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Sex Differences in the Neurokinin B System in the Human Infundibular Nucleus

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Context: The recent report that loss-of-function mutations in either the gene encoding neurokinin B (NKB) or its receptor (NK3R) produce gonadotropin deficiencies in humans strongly points to NKB as a key regulator of GnRH release.

Objectives: We used NKB immunohistochemistry on postmortem human brain tissue to determine: 1) whether the human NKB system in the infundibular nucleus (INF) is sexually dimorphic; 2) at what stage in development the infundibular NKB system would diverge between men and women; 3) whether this putative structural difference is reversed in male-to-female (MtF) transsexual people; and 4) whether menopause is accompanied by changes in infundibular NKB immunoreactivity.

Methods: NKB immunohistochemical staining was performed on postmortem hypothalamus material of both sexes from the infant/pubertal period into the elderly period and from MtF transsexuals.

Results: Quantitative analysis demonstrated that the human NKB system exhibits a robust female-dominant sexual dimorphism in the INF. During the first years after birth, both sexes displayed a moderate and equivalent level of NKB immunoreactivity in the INF. The adult features emerged progressively around puberty until adulthood, where the female-dominant sex difference appeared and continued into old age. In MtF transsexuals, a female-typical NKB immunoreactivity was observed. Finally, in postmenopausal women, there was a significant increase in NKB immunoreactivity compared to premenopausal women.

Conclusion: Our results indicate that certain sex differences do not emerge until adulthood when activated by sex steroid hormones and the likely involvement of the human infundibular NKB system in the negative and positive feedback of estrogen on GnRH secretion. **(*J Clin Endocrinol Metab* 97: 0000–0000, 2012)**

Because immunohistochemical studies have failed to show colocalization of estrogen receptor α in GnRH neurons in different species [rat (1), sheep (2), primates (3)], 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. Human genetic studies demonstrated that kisspeptin (4) as well as neurokinin B (NKB) signaling (5) are both potent regulators of GnRH secretion and are therefore thought to be essential for the onset of puberty and the maintenance of adult reproductive function. In humans, infundibular kisspeptin neurons are sexually dimorphic (6), but no information is available for the NKB system, although it has been shown to be sexually dimorphic in rat

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Abbreviations: ARC, Arcuate nucleus; AVH, anteroventral hypothalamus; BST, bed nucleus of the stria terminalis; BSTc, BST central part; DAB, 3,3-diaminobenzidine; IBAS, image-based analysis system; INF, infundibular nucleus; ME, median eminence; MPO, medial preoptic nucleus; MtF, male-to-female; NBM, nucleus basalis of Meynert; NKB, neurokinin B; NKB-ir, NKB immunoreactive; NPY, neuropeptide Y; PT, pars tuberalis; TBS, Tris-buffered saline.

(7, 8) and sheep (9, 10). Therefore, in the present study, we first asked whether the NKB system is sexually dimorphic in the human hypothalamus. By using postmortem brain tissues, we mapped the distribution of NKB fibers and cell bodies in the human hypothalamus and investigated the presence of putative sex differences. Next, we determined whether these sex differences are potentially the result of activational actions of sex steroid hormones by analyzing NKB expression at different stages of life (postnatal, puberty, adulthood, old age) in both sexes. We also included brain material from male-to-female (MtF) transsexuals who had undergone estrogen treatment in adulthood.

Subjects and Methods

Human brain tissue

Hypothalami of 42 subjects (Table 1) were obtained through autopsies by The Netherlands Brain Bank following the required permissions for brain autopsy and the use of tissue and medical information for research purposes. The subjects were categorized in an infant/pubertal period, an adult period, and an elderly period. The MtF transsexual group consisted of four sex-reassigned and estrogen-treated individuals and one individual who was not orchidectomized but was hormonally treated (see Table 2 for detailed hormonal profiles). A nontreated individual with strong cross-gender identity feelings, which were already present since his earliest childhood, was also analyzed. Exclusion criteria for the subjects were a history of endocrine deregulation (ovarian, uterine, or breast cancer; recent abortion; or pregnancy), use of corticosteroids or drugs affecting the hypothalamo-pituitary-gonadal axis during at least the last month before death, and neurodegenerative or psychiatric diseases. The subjects were matched for age, postmortem delay, and the duration of the formalin fixation.

Histology

Hypothalami were formalin-fixed, paraffin-embedded, and cut serially in 6-μm coronal sections from rostral to caudal. Every 100th section was collected on a SuperFrost/Plus (Menzel, Braunschweig, Germany) slide and stained with 0.5% thionin for general orientation. Every 50th section was then collected over the whole length of the hypothalamus for NKB immunocytochemistry to map the distribution of NKB-immunoreactive (NKB-ir) cells and fibers. To determine the border of the INF, every 50th section—adjacent to the NKB-stained ones— of the putative INF was mounted and stained for neuropeptide Y (NPY) (11).

NKB and NPY immunohistochemistry

Unless mentioned otherwise, all incubations were carried out at room temperature, and all washes were performed using Tris-buffered saline (TBS; 0.05 M Tris and 0.9% NaCl; pH 7.6). Sections were deparaffinized and rehydrated. Antigen retrieval was used for NKB (but not NPY) staining by placing sections in citrate buffer (0.1 M citric acid, 0.1 M trisodium citrate, pH 6.0) in a microwave (10 min at 700 W). Next, sections were saturated in TBS-milk [5% nonfat dry milk (Elk; Campina Melkunie, Eindhoven, The Netherlands)] for 1 h to decrease nonspecific binding. Sections were then incubated overnight at 4 C with a rabbit polyclonal anti-NKB antibody (1:2000; Peninsula Laboratories, San Carlos, CA; T-4450) or a rabbit polyclonal anti-NPY antibody (1:1000; Niepke 26/11/1988; Netherlands Institute for Neuroscience, Amsterdam, The Netherlands) diluted in Supermix-milk (0.5% Triton X-100, 0.25% gelatin, 5% milk powder in TBS; pH 7.6). After rinsing in TBS-milk and TBS, sections were incubated with a biotinylated goat-antirabbit antibody (1:400; Vector Laboratories, Burlingame, CA) in Supermix (0.5% Triton X-100, 0.25% gelatin in TBS; pH 7.6) for 1 h. The antibody-antigen complex was amplified with the avidin-biotin complex method (1:800; Kit ABC Vectastain Elite PK-6100; Vector Laboratories PLC, Cambridge, UK). Finally, sections were incubated in nickel-DAB (3,3-diaminobenzidine) solution (0.5 mg/ml; Sigma Chemical, St. Louis, MO; 0.01% H_2O_2; 2.33 mg/ml ammonium nickel sulfate in TBS), rinsed in distilled water, dehydrated, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

NKB and NPY specificity test

Specificity of the NKB antibody was confirmed by a solid phase preadsorption test. The synthetic human NKB peptide (Bachem) was dissolved (1 μg/μl) in isoelectric focusing medium (10% glycerol, 10% dimethylformamide, 2.5% Nonidet; Sigma Chemical) and spotted on five separate strips of gelatin (0.2%) -coated nitrocellulose (0.1 μm pore size, BA45; Schleicher & Schull, Dassel, Germany), each containing 30 μg of peptide. Fixation of the peptide to the nitrocellulose was performed overnight with 4% formaldehyde in a press block, followed by rinses in distilled water, Tris/HCl (0.05 M; pH 7.6), and Supermix, respectively. The anti-NKB antibody was adsorbed at 1:2000 in Supermix-milk 5% during five successive cycles. Binding of the antibody to the peptide was visualized by staining the spotted strips using the ABC method. No staining was observed after absorption of NKB to its blocking peptide. Cross-reactivities of the NKB antibody with other tachykinins were previously excluded (11). The specificity of the NPY antibody was previously demonstrated (12, 13).

Estimation of the total NKB-ir volume of the INF

Digital images of NPY-stained sections throughout the rostrocaudal axis of the INF were made using a 5× objective (Plan-Neofluar lenses) on a Zeiss Axioscope microscope mounted with a Sony B/W CCD camera (XC77CE) and connected to an ImageProPlus version 5.1 image analysis system (MediaCybernetics, Silver Spring, MD). Serial NKB-stained sections (300 μm between two successive sections) were digitized at 10× objective. By referring to the NPY staining images, the contour of the INF was manually outlined on the NKB staining images by an investigator (M.T.) who was blind to the nature of the patients. A standard threshold (corresponding to 2-fold the background) was used for discriminating the labeled material from the background and was quantified by a homemade OD program for DAB-nickel staining. To estimate the volume of the DAB-nickel precipitate as a measure of NKB immunoreactivity (cells and fibers together) throughout the INF, the surface of the outlined area and the masking area of NKB immunoreactivity within the outlined area were calculated in each section. All data were stored, and volume estimates of the INF (INF volume) and of total NKB immunoreactivity within the INF (NKB-ir INF vol-

TABLE 1. Clinicopathological data

Netherlands Brain Bank no. (patient no.)	Age	Brain weight (g)	Postmortem delay (h:min)	Fixation time (d)	Clinicopathological information
Females (n = 18)					
Infant/pubertal period (n = 5)					
86-027	5 months	735	10:00	40	Sudden infant death syndrome
89-027	6 months	780	<17:00	28	Cardiomyopathy
89-036	1 yr	820	NA	31	Hypoglycemia
87-077	7 yr	1320	<9:45	33	Astrocytoma
87-035	13 yr	1250	<13:00	48	Histiocytic lymphoma, cardiac failure
Adult period (n = 7)					
01-009	21 yr	975	19:35	65	Myocardial infarction
84-025	23 yr	1300	<10:00	35	Acute myeloid leukemia
01-072	25 yr	1500	<17:00	31	Found dead (epileptic seizure)
99-058	34 yr	1395	72:00	132	Cardiac abnormalities
91-009	36 yr	1348	<71:30	61	Fecal peritonitis
01-023	40 yr	1279	<41:00	54	Pulmonary carcinoma
97-131	43 yr	1345	<92:00	63	Myocardial infarction
Elderly period (n = 6)					
97-037	58 yr	1221	6:45	28	Epileptic convulsions after craniotomy
98-036	69 yr	1264	6:15	31	Cardiogenic shock
98-104	74 yr	1207	7:25	31	Necrosis of the intestines
97-156	77 yr	1235	2:40	47	Septic shock; metastasized pancreas carcinoma
98-016	82 yr	1078	10:45	35	Congestive cardiac failure
98-089	90 yr	1047	7:15	39	Ruptured abdominal aorta aneurysm
Males (n = 18)					
Infant/pubertal period (n = 5)					
86-041	6 months	800	<6:30	14	Sudden infant death syndrome
88-058	1 yr	1070	<35:35	28	Bacterial meningitis, sepsis
84-016	4 yr	1565	23:55	100	Sepsis
87-057	6 yr	1550	3:30	41	Peritonitis
87-036	14 yr	1640	<41:00	32	Lymphadenopathy
Adult period (n = 7)					
97-083	22 yr	1334	16:29	26	Hypertrophic cardiomyopathy
02-076	27 yr	1520	NA	NA	Drowning
98-031	33 yr	1588	46:25	72	Hemothorax after car accident
99-071	39 yr	1400	<16:30	130	Heart infarction
96-253	41 yr	1150	<17:00	1231	Kidney failure
99-141	44 yr	1565	<10:00	149	Myocardial infarction
99-035	44 yr	1434	<41:00	123	Multiorgan failure
Elderly period (n = 6)					
98-090	58 yr	1528	41:00	72	Collapsed; unsuccessful resuscitation
08-032	71 yr	1600	8:55	70	Pancreas and rectum carcinoma with hepatic metastases
06-028	76 yr	1514	19:35	27	Cardiac arrest
97-143	79 yr	1392	6:00	31	Pulmonary embolism
97-116	80 yr	1380	6:56	33	Respiratory insufficiency, lung emphysema
98-189	81 yr	1276	5:20	33	Unspecified respiratory problems
98-033	82 yr	NA	<89:00	63	Carcinoma of the lung, respiratory problems
MtF transsexuals (n = 5)					
98-137 (T1)	26 yr	1500	NA	NA	Suicide
93-042 (T2)	36 yr	1145	21:43	31	Pneumonia
88-064 (T3)	43 yr	1540	NA	NA	Neurosarcoma
95-018 (T4)	48 yr	1198	<40:20	36	Cardiac arrest
84-020 (T5)	50 yr	1380	NA	30	Suicide
Nontreated male with cross-gender identity feelings (n = 1)					
96-088	84 yr	1433	41	38	Lung carcinoma

NA, Not available.

TABLE 2. Hormonal profile of MtF transsexuals

Netherlands Brain Bank no. (patient no.)	Age (yr)	Age at beginning of hormonal treatment (yr)	Age at castration (yr)	Estrogen treatment	Antiandrogen treatment (cyproterone acetate)
98-137 (T1)	26	21	26	EE 5 μg 2 dd	50 mg 2 dd
93-042 (T2)	36	36	Not operated	Therapeutic doses (at least 5 yr b.d.)	50 mg 1 dd (at least the last 10 months b.d.)
88-064 (T3)	43	36	39	Age 39, EE 50 μg 2 dd (stopped 3 months b.d.)	50 mg 2 dd (stopped 2 yr b.d.)
95-018 (T4)	48	35	36	Age 35, EE 50 μg 2 dd. Age 40, EE 50 μg 1 dd (lasted until death)	50 mg 2 dd (stopped 10 months b.d.)
84-020 (T5)	50	42	44	Age 42, stilbestrol 5 mg 1 dd; after 2 months to 5 mg 2 dd. Age 44, EE 50 μg 2 dd (lasted until death)	Age 44, 50 mg 2 dd (stopped 2 yr b.d.)

NA, Not available; b.d., before death; EE, ethinyl estradiol; dd, dose per d.

ume) were performed by an image-based analysis system (IBAS) conversion program based upon multiplication of the outlined areas and masking area, respectively, by sample frequency corrected for section thickness (6 μm) over the whole length of the INF. The mean ± SEM number of NKB-stained sections quantified per subject was 17.4 ± 3.4.

Estimation of the total number of NKB-positive neurons within the INF

Every other NKB-stained section (600 μm between two successive sections) throughout the INF in the rostrocaudal direction of each subject was used for analysis. Estimates were made using an IBAS (Kontron Elektronik, Chichester, UK), connected to a Sony B/W CCD camera (XC77CE) mounted on a plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives). From the section to be measured, an image covering the INF (×2.5 objective) was loaded into the IBAS and displayed on the computer monitor. In this image, the INF was outlined manually (based on the adjacent NPY staining), and over this outlined area a grid was superimposed. From the respective grid fields, x and y coordinates were stored, and all individual images were retrieved using a 40× objective on the image analysis monitor. For analysis, 100% of the rectangular fields (each field covering at least 50% of the outlined area) were analyzed. To prevent double counting, only neurons containing a nucleolus (~2 μm diameter) were counted. The number of neurons per section was multiplied by the sampling frequency (the interval distances between individual sections) to obtain an estimation of the total number of NKB-ir neurons in the INF from 8.7 ± 1.7 sections per subject (mean ± SEM).

Statistical analysis

Two-way ANOVAs with the stage of life (infant/pubertal *vs.* adult *vs.* elderly period) and the sex (male *vs.* female) as independent factors were used to analyze the overall difference in infundibular NKB-ir volume and NKB-positive cells as well as possible confounding factors. A separate one-way ANOVA was performed to compare the volume occupied by NKB immunoreactivity and the number of NKB-positive cells in the INF among only adult men, adult women, and adult MtF transsexuals. All ANOVAs were followed when appropriate by *post hoc* tests using Fisher's protected least significant difference tests. The Fisher's exact probability test was used to compare the presence of NKB innervation in the pars tuberalis (PT) *vs.* its absence between adult men and women. Differences were considered significant for $P < 0.05$.

Results

NKB expression in the human hypothalamus

NKB-ir fibers are widely distributed throughout the human hypothalamus, whereas cell bodies were confined to a few specific nuclei (Fig. 1). NKB immunoreactivity was found in the cytoplasmic compartment of cell bodies, which exhibited varying size depending on their localization. Small, oval-to-round NKB neurons were numerous in the central and medial portions of the bed nucleus of the stria terminalis (BST) and in the INF/median eminence (ME) complex. The distribution of the neuronal population in the INF exhibited a rostrocaudal topography, with the majority of NKB-ir cells being found in the middle and caudal part of the INF. Less intensely labeled cell bodies were also found scattered periventricularly throughout the caudal extent of the hypothalamus. A small number of medium-sized round NKB neurons were observed in the nucleus basalis of Meynert (NBM), the anteroventral hypothalamus (AVH), the medial preoptic nucleus (MPO), and the posterior BST. Immunoreactive fibers for NKB exhibited varicosities and were abundantly identified throughout the hypothalamus, notably in the MPO, lateral septum, AVH, NBM, the ventromedial hypothalamus, the dorsomedial hypothalamus, and in the periventricular area, but they were particularly prominent in the BST (central, medial, ventral, and posterior portions; Fig. 2, A and B), the INF/ME complex (Fig. 2D), and the ME, where NKB-ir fibers ran spirally around the capillary vessels (Fig. 2E).

Sexual dimorphism of the NKB system

Systematic examination of NKB staining yielded obvious sex differences in the PT and the INF. Although the PT

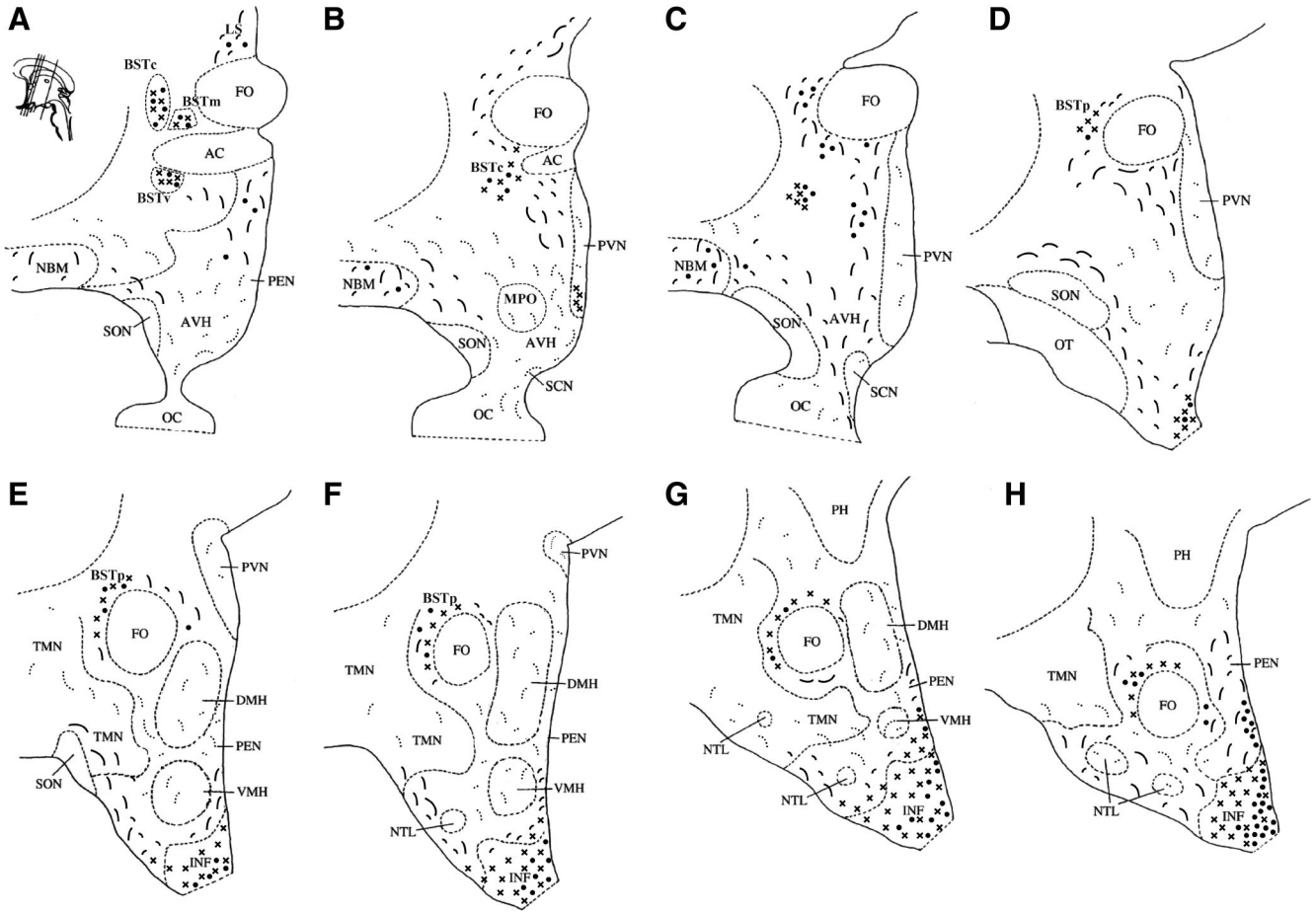

FIG. 1. Schematic drawing from anterior to posterior (A–H) in a representative female hypothalamus to illustrate the distribution of NKB-ir cells and fibers. NKB-ir cells are represented by *closed circles*, whereas NKB-ir fibers are represented by *dotted lines* (single fibers or low density), *continuous lines* (moderate density), and *crossed lines* (high density). AC, Anterior commissure; BSTm, BST, medial part; BSTp, BST, posterior part; BSTv, BST, ventral part; DMH, dorsomedial nucleus of the hypothalamus; FO, fornix; LS, lateral septum; 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, tuberomamillary nucleus; VMH, ventromedial nucleus of the hypothalamus.

of both sexes is totally devoid of NKB immunoreactivity during the infant/pubertal period, 100% of adult men examined in this study displayed a dense NKB innervation in the PT (seven of seven), whereas it was totally absent in adult women (seven of seven). A Fisher's exact probability test comparing the presence of NKB innervation in the PT *vs.* its absence between adult men and women revealed a highly significant effect ($P < 0.001$; see Fig. 3 for representative pictures). However, in elderly subjects, both sexes showed a high level of NKB immunoreactivity in this region; the same was true in MtF transsexuals (hormonally treated or not).

Another robust sexual dimorphism of the NKB system was observed in the INF; adult women consistently displayed more robust NKB staining and denser NKB innervation in the INF than adult men (Fig. 4). This sex difference only appeared in adulthood and continued into old age when NKB immunoreactivity was up-regulated after menopause (Fig. 5A). A two-way ANOVA on NKB-ir volume in the INF revealed an almost significant effect of the stage of life ($F_{2,30} = 3.163$; $P = 0.0567$), a significant effect of sex ($F_{1,30} = 19.134$; $P = 0.0001$), and a significant interaction effect between the two factors ($F_{2,30} = 7.771$; $P = 0.0019$). *Post hoc* tests confirmed that the volume occupied by NKB immunoreactivity in the INF of adult women is larger relative to adult men ($P = 0.032$). This female-dominant sexual dimorphism was heightened at menopause, *i.e.* elderly women have a larger NKB-ir volume in the INF compared with elderly men ($P = 0.012$) and compared with premenopausal women ($P = 0.0455$), whereas the NKB-ir volume in the INF in adult men did not differ from elderly men ($P = 0.8788$). No significant differences in NKB-ir volume were found in the INF between boys and girls during the infant/pubertal period ($P = 0.749$). Likewise, NKB-ir volume in the INF did not significantly differ between girls and adult women ($P = 0.372$), or between boys and adult men ($P = 0.268$). A qualitative examination of the neuronal NKB population in the INF showed a hypertrophy of NKB-ir neurons in

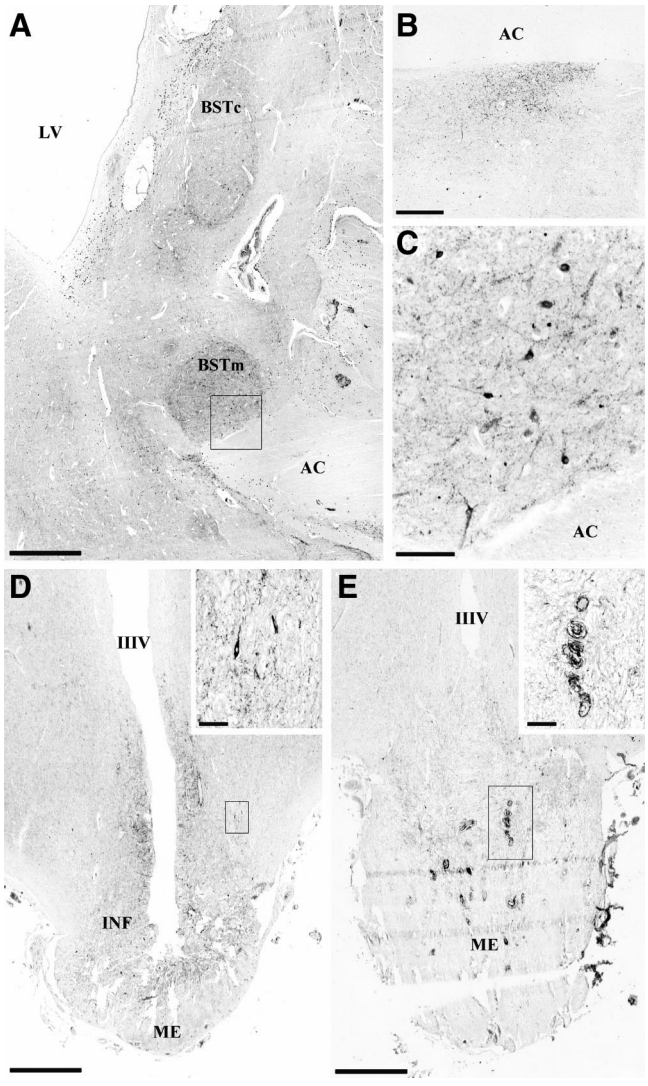

FIG. 2. Representative photomicrographs illustrating the main localization of NKB-ir cells and fibers in the human hypothalamus: BSTm and BSTc (A), BSTv (B), INF (D), and ME (E). Panel C and the *insets* of D and E show higher magnifications. *Scale bars*, 1 mm in A, D, and E; 0.5 mm in B; 0.05 in C and in the *insets*. AC, Anterior commissure; BSTm, BST, medial part; BSTv, BST, ventral part; LV, lateral ventricle; IIIV, third ventricle.

postmenopausal compared with premenopausal women (Fig. 5F).

An estimation of the total number of NKB-positive cells in this nucleus was also performed. Although the pattern of NKB-positive cells was very similar to that of NKB-ir volume (Fig. 5B), a two-way ANOVA failed to reveal any significant difference between the different factors (all $P > 0.10$).

The developmental pattern in NKB expression in the INF throughout life is clearly different between men and women. Regression analysis showed that NKB-ir volume in the INF is positively correlated with age in female subjects from the infant/pubertal period to the elderly period ($r = 0.548$; $P = 0.0185$; $n = 18$), whereas no significant correlation was observed between NKB-ir volume in the INF and age in male subjects ($r = 0.365$; $P = 0.1369$; $n = 18$; Fig. 5C). When the regression analysis was conducted using the total number of NKB-positive cells (Fig. 5D), a just significant negative correlation was observed with age in male subjects ($r = 0.473$; $P = 0.0473$; $n = 18$), but no correlation was found in female subjects ($r = 0.254$; $P = 0.3089$; $n = 18$).

In a separate analysis, we compared the volume occupied by NKB immunoreactivity and the number of NKB-positive cells in the INF among adult men, adult women, and adult MtF transsexuals (Fig. 5E). One-way ANOVA revealed that NKB-ir volume in the INF is significantly different between the three groups ($F_{2,16} = 3.994$; $P = 0.0392$). *Post hoc* tests showed that the volume occupied by NKB immunoreactivity in the INF of adult men (0.021 ± 0.011 mm^3) is significantly lower compared with adult women (0.072 ± 0.038 mm^3; $P = 0.0232$) and the MtF transsexuals (0.072 ± 0.058 mm^3; $P = 0.0351$). However, the NKB-ir volume of adult women and that of MtF transsexuals did not differ ($P = 0.9919$). It is interesting to note that the 84-yr-old gender dysphoric nontreated male subject had an NKB-ir volume (0.152 mm^3) in the postmenopausal female range (0.120 ± 0.045 mm^3). The number of NKB-positive cells was not statistically different between adult men and women and MtF transsexuals (one-way ANOVA; all $P > 0.5$).

Confounding factors

As expected, two-way ANOVA on brain weights revealed a significant effect of sex ($F_{1,19} = 11.660$; $P = 0.0019$) but no significant effect of stage of life ($F_{2,29} = 2.865$; $P = 0.0732$) and no interaction between the two factors ($F_{2,29} = 1.272$; $P = 0.2955$). Although a sex difference in brain weight was detected, such effect was not observed in the volume estimate of the INF (NPY delineated volume of the INF), suggesting that the sex difference observed in NKB-ir volume in the INF was not consecutive to a structural sex difference of the brain area analyzed. Indeed, a two-way ANOVA showed that the volume estimate of the INF is not different at different stages of life ($F_{2,30} = 0.087$; $P = 0.9166$) or between males and females ($F_{2,30} = 0.021$; $P = 0.8853$), and no interaction between the two factors was found ($F_{2,30} = 1.638$; $P = 0.2113$). Moreover, no effects of postmortem delay or fixation time on NKB immunocytochemical staining (NKB-ir volume in the INF and total number of NKB-positive cells in the INF) were observed (all $P > 0.10$).

Discussion

We showed here that the sex difference in NKB expression in the INF reached only significance in adulthood and that

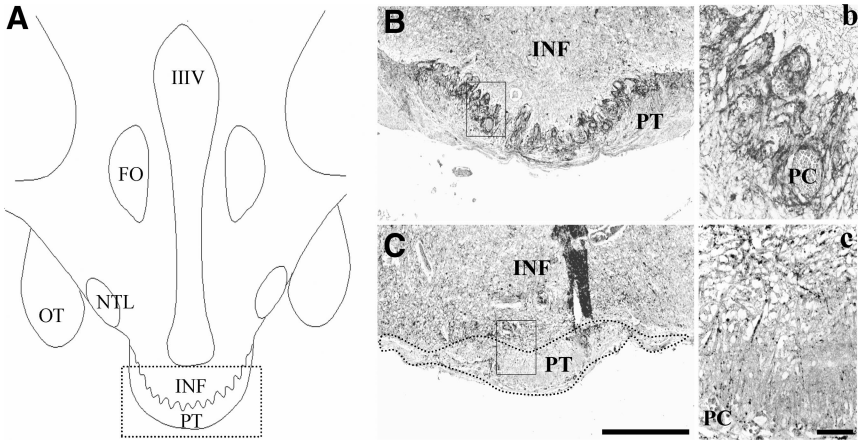

FIG. 3. Sexually dimorphic NKB innervation in the PT. A, Schematic drawing depicting the location of the human PT. The *box* in panel A illustrates the area photographed from a 39-yr-old man (B) and a 34-yr-old woman (C). The *boxes* in B and C illustrate the area rephotographed at higher magnification in panels b and c. The boundaries of the PT were drawn in a *dotted line* (C). *Scale bars*, 0.5 mm in A and B; 0.05 in a and b. FO, Fornix; NTL, lateral tuberal nucleus; OT, optic tract; PC, portal capillaries.

MtF transsexuals had a female-typical infundibular NKB system. These results suggest that: 1) in addition to the well-known perinatal period of steroid-dependent brain organization, sex steroid hormones during puberty might also contribute to the emergence of sex differences in adulthood; and 2) the sex-reversal observed in MtF transsexuals may reflect, at least in part, an atypical sexual differentiation of the hypothalamus.

Distribution of NKB immunoreactivity in the human hypothalamus

We observed that neurons and fibers expressing NKB immunoreactivity were found predominantly in all parts of the BST and in the INF/ME region, where NKB-ir fibers richly innervated portal capillaries. The neuronal distribution is in agreement with the distribution of NKB mRNA (14) and of preprotachikinin B protein (6) in the human hypothalamus, which strengthens antibody specificity as we showed by the preadsorption procedure. NKB neurons have also been described in the INF/arcuate nucleus (ARC) of the monkey (15, 16), sheep (9, 10, 17), goat (18), rat (7, 8, 19), and mouse (20).

Sexual dimorphisms of the human NKB system

A dense plexus of NKB-ir fibers in the PT was consistently found in adult men but was completely absent in adult women. This sex difference is only visible in adulthood, disappears into old age and cannot be attributed to low circulating sex steroid levels given that all orchidectomized and estrogen-treated MtF transsexuals displayed a dense NKB innervation similar to that observed in adult men. Similar observations were reported in the rat, in which a male-specific NKB innervation was observed around blood vessels of the external zone of the ME, compared with a more diffuse axonal wiring in the female (7). Moreover, the masculine phenotype emerges only in puberty and is activated by nonaromatizable androgen (21). Based upon the adult pattern of NKB innervation that we observed in humans, it is likely that androgens stimulate, whereas estrogens inhibit this innervation in the PT in adulthood. Although the PT can be considered as a gateway uniquely placed to influence hypothalamic-pituitary communication and function (22), there is currently no insight into the function of the male-specific NKB innervation of the PT.

Subsequently, we demonstrated that the NKB-ir volume in the INF is sexually dimorphic in adulthood (women > men). Surprisingly, no such significant sex difference was found in the total number of NKB-positive cells in the INF, suggesting a sexually dimorphic NKB innervation rather than a sex difference in the number of NKB-expressing cells. A female-dominant number of NKB neurons has been identified in the rat (8) and sheep (9, 10) ARC. It is interesting to note that although a sex difference was observed in the number of kisspeptin-immunoreactive neurons in humans, the most prominent sex difference was observed in the density of kisspeptin-immunoreactive fibers (6). The statistical discrepancy observed between our two measures of NKB expression could be partly explained by limitations related to the use of postmortem brain tissue, whose conditions at death cannot be tightly controlled [*i.e.* a long postmortem delay may cause fast axonal transport of the neuropeptide between death and fixation (23)]. It should also be noted that NKB gene expression varies with the rat estrous cycle (19), which could partly explain the lack of significant differences in NKB-positive cells between adult men and women, given that the stage of the menstrual cycle at death was not reported in the patient's medical folder.

Finally, the sex difference found in NKB-ir volume in the INF between adult men and women continues during the elderly period, where we also observed an increased infundibular NKB-ir volume and a hypertrophy of NKB-ir neurons in postmenopausal compared with premenopausal women. Our results thus confirm a previous study showing an increase and hypertrophy of neurons expressing NKB mRNA after menopause (24). These changes in NKB mRNA were duplicated in perimenopausal, postmenopausal, and young ovariectomized rhesus macaques (25). Importantly, these changes are reversed by estradiol

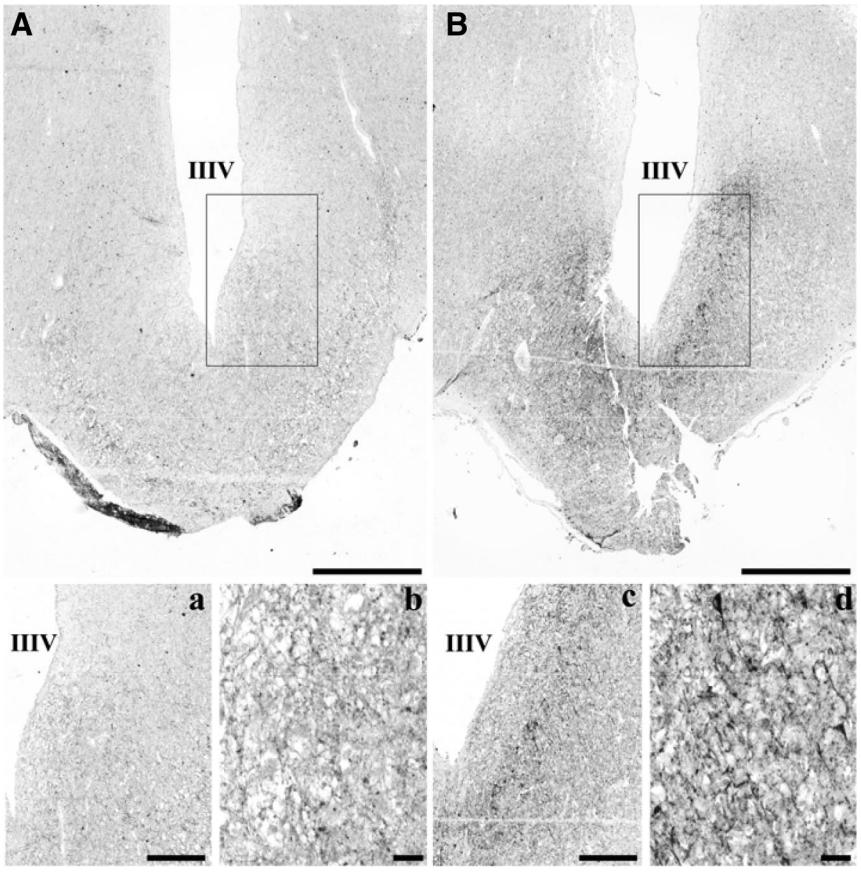

FIG. 4. Representative photomicrographs of the sexually dimorphic NKB immunoreactivity in the INF between adult men (A) and women (B). The *boxes* in panels A and B illustrate the area rephotographed at higher magnification in a and b. *Scale bars*, 0.5 mm in A and B; 0.25 mm in a and c; 0.05 mm in b and d. IIIV, Third ventricle.

replacement (15), suggesting that these changes are likely due to the loss of ovarian estradiol.

The apparent discrepancy between a female-like INF *vs.* a male-like PT observed in MtF transsexuals is in favor of the "brain-sex theory of transsexualism," suggesting atypical organizational effects of sex hormones during development in MtF. Although certain aspects of sexual differentiation might have been altered in MtF transsexuals (such as NKB expression in the INF), they were exposed to androgens throughout life, which could still have masculinized other brain structures and functions, such as the NKB innervation in the PT.

Development of the human infundibular NKB system

On the basis of the ontogenetic profile for GnRH release in primates (26), we hypothesized that the pubertal period might be the time when sex differences could develop in NKB expression. Indeed, we demonstrated that the NKB system is immature in both sexes during the first years after birth and starts to differentiate progressively from puberty to adulthood, where the female-dominant sex difference appears for the first time and continues over the years. Although it is generally accepted that sexual differentiation of the neuroendocrine hypothalamus does not proceed beyond the early postnatal period, puberty has been recently recognized as another period of development during which gonadal hormones organize the nervous system (27). Other human hypothalamic structures seem to differentiate later in life, such as the sexually dimorphic nucleus of the preoptic area [between 4 yr and puberty (28)], the darkly staining posteromedial components of the BST [around puberty (29)], and the BST central part (BSTc) [in adulthood (30)]. Furthermore, the sexually dimorphic NKB innervation in the rat ARC does not become visible before puberty (21). It is likely that the sex difference in human NKB expression also reflects organizational actions of testosterone during the prenatal period. However, this sex difference is only revealed postpubertally, suggesting that it needs to be activated by sex steroid hormones.

Sex reversal of NKB immunoreactivity in MtF transsexuals

Our data revealed a female-like infundibular NKB-ir volume in MtF transsexuals who had undergone estrogen treatment and sex reassignment in adulthood. The feminization of the NKB system of MtF transsexuals might be explained by either the presence of a higher estrogen concentration in the blood due to estrogen treatment or the lack of androgens due to orchidectomy. However, there is some evidence against these explanations: 1) subject T3 showed a large infundibular NKB-ir volume, despite the fact that estrogen treatment was stopped about 3 months before death; 2) subjects T4 and T5 continued to take estrogens until death but had smaller infundibular NKB-ir volumes than T3; and 3) subject T2, who was never orchidectomized, had the largest infundibular NKB-ir volume of the MtF transsexual group. Additional evidence comes from the 84-yr-old man who also had very strong cross-gender identity feelings but remained untreated, whose NKB-ir volume was in the range of postmenopausal women. In line with previous human postmortem studies showing a female-like BSTc (31, 32) and a female-like third interstitial nucleus of the anterior hypothalamus (33) in MtF transsexuals, our results may suggest an atypical sexual differentiation of the

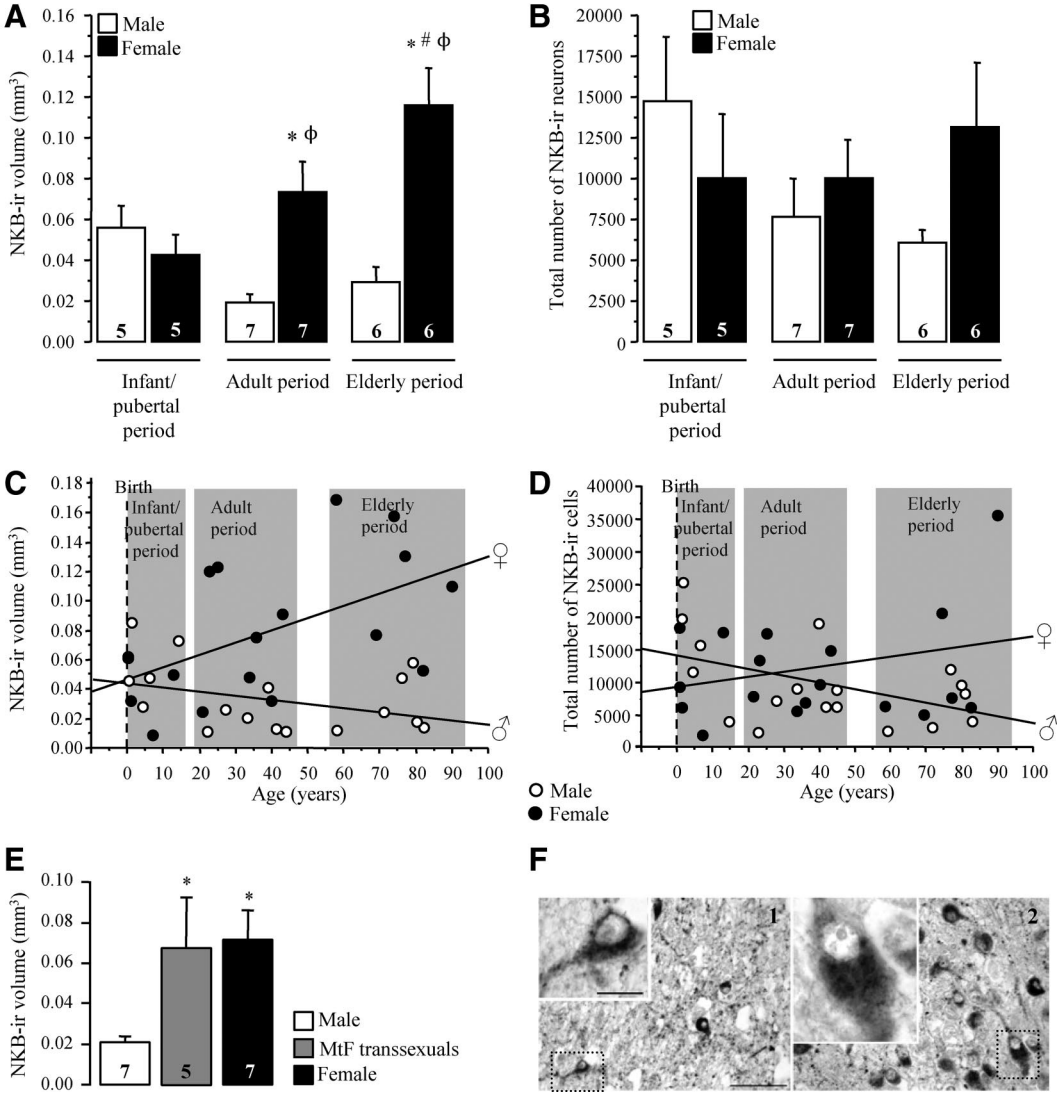

FIG. 5. Estimation of the volume occupied by NKB immunoreactivity (A) and of the total number of NKB-ir cells (B) in the INF of males and females during the infant/pubertal period (between 5 months and 14 yr), the adult period (between 22 and 44 yr), and the elderly period (between 58 and 90 yr). *, $P < 0.05$ *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 yr). The *lines* represent the regression line for each sex. E, Estimation of the volume occupied by NKB immunoreactivity in the INF of men, women, and MtF transsexuals in adulthood. *, $P < 0.05$ *vs.* male. F, Representative microphotographs illustrating the increase and hypertrophy of neurons expressing NKB in postmenopausal women (panel F2, 90 yr old) compared with premenopausal women (panel F1, 34 yr old) in the INF (40× 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 F1 and F2; 0.01 mm in the *insets*.

hypothalamus in transsexual people. Because the sex reversal does not seem to be influenced by circulating hormone levels in adulthood, the sexual differentiation of the infundibular NKB system is likely due to an organizational action of sex steroid hormones (presumably testosterone) during a critical period of development, which is likely to emerge postpubertally in humans as evidenced by the developmental curve of NKB expression over the different stages of life. Nevertheless, due to the small sample of MtF transsexuals and differences in estrogen levels, we cannot rule out an effect of the hormonal milieu in adulthood on NKB expression. In contrast with rodents where the LH surge mechanism is irrevocably fixated perinatally by exposure to androgen (34), the debate about a "permissive" *vs.* "deterministic" hypothalamic control of GnRH release is still open in humans (35). Although both sexes can show estrogen-induced LH surges (36), the evocability of the LH surge in men requires prolonged estrogen priming, and the amplitude of the surge is smaller compared with women (37). Nevertheless, the LH surge in MtF transsexuals was male-typical before sex reassignment and almost female-typical afterward, suggesting that long-term estrogen treatment to men could partly feminize the gonadotropin response (38). However, the best evidence of an atypical

sexual differentiation in MtF transsexuals came from the nontreated individual with strong cross-gender identity feelings, who displayed a female-like NKB expression.

Although the rodent model offers advantages to study the neuroendocrine control of steroid feedback on GnRH regulation, the neural mechanisms that govern the preovulatory LH surge in women seem to differ from those in rodents (35). Studies of functioning and anatomical localization of neuropeptides involved in GnRH regulation such as NKB in the human brain are critical to validate the animal experimental data and increase our understanding of the human reproductive axis and related neuroendocrine disorders.

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