to the ME (Goldsmith et al., 1990), highlighting additional functions of GnRH within the brain other than regulating the secretion of gonadotropins from the pituitary. However, it cannot be ruled out that the POA might play some role in reproductive function in primates.

The neuropeptide neurokinin B (NKB) has also received attention recently since human genetic studies have been linking loss-of-function mutations in *TAC3* and *TACR3* genes (encoding NKB and its receptor, NK3R, respectively) with hypogonadotropic hypogonadism and infertility (Topaloglu et al., 2009). Anatomically, NKB is coexpressed in the infundibular (arcuate) nucleus with KP and dynorphin A in a population of neurons, also termed KNDy (kisspeptin/neurokinin B/dynorphin), in several species (sheep: Goodman et al., 2007; mice: Navarro et al., 2009; rat: Krajewski et al., 2005; goat: Wakabayashi et al., 2010). KNDy neurons express both estrogen receptor-α and the androgen receptor and can be directly regulated by sex steroids (Dellovade and Merchenthaler, 2004; Smith et al., 2005a,b; Navarro et al., 2011). Recent experimental evidence suggests that NKB from this neuronal population participates in the (auto) regulation of KP output from KNDy neurons and thereby critically contributes to two key physiological events: the timing of puberty onset and the shaping of KP and by consequence GnRH pulsatile release (Moore et al., 2018). As for KP, sex differences have been observed in the number of (arcuate) NKB neurons. In sheep, a greater number of NKB neurons was observed in females, which was reversed by prenatal treatment with androgens (Goubillon et al., 2000; Cheng et al., 2010). In the rat, NKB neurons exhibit an unusual, qualitative sex difference in their ventral axonal projections, i.e., to the neuropil in females, but rather to capillary vessels in males (Ciofi et al., 2006), as well as in the number of NKB neurons (female > male; Ruiz-Pino et al., 2012). Interestingly sex differences in NKB expression and projections emerge progressively

during puberty and appear to be dependent on pubertal exposure to the rising levels of estradiol in females and testosterone in males (Ciofi et al., 2007).

In this chapter, an overview will be provided on KP and NKB expression in the human hypothalamus and their potential role in human reproductive function.

KISSPEPTIN EXPRESSION IN THE HUMAN HYPOTHALAMUS

In humans, immunohistochemistry on postmortem tissues have revealed a dense network of KP immunoreactive (−ir) fibers in the medial hypothalamus (Hrabovszky et al., 2010; Taziaux et al., 2016). The highest fiber densities are observed around the third ventricle, including the organum vasculosum of the lamina terminalis (OVLT), ventral periventricular area, anteromedial and anterolateral POA, paraventricular nucleus (PVN), at both magnocellular and parvocellular subdivisions, INF and stalk, dorsomedial hypothalamic nucleus, and the dorsal hypothalamic area. Overall, the density of KP-ir fibers diminishes with increasing distance from the third ventricle and labeled fibers become scarce in the ventromedial hypothalamic nucleus (VMN) and the lateral hypothalamic area.

The majority of KP-ir cell bodies are observed in the INF. Some intensely labeled cell bodies are also found scattered periventricularly throughout the rostrocaudal extent of the hypothalamus. Furthermore, some less intensely immunostained KP cell bodies can be found in the PVN (Hrabovszky et al., 2010).

Strong sex differences have been observed in the number of KP fibers and cell bodies (Hrabovszky et al., 2010; Taziaux et al., 2016; Fig. 18.1). Overall, consistently fewer ir fibers are observed in the hypothalamic sections of male individuals, compared with the patterns found in female brains. Furthermore, whereas KP-ir cell bodies are consistently observed in the rostral periventricular zone of female brains, no such labeled cells are found in males in this region (Hrabovszky et al., 2010). A further obvious sex difference is that the INF contain very few, if any, rather faintly labeled cells in males, compared with many heavily labeled cells in females (Hrabovszky et al., 2010; Taziaux et al., 2016). These sex differences are present in young adults (Hrabovszky et al., 2010) as well as in more aged subjects (Hrabovszky et al., 2011). Furthermore, KP expression appears to change over the lifetime, with a greater number of KP cell bodies in the infant/prepubertal and elderly period compared to the adult period (Taziaux et al., 2016; Figs. 18.1 and 18.2A–C). Thus the developmental pattern of INF KP expression throughout life seems to be quite similar between men and women, i.e., a moderate number of KP neurons in the infant/prepuberal period followed by a lower number in the adult period and an increasing number in the elderly period. It is difficult to discern at which stage of life these sex differences emerge, but based on mean numbers, females have quantitatively more KP cell bodies compared to age-matched males at all stages of life analyzed (Taziaux et al., 2016), raising the possibility that this sex difference is already present before birth and might reflect organizational effects of sex steroids during early development. KP expression (as well as that of its receptor) has already been observed in fetuses at 15 weeks of gestation (Guimiot et al., 2012), but no sex comparison was made in this particular study.

The changes in KP expression in the INF likely reflect fluctuations in the circulating concentration of sex steroid hormones throughout life. This is most obvious in females, with KP expression following a U-shaped curve, i.e., greater numbers of KP-expressing neurons in the infant/prepubertal and elderly periods compared to the adult period, thereby showing an inverted relationship with circulating estrogen levels. Accordingly, low KP mRNA levels are observed in estradiol-treated ovariectomized monkeys and in premenopausal women, whereas they are elevated in untreated ovariectomized monkeys and postmenopausal women (Rometo et al., 2007). Remarkably, in postmenopausal women, KP neurons show a hypertrophied morphology. This is observed by using in situ hybridization to measure KP mRNA levels (Rometo et al., 2007) as well as by using immunohistochemistry to measure KP peptide levels (Taziaux et al., 2016). This cellular hypertrophy suggests increased neuronal activity as well as that they are part of the neural circuitry regulating estrogen negative feedback. Since there does not seem to be a homologue of the rodent AVPv/PeN KP population in humans (Hrabovszky et al., 2010; Taziaux et al., 2016), it is very likely that the INF represents the hypothalamic site that mediates both negative and positive feedback actions of estradiol in humans, as was already suggested for primates (Knobil, 1980). This is further supported by the fact that GnRH neurons can predominantly be found in this same brain region, whereas in rodents, the majority of the GnRH neurons are located in the OVLT/POA. In both monkeys (Shahab et al., 2005) and humans (Guimiot et al., 2012), the KP receptor GPR54 has also been identified in the mediobasal hypothalamus.

NEUROKININ B EXPRESSION IN THE HUMAN HYPOTHALAMUS

NKB-ir fibers can be found widely distributed throughout the human hypothalamus, whereas cell bodies are confined to a few specific nuclei (Taziaux et al., 2012; Fig. 18.3). Cell bodies expressing NKB vary in size

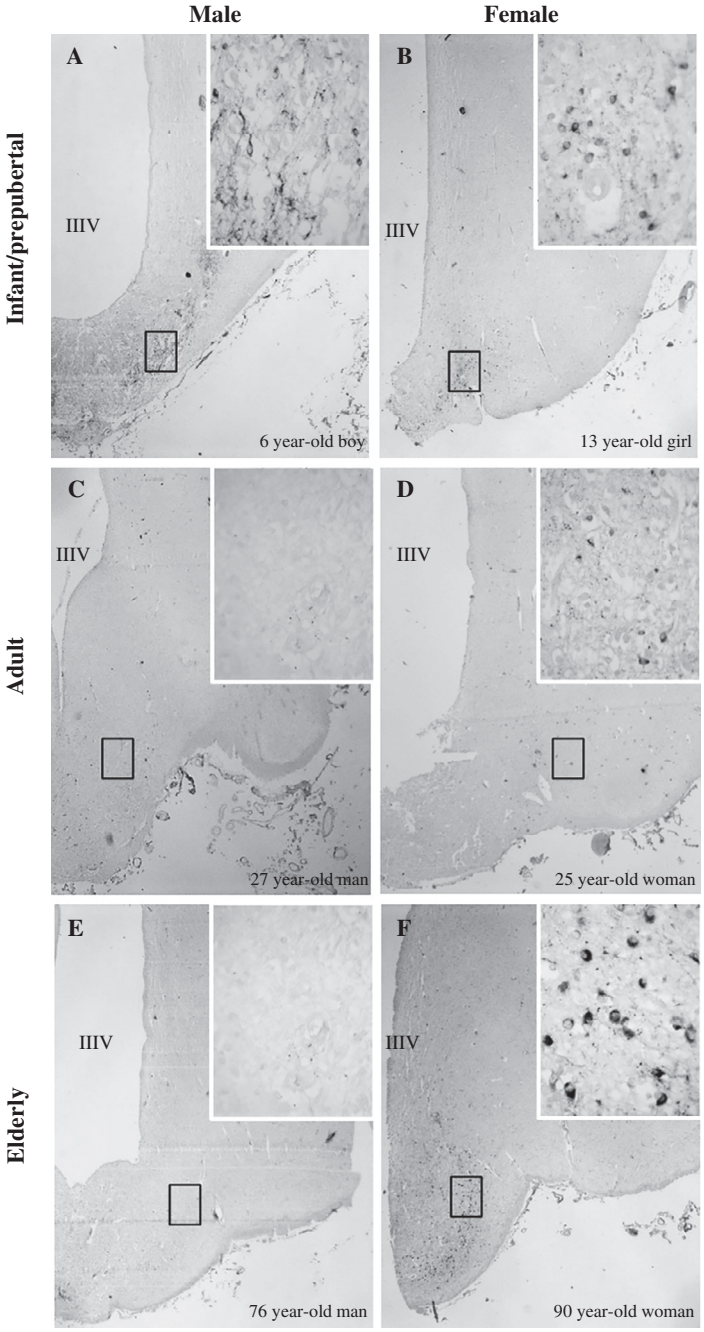

Fig. 18.1. Representative photomicrographs of kisspeptin expression in infundibular nucleus (INF) during the infant/prepubertal (A, B), adult (C, D), and elderly (E, F) periods in both sexes. The *boxes* in panels illustrate the area photographed at higher magnification. Note the numerous and intensely labeled kisspeptin neurons in the infant/prepubertal (B) and elderly periods (F) compared to the adult period (D) in the female INF. *IIIV*, third ventricle. Reproduced from Taziaux M, Staphorsius AS, Ghatei MA et al. (2016). Kisspeptin expression in the human infundibular nucleus in relation to sex, gender identity, and sexual orientation. J Clin Endocrinol Metab 101: 2380–2389, with permission of Endocrine Society, Oxford University Press.

depending on their localization. Small, oval-to-round NKB neurons are numerous in the central and medial portions of the bed nucleus of the stria terminalis (BST) and in the INF/ME complex, with the majority of the NKB cells being found in the middle and caudal part. Less intensely labeled cell bodies are also found scattered periventricularly throughout the caudal extent of the hypothalamus. A small number of medium-sized round NKB neurons are observed in the nucleus basalis of Meynert (NBM), the anteroventral hypothalamus, the medial POA, and the posterior BST. Immunoreactive fibers for NKB exhibit varicosities and are abundantly

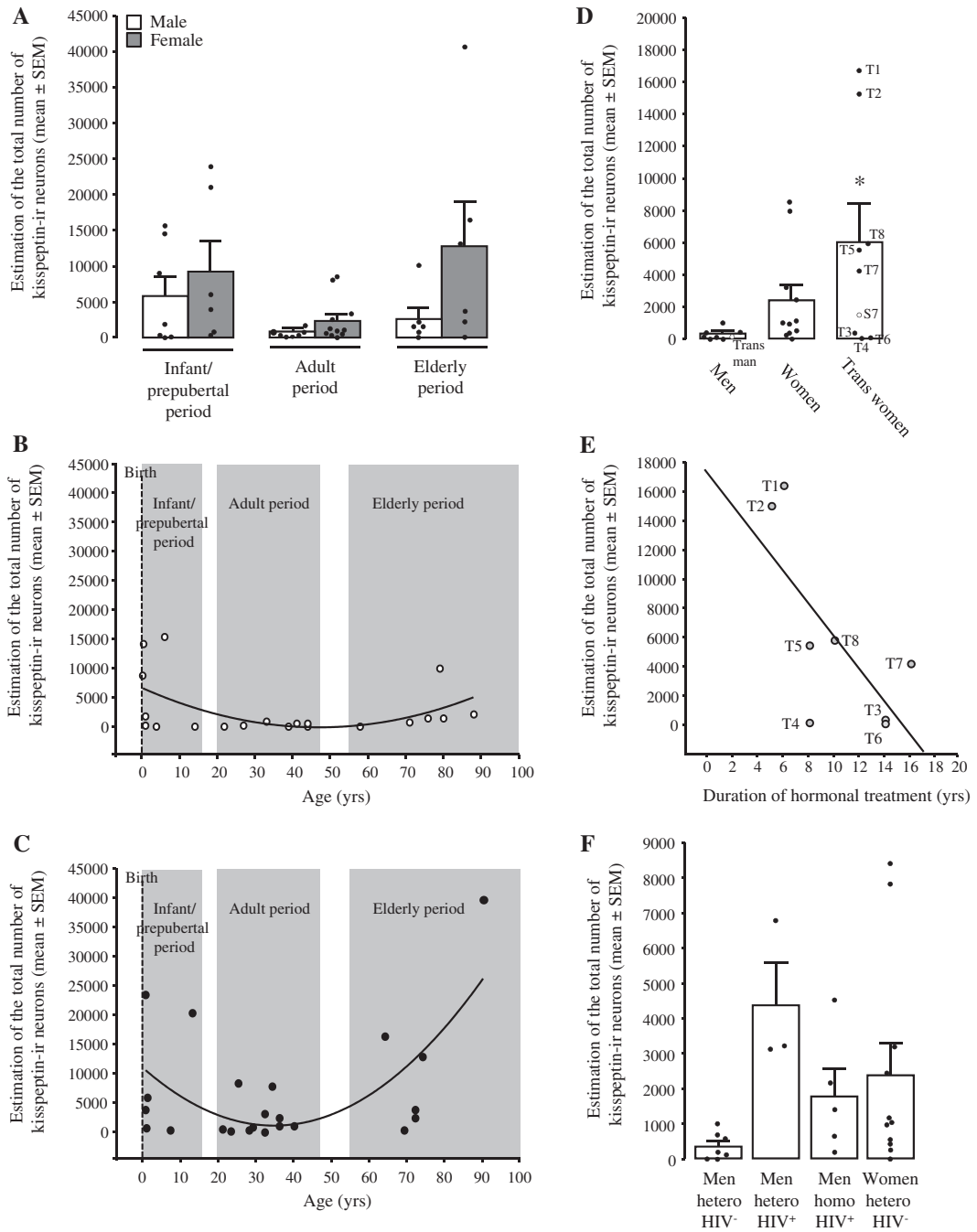

Fig. 18.2. (A) Estimation of the total number of kisspeptin-ir neurons in the INF of males and females during the infant/pubertal period (between 5 months and 14 years), the adult period (between 22 and 44 years), and the elderly period (between 58 and 90 years). Relationship between the total number of kisspeptin-ir neurons in the INF and age in females (B) and in males (C). The lines represent the quadratic regression curve. (D) Estimation of the total number of kisspeptin-ir neurons in the INF of adult men, women, and trans women. The open circle represents the number of KP neurons in the trans man sample. (E) Relationship between the total number of kisspeptin-ir neurons in the INF and duration of the hormonal treatment in transwomen. (F) Estimation of the total number of kisspeptin-ir neurons in the INF of HIV$^-$ heterosexual men, HIV$^-$ heterosexual women, HIV$^+$ heterosexual men, and HIV$^+$ homosexual men. *Black dots* represent individual data. * $P < 0.05$ vs adult men. *S7,* nontreated trans woman; *T1–T8*, transgender individuals. Panels (B) and (C) reproduced from Taziaux M, Staphorsius AS, Ghatei MA et al. (2016). Kisspeptin expression in the human infundibular nucleus in relation to sex, gender identity, and sexual orientation. J Clin Endocrinol Metab 101: 2380–2389, with permission of Endocrine Society, Oxford University Press.

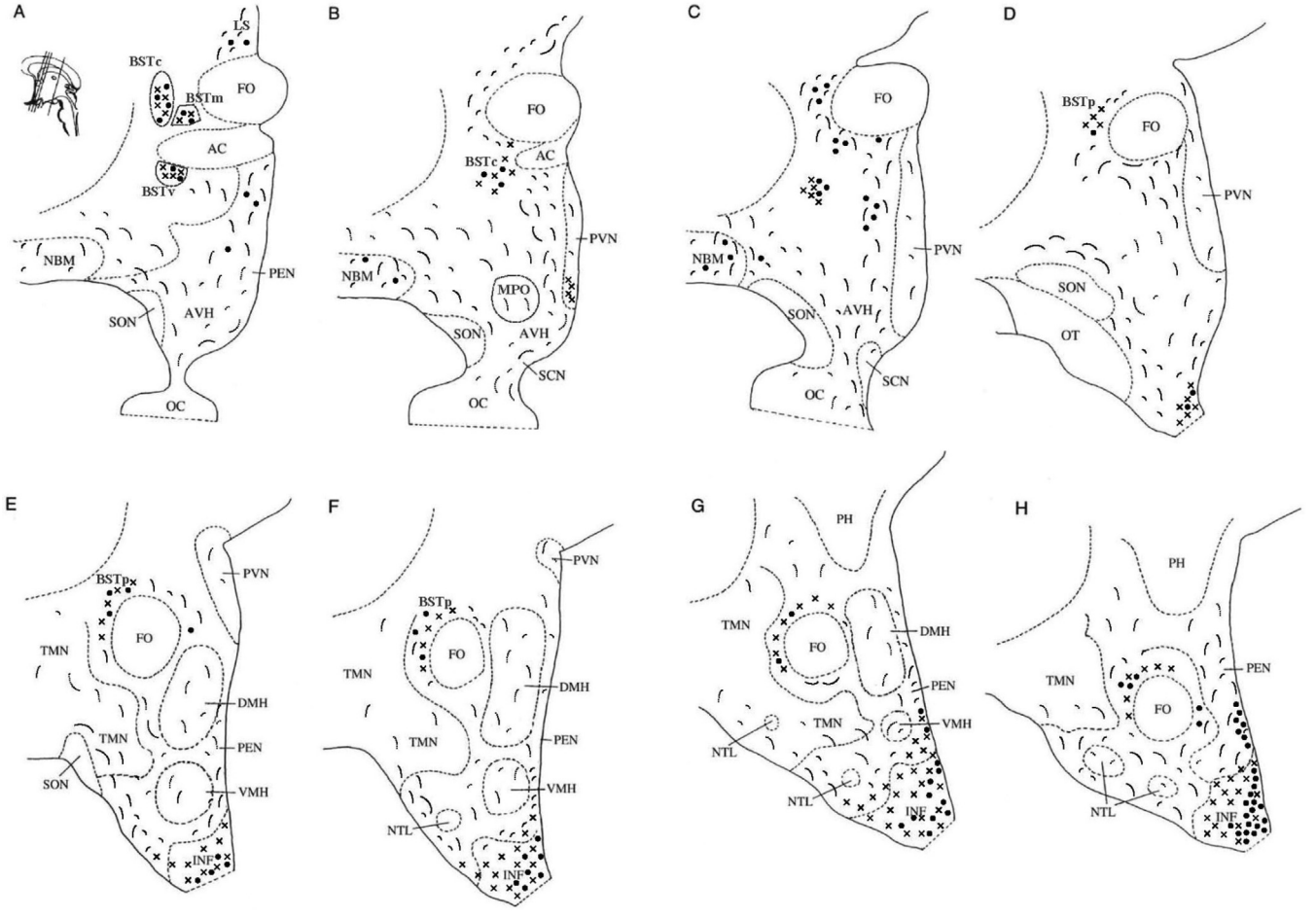

Fig. 18.3. Schematic drawing from anterior to posterior (A–H) in a representative female hypothalamus to illustrate the distribution of neurokinin-B immunoreactive (NKB-ir) cells and fibers. NKB-ir cells are represented by close circles, whereas NKB-ir fibers are represented by *dotted lines* (single fibers or low density), *continuous lines* (moderate density), and *crossed lines* (high density). Abbreviations: *AC*, anterior commissure; *AVH*, anteroventral hypothalamic area; *BSTc*, bed nucleus of the stria terminalis, central part; *BSTm*, bed nucleus of the stria terminalis, medial part; *BSTp*, bed nucleus of the stria terminalis, posterior part; *BSTv*, bed nucleus of the stria terminalis, ventral part; *DMH*, dorsomedial nucleus of the hypothalamus; *FO*, fornix; *INF*, infundibular nucleus; *LS*, lateral septum; *MPO*, medial preoptic nucleus; *NBM*, nucleus basalis of Meynert; *NTL*, lateral tuberal nucleus; *OC*, optic chiasm; *OT*, optic tract; *PEN*, periventricular nucleus; *PH*, posterior hypothalamic nucleus; *PVN*, paraventricular nucleus; *SCN*, suprachiasmatic nucleus; *SON*, supraoptic nucleus; *TMN*, tubero mamillary nucleus; *VMH*, ventromedial nucleus of the hypothalamus. Reproduced from Taziaux M, Swaab DF, Bakker J (2012). Sex differences in the neurokinin B system in the human infundibular nucleus. J Clin Endocrinol Metab 97: E2210–2220, with permission of Endocrine Society, Oxford University Press.

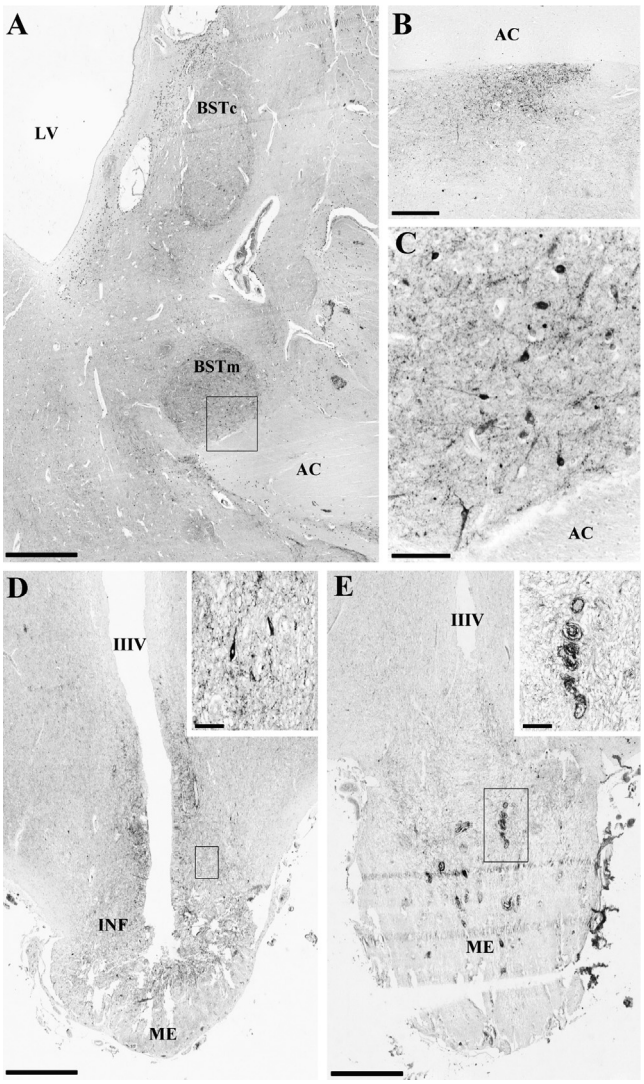

Fig. 18.4. Representative photomicrographs illustrating the main localization of NKB-ir cells and fibers in the human hypothalamus: BSTm and BSTc (A), BSTv (B), INF (D), and ME (E). (C) and the insets in (D) and (E) show higher magnifications. Scale bars: 1 mm in (A), (D), and E; 0.5 mm in (B); 0.05 in (C) and in the insets. Abbreviations: *AC*, anterior commissure; *BSTc*, bed nucleus of the stria terminalis, central part; *BSTm*, bed nucleus of the stria terminalis, medial part; *BSTv*, bed nucleus of the stria terminalis, ventral part; *INF*; infundibular nucleus; *LV*, lateral ventricle; *ME*, median eminence; *IIIV*, third ventricle. Reproduced from Taziaux M, Swaab DF, Bakker J (2012). Sex differences in the neurokinin B system in the human infundibular nucleus. J Clin Endocrinol Metab 97: E2210–2220, with permission of Endocrine Society, Oxford University Press.

present throughout the hypothalamus, notably in the medial POA, lateral septum, anteroventral hypothalamus, NBM, VMN, the dorsomedial hypothalamus, and in the periventricular area but are particularly prominent in the BST (Fig. 18.4A–C) and the INF/ME complex (Fig. 18.4D and E). The distribution of NKB peptide is in agreement with the distribution of NKB mRNA (Chawla et al., 1997) and that of preprotachikinin B protein (Hrabovszky et al., 2010).

Prominent sex differences can be observed at two sites, i.e., the INF and the pars tubularis (PT). Although the PT is totally devoid of NKB-ir during the infant/pubertal period in both sexes, adult men display a dense NKB innervation in the PT, which is totally absent in women (Taziaux et al., 2012; Fig. 18.5). By contrast, in elderly subjects, both men and women show high levels of NKB-immunoreactivity in this area. This sex difference is thus only visible in adulthood. Similar observations have been reported in the rat, in which a male-specific NKB innervation is observed around blood vessels of the external zone of the ME, compared with a more diffuse axonal wiring in the female (Ciofi et al., 2006). Moreover, in rats, the masculine phenotype emerges only at puberty and is activated by androgens (Ciofi et al., 2007). Based upon the pattern observed in adults, it is likely that androgens stimulate, whereas

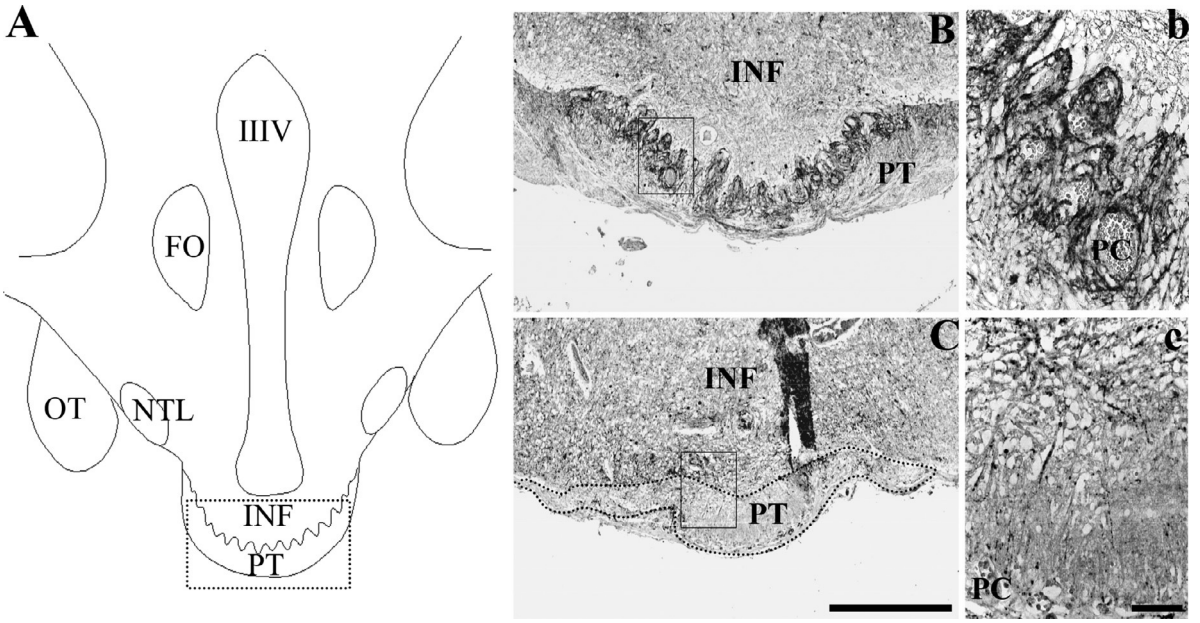

Fig. 18.5. Sexually dimorphic NKB innervation in the PT. (A) Schematic drawing depicting the location of the human PT. The *box* in (A) illustrates the area photographed from a 39-year-old man (B) and a 34-year-old woman (C). The *boxes* in (B) and (C) illustrate the area rephotographed at higher magnification in panels (b) and (c). The boundaries of the PT are drawn in *dotted line* (C). Scale bars: 0.5 mm in (A) and (B); 0.05 in (a) and (b). *FO*, fornix; *INF*, infundibular nucleus; *NTL*, lateral tuberal nucleus; *OT*, optic tract; *PC*, portal capillaries; *PT*, pars tuberalis. Reproduced from Taziaux M, Swaab DF, Bakker J (2012). Sex differences in the neurokinin B system in the human infundibular nucleus. J Clin Endocrinol Metab 97: E2210–2220, with permission of Endocrine Society, Oxford University Press.

estrogens inhibit this NKB innervation in the PT. Although the PT can be considered as a gateway uniquely placed to influence communication between the hypothalamus and the pituitary, there is currently no insight into the function of the male-specific NKB innervation of the PT.

In the INF, there are strong sex differences in NKB-ir fibers with females showing a much higher density of NKB-ir fibers than men (measured as total volume of NKB-ir), but no significant sex difference in the number of NKB-expressing cell bodies (Taziaux et al., 2012; Figs. 18.6 and 18.7A–D). However, one study reported a somewhat higher regional density of NKB-ir somata in women compared to men (Hrabovszky et al., 2011). In contrast to KP expression, sex differences in NKB expression seem to emerge later in life, i.e., progressively from puberty to adulthood, where the female-dominant sex difference appears for the first time and continues over the years, well into the elderly period (Taziaux et al., 2012). Although it is generally thought that the sexual differentiation of the neuroendocrine hypothalamus does not proceed beyond the early postnatal period, puberty has been recognized as another period of development during which sex steroid hormones organize the nervous system (Sisk and Zehr, 2005). Several hypothalamic structures seem to differentiate later in life, such as the sexually dimorphic nucleus of the POA (between 4 years of age and puberty; Swaab and Hofman, 1988), the darkly stained posteromedial components of the BST (around puberty; Allen and Gorski, 1990), and the central part of the BST (into adulthood; Chung et al., 2002). Furthermore, the sexually dimorphic NKB innervation in the rat does not become visible before puberty (Ciofi et al., 2007). Therefore, it is likely that the sex difference in human NKB expression also reflects organizational actions of sex steroid hormones during the prenatal period. However, this sex difference is only revealed postpubertally, suggesting that it needs to be activated by sex steroid hormones as well.

As was also shown for KP-ir cell bodies (Taziaux et al., 2016), a hypertrophy of NKB-ir neurons was observed in postmenopausal women compared to premenopausal women, most likely reflecting the loss of ovarian estradiol (Taziaux et al., 2012; Fig. 18.7F).

Taken together, sex differences in NKB expression can be observed (female > male) but are clearly less prominent in comparison to KP expression.

KDNy NEURONS IN HUMANS?

In many species, KP and NKB can be found to be co-expressed with dynorphin A in a population of neurons in the ARC (or INF), also known as the KNDy neurons (Krajewski et al., 2005; Goodman et al., 2007;

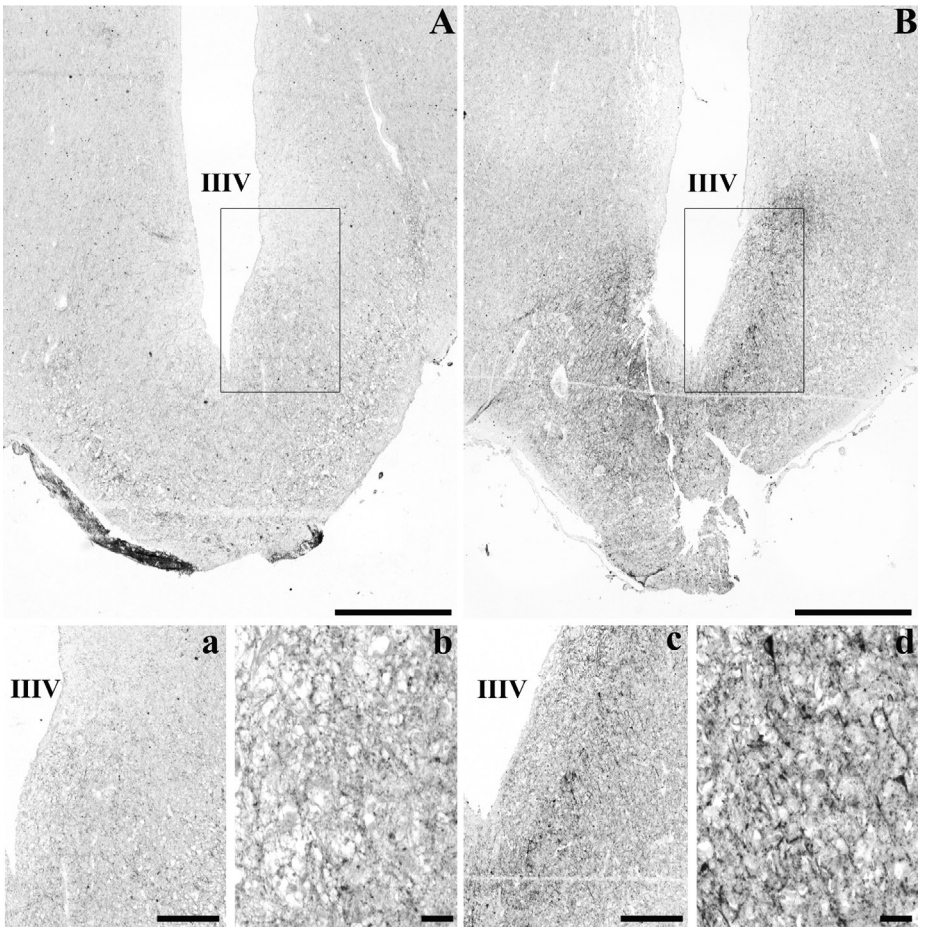

Fig. 18.6. Representative photomicrographs of the sexually dimorphic NKB immunoreactivity in the INF between adult men (A) and women (B). The *boxes* in panels (A) and (B) illustrate the area rephotographed at higher magnification in (a) and (b). Scale bars: 0.5 mm in (A) and (B); 0.25 mm in (a) and (c); 0.05 mm in (b) and (d). *IIIV*, third ventricle. Reproduced from Taziaux M, Swaab DF, Bakker J (2012). Sex differences in the neurokinin B system in the human infundibular nucleus. J Clin Endocrinol Metab 97: E2210–2220, with permission of Endocrine Society, Oxford University Press.

Navarro et al., 2009; Wakabayashi et al., 2010). Indeed, overlap between NKB-ir and KP-ir perikarya have been observed when using dual fluorescence immunohistochemistry (Molnar et al., 2012). In this particular study, in which brain tissues from young men (<50 years), aged men (>50 years) and aged, postmenopausal women (>55 years) were compared, 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 contain NKB-ir. 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) contain KP-ir. However, in young men, most of the NKB-ir perikarya are single-labeled and only 35.8%± 5.1% contain KP-ir. This suggests that KP expression in NKB neurons is highly sex and age dependent.

Remarkably, additional colocalization studies have revealed unexpectedly low levels (if any) of dynorphin (A or B) signal in neuronal cell bodies in the INF from young men (Hrabovszky et al., 2012) or in postmenopausal women (Skrapits et al., 2015). Dynorphin signal is absent from most KP neurons and fibers, in contrast with the extensive coexpression previously reported in rodents (Navarro et al., 2009), sheep (Goodman et al., 2007), and goats (Wakabayashi et al., 2010). By contrast, using in situ hybridization, Dynorphin expressing cells show a similar postmenopausal hypertrophy as KP and NKB neurons in the INF (Rometo and Rance, 2008), suggesting a possible role for Dynorphin in the negative feedback regulation of GnRH and LH release. Whether the absence of dynorphin in KP/NKB neurons indeed represents important species differences or whether it is caused by an increased postmortem degradation of dynorphin in KP/NKB neurons still needs to be clarified.

By contrast, the tachykinin peptide substance P (SP) shows important sex differences in the INF, with greater numbers of SP-ir neurons in postmenopausal women compared to age-matched men (Hrabovszky et al., 2013). Furthermore, SP-ir can be detected in large

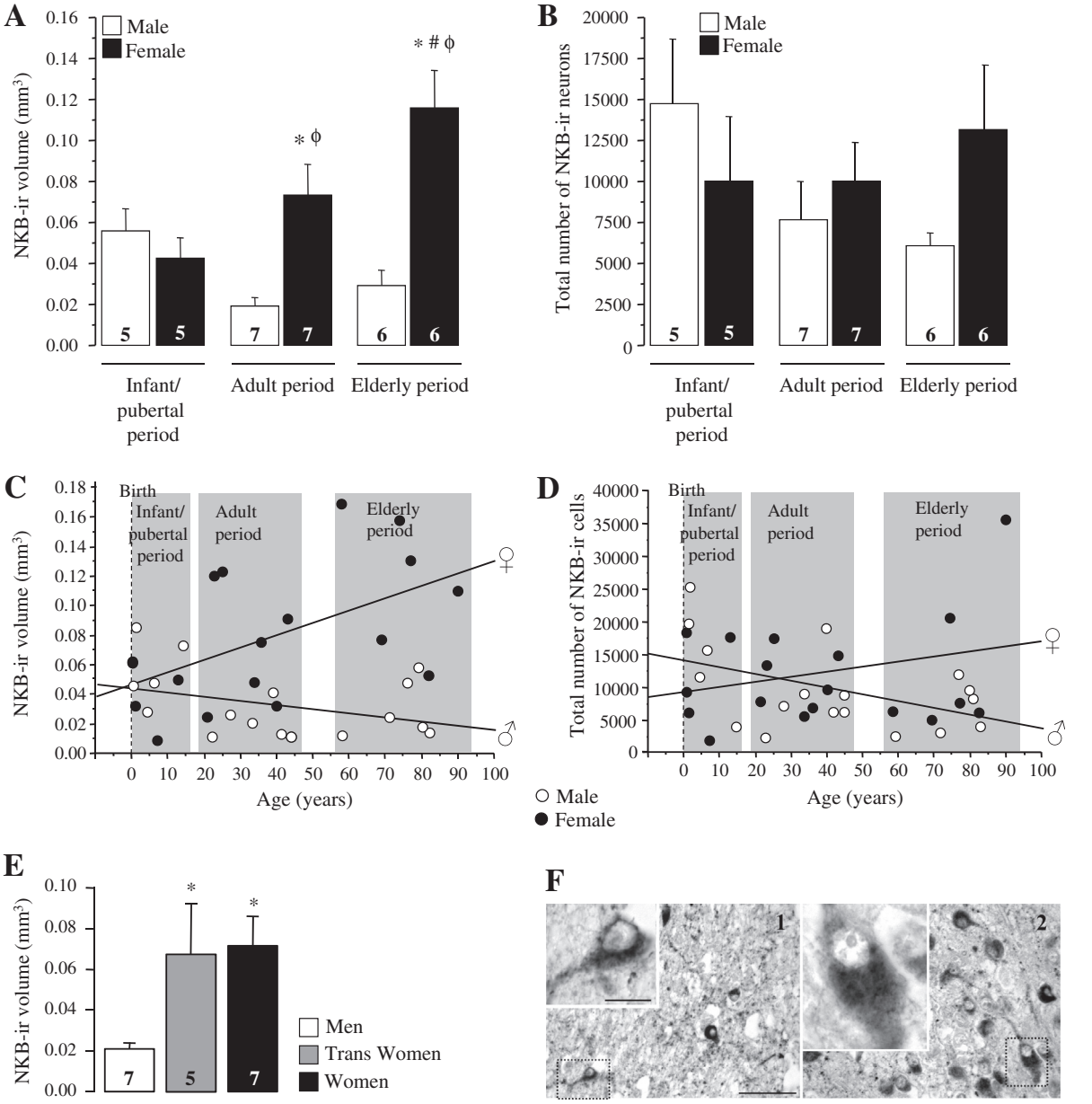

Fig. 18.7. Estimation of the volume occupied by NKB-ir (A) and of the total number of NKB-ir cells (B) in the INF of males and females during the infant/pubertal period (between 5 months and 14 years), the adult period (between 22 and 44 years), and the elderly period (between 58 and 90 years). * $P < 0.05$ vs male from the adult period; # $P < 0.05$ vs female from the adult period; ϕ $P < 0.05$ vs male from the elderly period. Estimation of the total NKB-ir volume (C) and of the total number of NKB-ir cells (D) in the INF of both sexes from the postnatal period (5 months) to the elderly period (90 years). The lines represent the regression line for each sex. (E) Estimation of the volume occupied by NKB-ir in the INF of men, women, and trans women in adulthood. * $P < 0.05$ vs male. (F) Representative microphotographs illustrating the increase and hypertrophy of neurons expressing NKB in postmenopausal women (panel 2; 90-year-old) compared to premenopausal women (panel 1; 34-year-old) in the INF (40x objective). The *boxes* in panels (1) and (2) illustrate the area rephotographed at higher magnification (100 × objective) in the insets. Scale bars: 0.05 mm in panels 1 and 2; 0.01 in the insets. Reproduced from Taziaux M, Swaab DF, Bakker J (2012). Sex differences in the neurokinin B system in the human infundibular nucleus. J Clin Endocrinol Metab 97: E2210–2220, with permission of Endocrine Society, Oxford University Press.

subsets of KP-ir and NKB-ir neurons in the INF: 31% of KP-ir and 25% of NKB-ir perikarya contain SP, whereas 16.5% of all labeled cell bodies exhibit all three neuropeptides (Skrapits et al., 2015). Dual- and triple-labeled fibers are also detected in the infundibular stalk, raising the possibility that these peptides are coreleased into the portal circulation. The absence of dynorphin, but the presence of SP in KP/NKB neurons suggests that

compared to rodents and sheep, humans might use different neuropeptide signaling mechanisms to regulate sex steroid feedback on GnRH secretion.

CONNECTIONS OF KP AND NKB NEURONS WITH GnRH NEURONS

Double immunohistochemistry for KP and GnRH has shown that KP-ir neurons in the INF are in contact with other KP-ir neurons (cell bodies and dendrites) as well as that they innervate somatic and dendritic compartments of GnRH neurons (Hrabovszky et al., 2010). There is a robust sex difference in the number of KP-ir contacts with higher innervation in postmenopausal women compared to age-matched men. Furthermore, 26% of these contacts of KP-ir neurons on GnRH bodies also contain NKB-ir in postmenopausal women, whereas it is lower (10%) in age-matched men (Hrabovszky et al., 2013). At present there is no immunohistochemical evidence for the presence of GPR54 on GnRH axons, albeit that NK3 receptors have been localized on GnRH axons in the rat (Krajewski et al., 2005).

Furthermore, KP-ir axons have been found to form sporadic appositions onto GnRH-ir fibers in the INF stalk and around the portal capillary vessels of the postinfundibular ME (Hrabovszky et al., 2010). In contrast to rodents, in which most GnRH axons in the ME terminate in the external zone, many GnRH neurons in humans and monkeys travel long distances in the INF stalk and descend all the way down to the posterior pituitary (King and Anthony, 1984). GnRH fibers in this descending tract are also accompanied and occasionally contacted by KP-ir axons (Hrabovszky et al., 2010). The observation that KP-ir axons can be found around the portal vasculature of the human postinfundibular ME suggests that KP might be secreted into the hypophysial portal circulation and could act directly on gonadotrophs to affect LH secretion from the pituitary.

CLINICAL IMPLICATIONS OF KP AND NKB IN HUMAN REPRODUCTION

The discovery of KP and NKB being potent regulators of GnRH secretion and thus critically involved in pubertal maturation and the maintenance of adult reproductive function has led to a huge scientific interest in the role of KP and NKB in controlling the reproductive axis in numerous animal models as well as in humans. (Hunjan and Abbara, 2019). This has opened up the possibility of manipulating KP signaling in disorders related to decreased GnRH signaling, such as hypogonadotropic hypogonadism and subfertility but also in disorders in which the reproductive axis needs to be suppressed, such as hormone-sensitive cancers.

A variety of clinical studies have shown that exogenously administered KP, either intravenously or through a bolus injection subcutaneously, reliably increases LH secretion in both men and women (reviewed in Clarke et al., 2015). This effect seems to be more pronounced in the preovulatory phase of the menstrual cycle (Dhillo et al., 2007), suggesting that it is dependent on circulating estradiol levels. Furthermore, it was found that KP increases LH pulsatility in women with hypothalamic amenorrhea (Jayasena et al., 2009), although chronic administration induces tachyphylaxis, i.e., desensitization due to a downregulation of the KP receptor. Furthermore, KP administration induces egg maturation in a dose-dependent manner in women undergoing in vitro fertilization treatment providing hope that KP may be successfully used to develop new or to improve existing fertility treatments.

By contrast, NKB infusion has been shown to induce hot flushes in a group of healthy women (Jayasena et al., 2015) suggesting that NKB plays a role in thermoregulation which has also been shown in mice (Padilla et al., 2018). This opens the possibility of new treatments in which NKB signaling can be blocked pharmaceutically to reduce the appearance of hot flushes during the menopause or during treatment of sex-steroid sensitive cancers.

KP AND NKB EXPRESSION IN RELATION TO GENDER IDENTITY AND SEXUAL ORIENTATION

In many animal species, including humans, strong sex differences are evident in KP and to a lesser extent in NKB expression. Most likely these sex differences reflect organizational actions of sex steroids during early development. KP expression can already be detected in human fetuses, but there is currently no information available on the presence of any sex differences before birth. However, in many species, sex differences in KP expression do not become visible before puberty, such as the sexually dimorphic KP population in the AVPv/PeN in the mouse. Interestingly, it has been shown that perinatal exposure to testosterone or estradiol derived from neural aromatization of testosterone completely masculinizes this population in female rodents (Kaufmann et al., 2007). Conversely, estradiol is required during a specific prepubertal period to induce female-typical numbers of kisspeptin neurons (Clarkson et al., 2009; Bakker et al., 2010). Estradiol has thus both masculinizing and feminizing effects on this particular KP population depending on when it is present during development: when present perinatally in males, it induces masculinization but when present postnatally (after P15) in females, it induces feminization. This is highlighted by

results obtained in ArKO mice, i.e., both male and female ArKO mice show low numbers of KP neurons (Bakker et al., 2010), which suggests that this population has not been sexually differentiated in this mouse model due to the absence of estrogens in both sexes.

The observed sex difference in KP expression reflecting organizational actions of sex steroid hormones in animal models makes KP an interesting candidate neuropeptide to study also in relationship to the sexual differentiation of the human brain and subsequently for potential effects of sex steroid hormones on the human brain. This question has in particularly been raised with regard to fundamental features of human existence, such as gender identity and sexual orientation. Gender dysphoria or gender incongruence has been defined as a marked and persistent incongruence between an individual's experienced gender and the at birth assigned sex (DSM-5; ICD-11). A prominent hypothesis on its etiology proposes that the condition is related to the sexual differentiation of the brain and, specifically, to the fact that different critical periods exist for the development of the reproductive organs vs the brain, thereby positing that these processes have been affected differentially in individuals with gender incongruence (Swaab, 2007). Postmortem studies investigating the brains of individuals with gender incongruence have generally confirmed the sexual differentiation hypothesis. For example, a female-typical volume and number of neurons in the central subdivision of the bed nucleus of the stria terminalis and the third interstitial nucleus of the anterior hypothalamus (INAH-3) have been observed in transgender females (male sex assigned at birth and female gender identity) (Zhou et al., 1995; Kruijver et al., 2000; Garcia-Falgueras and Swaab, 2008). By contrast, functional and structural neuroimaging studies have shown more mixed results. Some studies reported a sex reversal, i.e., hypothalamic responses to the male-chemosignal androstadienone were in line with their experienced gender in both transgender boys and girls (Burke et al., 2014), as well as masculinized neural activity patterns while performing a mental rotation task in transgender boys (female sex assigned at birth, male gender identity, treated with GnRH agonists to inhibit puberty at the time of the study; Burke et al., 2016). By contrast, at the structural level, gray matter volumes were largely concordant with their sex assigned at birth (e.g., Hoekzema et al., 2015).

In two postmortem studies (Taziaux et al., 2012, 2016), KP and NKB expression were analyzed in transgender individuals. The MTF transsexual group consisted of seven sex-reassigned and estrogen-treated individuals and one individual who was not orchidectomized but hormonally treated. A nontreated individual with strong cross-gender identity feelings, which were already present since early childhood, and one trans man (female sex assigned at birth, male gender identity) were also analyzed. Interestingly, female-typical numbers of KP-ir and NKB-ir neurons were observed in the INF of trans women who had undergone estrogen treatment and sex-reassignment in adulthood (Taziaux et al., 2012, 2016; Fig. 18.2D and E, Fig. 18.7D). Linear regression analyses indicated that the number of KP-ir neurons was not correlated with age in transgender individuals but is negatively, albeit not significantly, correlated with the duration of hormonal treatment (Fig. 18.2E), such that long-term estrogen treatment was associated with lower number of KP-ir neurons. Finally, it is interesting to note that the one trans man subject had a number of KP-ir neurons (144 neurons) in the male range, while the untreated trans gender subject (S7) showed an intermediate number of KP-ir neurons (1465 neurons).

The sex reversal of the KP and NKB neuronal populations in the INF might be explained either by the presence of higher estrogen concentrations in the blood due to prolonged estrogen treatment or the lack of androgens due to orchidectomy (or antiandrogen treatment). Due to the low number of individuals as well as the strong variability in the duration of hormone treatment and KP/NKB expression, it is difficult to draw any strong conclusions on this particular observation of a female-typical KP and NKB population in trans women. It could indeed indicate a sex-atypical differentiation of the hypothalamus. However, it has previously been reported that the LH surge in trans women was male-typical, i.e., no LH surge, before sex reassignment and almost female-typical afterwards, i.e., significant rise in LH, suggesting that long-term estrogen treatment could feminize the gonadotropin response (Gooren, 1986). This could suggest that LH responses to estrogens in humans might not be as perinatally fixed as is the case in rodents but might depend on the nature of circulating sex steroids with androgens generally being inhibitory.

KP EXPRESSION IN RELATION TO SEXUAL ORIENTATION

One theory of homosexual orientation is that it results from low fetal exposure to testosterone and that the absence of organizational effects of testosterone in homosexual men is responsible for a feminization of certain brain regions. Indeed, hypothalamic differences in relation to sexual orientation have been observed. For example, in accordance with this theory, a smaller INAH-3 (LeVay, 1991) and a larger anterior commissure (Allen and Gorski, 1992), both thus "female-like," were observed in homosexual compared to heterosexual men.

By contrast, a larger suprachiasmatic nucleus (Swaab and Hofman, 1990) was observed in homosexual men compared to heterosexual men, whereas there is no sex difference observed in this nucleus. In addition, another hypothalamic nucleus that shows sex differences, i.e., the sexually dimorphic nucleus (SDN: male > female) did not differ between homosexual and heterosexual men. These findings do not support the hypothesis of a female-typical hypothalamus in homosexual men.

In the study of Taziaux et al. (2016), the number of KP-ir neurons was compared between homosexual and heterosexual men. The number of KP-ir neurons was higher in homosexual men compared to heterosexual men and appeared to be "female-like" (Fig. 18.2F). However, since all homosexual subjects died of acquired immunodeficiency syndrome (AIDS), which has been associated with subnormal testosterone levels and hypogonadism (Sellmeyer and Grunfeld, 1996) and thus most likely a reduced negative feedback, brain tissues from heterosexual men who died from AIDS were included as additional controls. Increased KP expression was observed in HIV+ heterosexual men and when compared to HIV+ homosexual men, there were no significant differences, suggesting that the number of KP neurons does not vary with sexual orientation. Furthermore, this particular finding is not in support of the hypothesis of a female-typical hypothalamus in homosexual men.

CONCLUDING REMARKS

As has been shown in many animal species, important sex differences can be observed in KP and NKB expression in the human hypothalamus. These sex differences might be related to sex differences in GnRH functioning, i.e., cyclical in women and tonic in men. It also most likely suggests that any positive feedback actions of estrogens on GnRH secretion are mediated by KP neurons in the INF and that there is no such role for more rostral KP neurons. Interestingly, although these sex differences probably reflect organizational actions of sex steroid hormones, the human INF KP system appears to remain sensitive to gonadal hormones throughout life since KP expression is higher in the infant/prepubertal and elderly periods, which are both characterized by low levels of circulating hormones. The sex reversal observed in KP and NKB expression in trans women might reflect, at least partially, an atypical sexual differentiation of the brain.

It is important to note that these observations are based on postmortem brain material derived from a rather heterogeneous patient population. The high variability in the number of KP- and NKB-ir neurons especially in females could be partially explained by limitations related to the use of postmortem brain tissue, for which conditions at death cannot be tightly controlled. Other confounding factors are the known sex differences in brain weights, but no such sex differences were observed for the INF between adult men and women.

Finally, postmortem studies using immunohistochemistry and/or in situ hybridization remain important tools to increase our understanding of the human reproductive axis and associated neuroendocrine disorders. Future studies should focus on a further characterization of INF KP neurons in humans, such as whether they express steroid hormone receptors and to which brain areas they project, i.e., whether they project to other areas in the brain in addition to the median eminence. Interestingly, GPR54, the kisspeptin receptor, has been shown to be expressed outside the hypothalamus as well (Muir et al., 2001). This is particularly interesting in light of the recent finding of a specific, stimulatory role for kisspeptin in female sexual behavior in the mouse (Hellier et al., 2018). This latest discovery leads to the question whether kisspeptin might play a very similar role in women.

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