Kisspeptin expression in the human infundibular nucleus in relation to sex, gender identity and sexual orientation.

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Context: Since the discovery of its central role in reproduction, our functional neuroanatomical knowledge of the hypothalamic kisspeptin system is predominantly based on animal studies. Although sex differences in kisspeptin expression have been shown in humans in adulthood, the developmental origin of this sex difference is unknown.

Objectives: Our objectives were to determine 1) when during development the sex difference in kisspeptin expression in the infundibular nucleus (INF) would emerge and 2) whether this sex difference is related to sexual orientation or transsexuality.

Design and setting: Post-mortem hypothalamic tissues were collected by the Netherlands Brain Bank and sections were stained for kisspeptin by immunohistochemistry.

Patients: Hypothalami of 43 control subjects were categorized into 3 periods: infant/prepubertal (6 girls/7 boys), adult (11 women/7 men) and elderly (6 aged women/6 aged men). Eight male-to-female (MTF) transsexuals, 3 HIV^+ heterosexual men and 5 HIV^+ homosexual men were also analyzed.

Main outcome measure: We estimated the total number of kisspeptin-immunoreactive neurons within the INF.

Results: Quantitative analysis confirmed that the human infundibular kisspeptin system exhibits a female-dominant sex difference. The number of kisspeptin neurons is significantly greater in the infant/prepubertal and elderly periods compared to the adult period. Finally, in MTF transsexuals – but not homosexual men -, a female-typical kisspeptin expression was observed.

Conclusions: These findings suggest 1) that infundibular kisspeptin neurons are sensitive to circulating sex steroid hormones throughout life and 2) that the sex-reversal observed in MTF transsexuals might reflect, at least partially, an atypical brain sexual differentiation.

The human brain is thought to be sexually differentiated under the influence of testosterone acting in the male fetus (1). 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 that participate in the neural control of reproduction, including the secretion of gonadotropin-releasing hormone (GnRH) (GnRH; (2)). In the human hypothalamus, postmortem and neuroimaging studies have identified several structural (3–8) and func-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received December 8, 2015. Accepted March 30, 2016.

Abbreviations:

tional (9–10) sex differences, some of which seemed to be related to gender identity and sexual orientation (1, 11–13).

Human genetic studies demonstrated that kisspeptin (14) and neurokinin B (NKB) signaling (15) are both potent regulators of GnRH secretion and are therefore critically involved in the onset of puberty and the maintenance of adult reproductive function. We recently reported that the NKB system exhibits a female-dominant sex difference in the human infundibular nucleus (INF) that reached only significance in adulthood as well as that male-to-female (MTF) transsexuals had a female-typical infundibular NKB system (16). At present, only a fragmented overview of kisspeptin expression throughout life is available in humans. Kisspeptin expression in the human brain has been reported in fetuses (17) and adult subjects (18–20). Sex differences were observed in adulthood with women having a greater number of kisspeptin neurons in the INF than men (19–20). In addition, an increase and hypertrophy of neurons expressing kisspeptin mRNA was found after menopause (18). However, in humans, it is unknown when sex differences in kisspeptin expression arise during development under the influence of sex hormones. Therefore, in the present study, we analyzed infundibular kisspeptin expression from birth to old age in both sexes. By including brain material from MTF transsexuals and homosexual men, we also investigated whether this sex difference was related to gender identity and sexual orientation or rather would reflect circulating sex steroid levels.

Materials and Methods

Human brain tissue

Hypothalami of 61 subjects (Table 1) were obtained through autopsies by the Netherlands Brain Bank following the required permissions for brain autopsy, use of tissue and medical information for research purposes. The subjects were categorized into 3 periods: infant/prepubertal, adult, and elderly periods. The MTF transsexual group consisted of 7 sex-reassigned and estrogen-treated individuals and one individual who was not orchidectomized but hormonally treated (see Table 2 for details). A nontreated individual (S7) with strong cross-gender identity feelings, which were already present since his earliest childhood, and one female-to-male transsexual were also analyzed. The effect of sexual orientation on kisspeptin expression was studied in 5 homosexual men. Since all homosexual subjects died of acquired immunodeficiency syndrome (AIDS) or related diseases, 3 heterosexual men with AIDS were included in the analyses as control. 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 hypothalamic-pituitary-gonadal axis during the last month before death and neurodegenerative or psychiatric diseases. For each period of life, males and females were matched for age, postmortem delay, and the duration of the formalin fixation (t-tests).

Histology

Hypothalami were formalin fixed, paraffin embedded, and cut serially in 6-μm coronal sections from rostral (when the anterior commissure, the lamina terminalis and the optic chiasm appeared) to caudal (until the posterior part of the mammillary bodies). Every 100th section was collected on a SuperFrost/Plus (Menzel, Germany) slide and stained with 0.5% thionin for general orientation. To determine the border of the INF, every 50th section of the putative INF was mounted from the rostral start of the median eminence until the appearance of the mammillary bodies and stained for neuropeptide Y (NPY; 11). Once the border of the INF was delimited by NPY staining, every 50th section - adjacent to the NPY-stained ones - was then collected over the whole length of the INF for kisspeptin immunohistochemistry. Sections were deparaffinized and rehydrated through xylene and decreasing grades of ethanol. Unless stated otherwise, all incubations were carried out at room temperature and all washes were performed using Tris-buffered saline (TBS; 0.05M Tris and 0.9% NaCl; pH 7.6).

NPY immunohistochemistry. Sections were incubated sequentially in 5% normal goat serum (NGS; 1 hour) in TBS-0.1% Triton-X 100 (TBST), and overnight at 4°C in a rabbit polyclonal anti-NPY antibody (1:1000; Niepke 26/11/1988; Netherlands Institute for Neuroscience, Amsterdam, The Netherlands). They were then incubated for 1 hour in a goat antirabbit biotinylated antibody (Dako, Denmark; 1: 1000 in TBST) followed by 1h in avidin–biotin complex (ABC; 1:800, Kit ABC Vectastain Elite PK-6100, Vector Laboratories PLC, Cambridge, UK). Finally, sections were incubated in nickel-DAB solution (0.5 mg/ml 3,3-diaminobenzidine; Sigma Chemical, St Louis, MO, USA; 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 Eukitt (Sigma).

Kisspeptin immunohistochemistry. Sections were first processed for antigen retrieval by placing them in citrate buffer (0.1m citric acid, 0.1m trisodium citrate, pH 6.0) in a microwave (5 minutes, 700W). Next, sections were incubated sequentially in 5% normal donkey serum (NDS; 1 hour) in TBST, and 2x overnight at 4°C in a sheep polyclonal antikisspeptin antibody (GQ2; 1:50000; kindly provided by Dr. Stephen Bloom [Imperial College London, London, UK]). They were then incubated for 1 hour in a donkey antisheep biotinylated antibody (Dako, Denmark; 1: 1000 in TBST) followed by 1h in ABC (1:800). Finally, sections were incubated in nickel-DAB solution, rinsed in distilled water, dehydrated, cleared in xylene and coverslipped with Eukitt.

Antibody characterization

Kisspeptin. The GQ2 was raised in sheep against the full-length human kisspeptin-54 peptide sequence. The specificity of the GQ2 antibody was previously addressed by Dhillo et al (21) for use in radioimmunoassay (RIA). The GQ2 antibody was used successfully in previous immunohistochemical experiments on hypothalamic sections from human (19–20) and monkey (22). In the latter, preabsorption of the GQ2 antibody with synthetic kisspeptin-54 peptide completely abolished kisspeptin immunoreactivity.

Table 1. Clinicopathological data

	NBB number	Age	Brain weight (g)	Post-mortem delay (h:min)	Fixation time (days)	Clinicopathological information
Control females (*n* = 23)						
Infant / Pubertal period (n = 6)	86–027	5 m	735	10:00	40	Sudden infant death syndrome
	89–027	6 m	780	<17:00	28	Cardiomyopathy
	97–153	7 m	760	1240	39	Sudden infant death syndrome
	89–036	1 yr	820	NA	31	Hypoglycaemia
	87–077	7 yr	1320	<9:45	33	Astrocytoma
	87–035	13 yr	1250	<13:00	48	Histiocystic lymphoma, cardiac failure
Adult period (n = 11)	01–009	21 yr	975	19:35	65	Myocard infarction
	84–025	23 yr	1300	<10:00	35	Acute myeloid leukemia
	01–072	25 yr	1500	<17:00	31	Found dead (epileptical seizure)
	85–041	28 yr	1365	325	44	Cardiogenic shock
	85–027	29 yr	1150	790	60	Corrected Fallots tetralogy; cardiac failure, hepatic coma
	02–006	32 yr	1287	2460	45	Severe pulmonary hypertension
	92–037	32 yr	1280	1800	45	Bronchopneumonia
	99–058	34 yr	1395	72:00	132	Cardial abnormalities
	91–009	36 yr	1348	<71:30	61	Faecal perotonitis
	84–002	36 yr	1420	5160	51	Multiple fractures; rupture of thoraci aorta
	01–023	40 yr	1279	<41:00	54	Pulmonary carcinoma
Elderly period (n = 6)	01–004	64 yr	1159	8:35	36	Probable ileus, perforation with acute stomach
	98–036	69 yr	1264	6:15	31	Cardiogenic shok
	98–080	72 yr	1041	<24:00	67	Respiratory failure secondary to cardiac abnormalities
	98–034	72 yr	NA	3930	56	Bronchopneumonia
	98–104	74 yr	1207	7:25	31	Necrosis of the intestins
	98–089	90 yr	1047	7:15	39	Ruptured abdominal aorta aneurism
Control males (*n* = 20)						
Infant / Pubertal period (n = 7)	85–036	2,5 m	635	<17:00	73	Cardiac failure, aortic stenosis, cerebral ischemia
	86–041	6 m	800	<6:30	14	Sudden infant death syndrome
	88–058	1 yr	1070	<35:35	28	Bacterial meningitis, sepsis
	88–092	1 yr	920	<41:00	31	Penthotal intoxication, hypoxia
	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	Haematothorax following 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	Myocard infarction
	99–035	44 yr	1434	<41:00	123	Multi-organ 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 cacinoma 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
	09–001	88 yr	1418	4:43	51	Gastro-intestinal bleeding
Heterosexual men HIV$^+$ (*n* = 3)	89–024	21 yr	1430	2940	25	AIDS: mycobacterial infections, pneumonia, cerebrovascular accident
	86–048	30 yr	1430	530	37	AIDS: pneumocystic carinii pneumonia, lung tuberculosis, toxoplasmosis, heroin addiction
	89–042	30 yr	1340	1785	25	AIDS: disseminated non-Hodkin's lymphoma
Homosexual men HIV$^+$ (*n* = 5)	86–046	32 yr	1440	2930	11	AIDS: pneumocystic carinii pneumonia
	87–080	39 yr	1320	5340	28	AIDS: progressive multifocal leukoencephalopathy
	88–087	41 yr	1240	490	34	AIDS: bronchopneumonia, cytomegalic infections and toxoplasmosis
	86–043	42 yr	1340	NA	24	AIDS: disseminated Kaposi's sarcoma
	88–121	42 yr	1340	1125	30	AIDS: cytomegalic meningoencephalitis
MTF transsexuals (*n* = 8)	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
	93–070 (T6)	53 yr	1500	5760	34	Acute fatty liver
	07–021 (T7)	58 yr	1358	445	37	Renal cell carcinoma
	98–141 (T8)	74 yr	1118	2455	33	Multiple cerebral infarction
FTM transsexual (*n* = 1)	98–245	51 yr	1171	4:15	32	Cachexia
Non-treated male with cross-gender identity feelings (*n* = 1)	96–088 (S7)	84 yr	1433	41	38	Lung carcinoma

MTF: male-to-female; NA = not available; NBB: Netherlands Brain Bank.

NPY. The specificity of the NPY antibody was previously demonstrated by solid-phase preabsorption procedures (23–24).

Estimation of the total number of kisspeptin-ir neurons within the INF. Estimates were made using Mercator image analysis software (Explora Nova, La Rochelle, France), connected to a Q-imaging Retiga EXi Fast 1294 camera mounted on a plain objective microscope (Zeiss ImagerZ1 with Plan-NEOFLUAR Zeiss objectives). From the section to be measured, a low magnification image (2.5x objective) covering the INF was obtained. 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 63× objective on the image analysis monitor. For analysis, 100% of the rectangular fields were analyzed. To prevent double counting, only kisspeptin-ir 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 kisspeptin-ir neurons in the INF from 16.75 ± 0.50 (mean ±S.E.M.) sections per subject.

Estimation of the INF volume

The INF volume was calculated with the trapezoidal computation method (25) that consists of two steps: 1) the measurement

Table 2. Hormonal profil of transsexual subjects

NBB number	Age (yr)	Age of hormonal treatment / Orchidectomy	Estrogen treatment	Anti-androgen treatment
Male-to female transsexuals				
(T1) 98–137	26	21 / 26	EE 50 μg 2 dd.	50 mg CPA 2 dd.
(T2) 93–042	36	NA / Not orchidectomized	Estradiol in combinaison with hydroxyprogesterone in therapeutical dosages. Exact period of treatment is not known but based on the significant testes atrophy she was most probably treated for a period of about 5 yr or more.	50 mg CPA 1 dd (at least the last 10 months b. d.).
(T3) 88–064	43	36 / 39	Age 39: 50 μg EE 2 dd (stopped 3 months b.d.).	Age 36: 50 mg CPA 2 dd (stopped 2 yr b.d.).
(T4) 95–018	48	35 / 36	Age 35–40: EE 50 μg 2 dd. Age 40: EE 50 μg 1 dd (treatment lasted until death).	Age 35–40: CPA 50 mg 2dd.
(T5) 84–020	50	42 / 44	Age 42: DES 5 mg 1 dd, after 2 months to 5 mg 2 dd. Age 44: EE 50 μg 2 dd (lasted until death).	Age 44: CPA 50 mg 2 dd (stopped 2 yr b.d.).
(T6) 93–070	53	40 / 50	Age 40: DES treatment (stopper after 1 yr). Age 43–47: Premarin 0625 mg 1 dd. Age 47–50: Premarin 3075 mg 1 dd. Age 50–53: Premarin 2,5 mg 1 dd and topical estrogen cream. (estrogen treatment stopped 3 months b.d.).	Age 50–53: CPA 50 mg 1 dd.
(T7) 07–021	58	43 / 46	Age 43: EE 50 mg 1 dd. Age 44–51: EE 50 mg 2 dd. Age 52–54: treatment has been stopped. Age 55–57: estradiol plasters 100 mg.	Age 43–48: cyproteron 50 mg 1 dd. Age 49–51: cyproteron 25 mg 1 dd.
(T8) 98–141	74	64 / 64	Age 64: EE 50 mg 2 dd. Age 67: Estraderm 100 mg 1 dd.	Age 64: 50 mg 2 dd.
(S7) 96–088	84	No hormonal treatment/ No orchidectomy		
Female-to male transsexual				
(FTM) 98–245	51	27 / 28		Age 27: Sustanon 250 mg, twice a month injections. Age 30: TU 40 mg 3 dd. Age 34: TU 40 mg 2 dd. Age 36: TU 40 mg 4 dd. Age 44: Sustanon 250 mg twice a month injections Age 47–48: Sustanon 250 mg, every 3 weeks. Age 48-death: no T treatment anymore

b.d., before death; DES, diethylstilbestrol; NBB, Netherlands Brain Bank; CPA, cyproterone acetate; EE, ethinyloestradiol; TU, testosterone undecanoate.

of the infundibular area in each section by manually tracing the border of the INF based on NPY staining by using the Mercator image analysis software; 2) the measurement of the volume between two consecutive sections using the interpolation of the area. The total volume of the INF is the summation of all the interpolated volumes between sections.

kisspeptin-ir neuronal morphology in females

In order to confirm previous data showing a hypertrophy of kisspeptin-expressing neurons after menopause (18), twenty to thirty kisspeptin-ir neurons were randomly selected for cell area measurement in females (n = 3 for each period of life). Images of these neurons were digitized through a video camera attached to the microscope (100x objective) and the cell perimeters were manually delineated for measurement of cell area with a Macintosh-based image analysis system using the NIH Image program (version 1.49; Wayne Rasband, NIH, Bethesda, MD, USA).

Statistical analysis. Data were first checked for normality using the Shapiro-Wilk test. A Box-Cox transformation was then applied to approach (fixation time, [GRAPHIC] = −0.550) or achieve (number of kisspeptin-ir neurons, [GRAPHIC] = 0.186; brain weight, [GRAPHIC] = 2.810; postmortem delay, [GRAPHIC] = −0.004) normality of non-normally distributed data prior to further analyses. Statistical analyses and box and whisker plots were generated from Box-Cox transformed data (see original and Box-Cox transformed data in Supplemental Table 1). Two-way ANOVAs with the stage of life (infant/prepubertal vs. adult vs. elderly) and the sex (male vs. female) as independent factors were used to analyze the overall difference in the number of kisspeptin-ir neurons. One-way ANOVAs were performed to assess 1) the effect of gender identity and sexual orientation on the number of kisspeptin-ir neurons and 2) kisspeptin-ir neuronal morphology in females. All ANOVAs were followed when appropriate by post hoc tests using Fisher's protected least significant difference tests. Age effects on the number of kisspeptin-ir neurons were addressed in a polynomial regression model for each sex separately. Analyses of covariance were used to control for possible confounding factors such as the volume of the INF, postmortem delay and fixation time. Differences were considered significant for $P < .05$.

Results

The neuronal distribution of infundibular kisspeptin-ir was similar to that previously reported by other groups in humans (18–20). These neurons were almost absent from the anterior part of the INF but were found in gradually increasing numbers as the nucleus extends caudally, with greatest numbers observed in the middle to caudal parts of the INF (Figure 1).

Overall, a greater number of kisspeptin-ir neurons was found in females compared to males (see Figure 2 for representative pictures). Moreover, kisspeptin expression appeared to change over the lifetime, with a greater number in the infant/prepubertal and elderly periods compared to the adult period (Figure 3A and Supplemental Table 2 for the original numerical values). Two-way ANOVA on the

number of kisspeptin-ir neurons confirmed a significant effect of sex (females > males; $F_{1,37} = 6.87$; $P = .013$), a significant effect of the stage of life ($F_{2,37} = 4.74$; $P = .015$), but no significant interaction effect between the two factors ($F_{2,37} = 0.096$, $P = .909$). Post hoc test revealed that the number of kisspeptin-ir neurons is significantly lower in the adult period compared to the infant/prepubertal ($P = .032$) and the elderly ($P = .020$) periods. No difference was found in the number of kisspeptin-ir neurons between the infant/prepubertal and the elderly period ($P = .81$).

The developmental pattern of infundibular kisspeptin expression throughout life seemed to be quite similar between men and women, ie, a moderate number of kisspeptin-ir neurons in the infant/prepubertal period followed by a lower number in the adult period and an increasing number in the elderly period (see Figure 3B and 3C). Quadratic polynomial regression analysis showed that the number of kisspeptin-ir neurons is significantly correlated with age in both males (r = 0.55; $P = .047$, n = 20) and females (r = 0.52; $P = .042$, n = 23).

Furthermore, as previously described (18), most kisspeptin-ir neurons in the INF of postmenopausal women showed hypertrophy (Figure 3E and F). One-way ANOVA showed that the mean cell area of kisspeptin-ir neurons was significantly different among the groups ($F_{2,6} = 79.09$; $P < .0001$). Post hoc tests revealed that kisspeptin-ir neurons exhibited a hypertrophied morphology in postmenopausal women (564.8 ± 33.2, mean ± SEM) compared to premenopausal women (175.4 ± 14.6, mean ± SEM) and infant/prepubertal girls (153.5 ± 26.7, mean ± SEM; all $P < .0001$). No difference was found between infant/prepubertal girls and premenopausal women ($P = .57$).

Finally, we assessed the effect of gender identity and sexual orientation on the number of kisspeptin-ir neurons (Figure 3D). One-way ANOVA indicated that the number of kisspeptin-ir neurons is significantly different between the groups ($F_{4,29} = 2.975$; $P = .036$). Post hoc tests showed that the number of kisspeptin-ir neurons of men is significantly lower compared to women ($P = .044$), to MTF transsexuals ($P = .005$) and to HIV$^+$ heterosexual men ($P = .011$). No significant difference was observed between HIV+ heterosexual men and HIV+ homosexual men ($P = .28$). Among MTF transsexuals, linear regression analyses indicated that the number of kisspeptin-ir

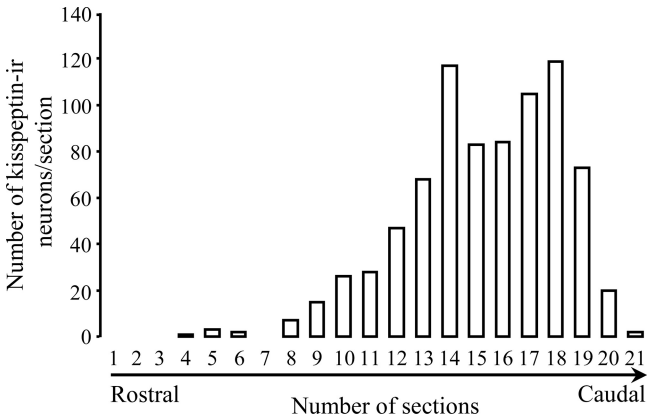

Figure 1. Distribution of kisspeptin-ir neurons throughout the rostrocaudal extent of the INF at one side of the brain in a representative 90 year-old women. The distance between 2 sections is 300 μm. Note the gradual increase of kisspeptin-ir neurons as the nucleus extends caudally.

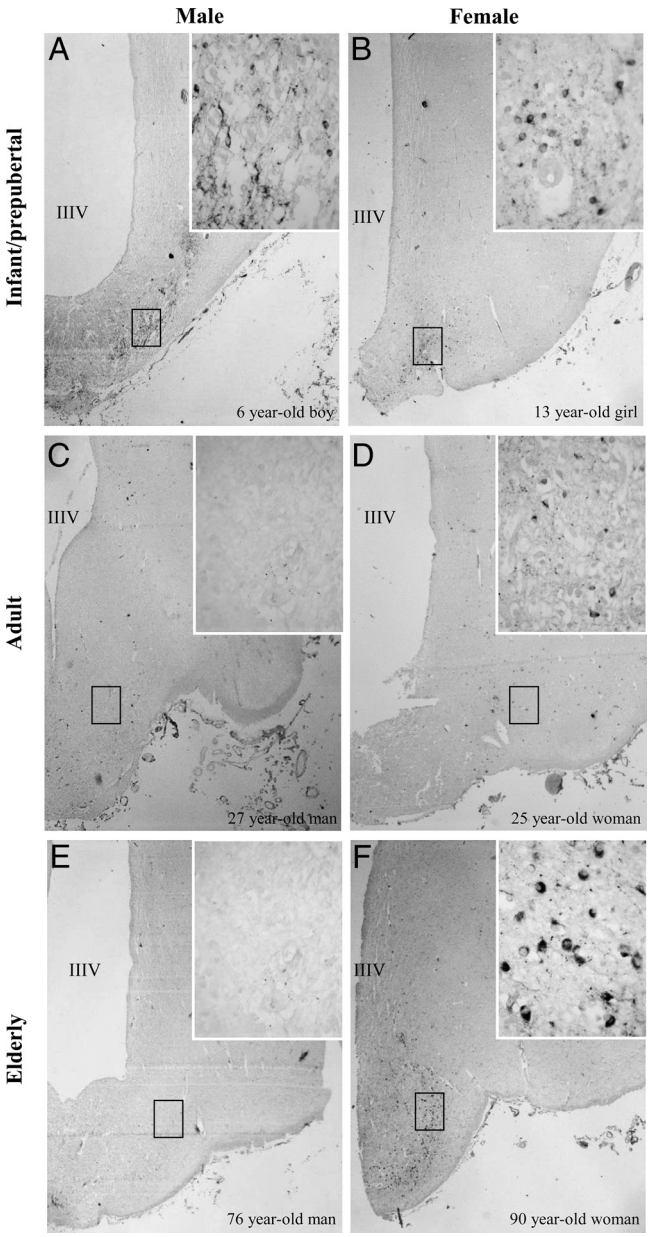

Figure 2. Representative photomicrographs of infundibular kisspeptin expression in the infant/prepubertal (A, B), adult (C, D) and elderly (E, F) periods in both sexes. The boxes in panels illustrate the area photographed at higher magnification. Note the numerous and intensely labeled kisspeptin neurons in the infant/prepubertal (B) and elderly periods (F) compared to the adult period (D) in the female INF.

neurons is neither correlated with age (r = –0.24; $P = .57$; n = 8) nor with the duration of hormonal treatment (r = –0.55; $P = .15$; n = 8). Finally, it is interesting to note that the female-to-male (FTM) transsexual subject had a number of kisspeptin-ir neurons (144 neurons) in the male range, while the untreated gender-identity disorder subject (S7) showed an intermediate number of kisspeptin-ir neurons (1465 neurons).

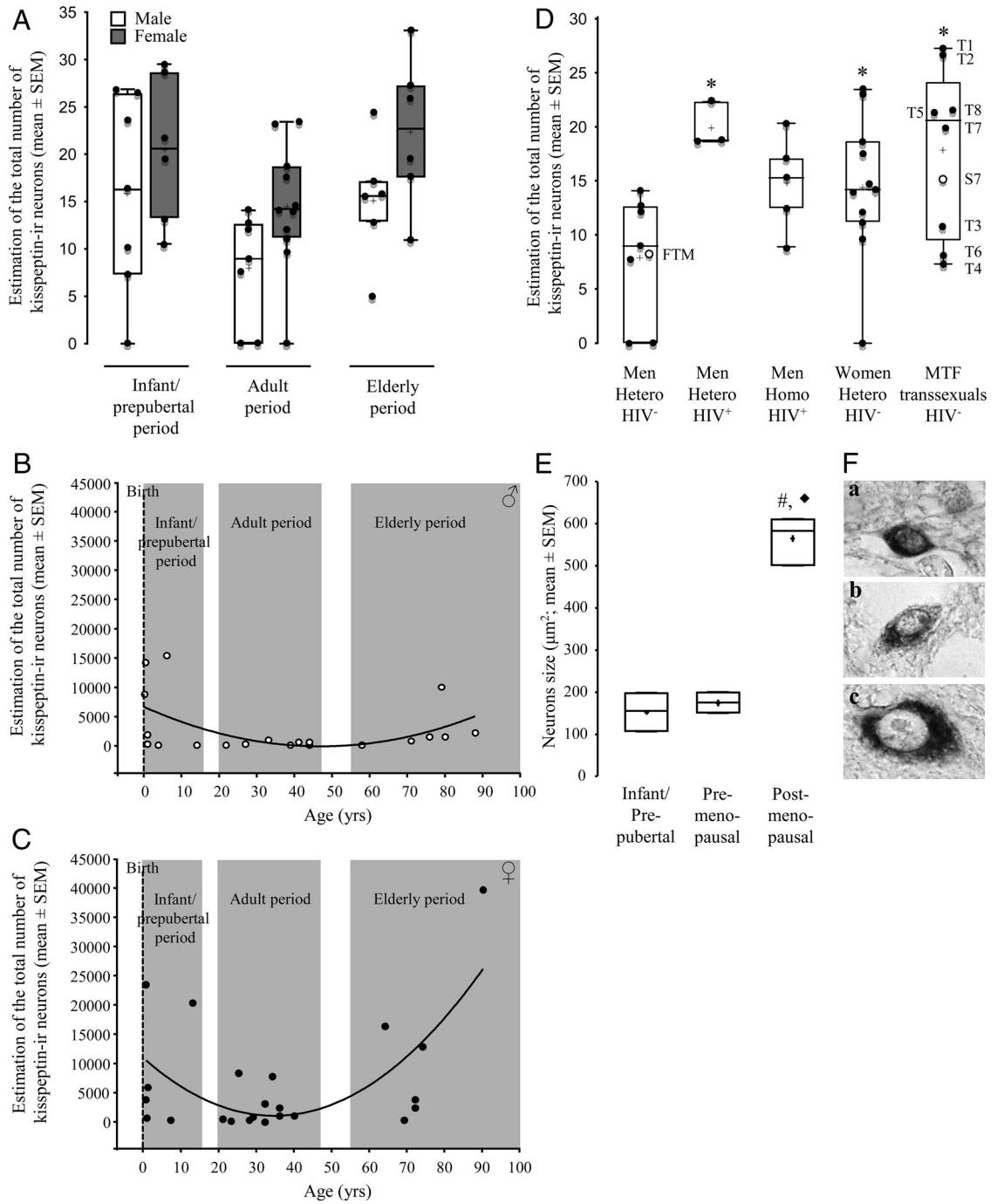

Figure 3. A, Box-and-whisker plots illustrating how the number of kisspeptin-ir neurons in the INF varies and differs between males and females during the infant/pubertal period (between 5 months and 14 years), the adult period (between 22 and 44 years) and the elderly period (between 58 and 90 years). The line represents the median and the cross represents the mean. Black dots represent individual data. 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, Box-and-whisker plots illustrating how the number of kisspeptin-ir neurons in the INF varies according to transsexuality and sexual orientation. E, Box-and-whisker plots representing the neuronal hypertrophy of kisspeptin neurons after menopause. F, Representative microphotographs illustrating the hypertrophy of neurons expressing kisspeptin in postmenopausal women (c) compared with premenopausal women (b) and infant/prepubertal girls (a) in the INF (100x objective). * $P < .05$ vs. HIV- heterosexual men; # $P < .05$ vs. infant/prepubertal girls; ◆ $P < .05$ vs. premenopausal women.

Confounding factors

As expected, two-way ANOVA on brain weights revealed a significant effect of sex ($F_{1,36} = 12.19$; $P = .001$), a significant effect of stage of life ($F_{2,36} = 4.11$; $P = .025$) but no interaction between the two factors ($F_{2,36} = 1.01$; $P = .38$). Post hoc analysis showed that the brain weights of infant/prepubertal subjects is lower compared to both adult ($P = .016$) and elderly ($P = .041$) subjects. A similar, although not statistically significant, effect of the stage of life ($F_{2,37} = 3.15$; $P = .055$) was observed in the volume of the INF whereas no significant effect was observed between males and females ($F_{1,37} = 0.036$; $P = .85$) and no interaction between the two factors was found ($F_{2,37} = 0.99$; $P = .38$). To assess whether the sex and stage of life differences observed in the number of kisspeptin-ir neurons was not consecutive to a structural sex difference of the brain area analyzed (INF) or affected by possible confounding factors such as postmortem delay and fixation time, analyses of covariance were carried out. When adjusted for the volume of the INF or fixation time, significant effects of sex ($F_{1,38} = 7.91$; $P = .0077$ and $F_{1,37} = 6.83$; $P = .013$, respectively) and stage of life ($F_{2,38} = 5.80$; $P = .0063$ and $F_{2,37} = 3.61$; $P = .0370$, respectively) on the number of kisspeptin-ir neurons were still detected. However, when controlled for postmortem delay, only a significant effect of sex ($F_{1,36} = 7.30$; $P = .0104$) was observed (stage of life effect: $F_{2,36} = 2.70$; $P = .081$). Moreover, no difference was found in brain weights or infundibular volume between adult men, women, MTF transsexuals and homosexual subjects ($P > .5$).

Discussion

Our study provided for the first time a systematic and quantitative analysis of kisspeptin-ir neurons along the rostro-caudal extent of the human INF throughout life in both sexes. We demonstrated that kisspeptin expression shows sex differences (females > males) and changes over lifetime (infant/prepubertal > adult < elderly). These changes in kisspeptin expression in the INF likely reflect the fluctuation in the concentration of sex steroid hormones throughout life. Moreover, MTF transsexuals, but not homosexual men, had a female-typical number of kisspeptin-ir neurons. This sex-reversal may thus reflect, at least in part, an atypical sexual differentiation of the hypothalamus.

Female-dominant sex difference in infundibular kisspeptin expression

Most our knowledge about a putative sex difference in kisspeptin expression in the human INF came from independent studies using adult subjects. Thus, sex differences in the number of kisspeptin neurons (female>male) were found in young adults (19) as well as in aged subjects (20). In the present study, we observed an overall female-dominant sex difference in the number of kisspeptin-ir neurons in the human INF. Unfortunately, the absence of a statistically significant interaction between the sex and the stage of life did not allow us to unravel at which stage of life this sex difference emerges. However, based on the mean number of kisspeptin-ir neurons, females have quantitatively more kisspeptin-ir neurons compared to age-matched males at all stages of life analyzed, raising the possibility that this sex difference is already present during childhood and might reflect organizational effects of sex steroids during early development. It is worth mentioning that the expression of NKB in the INF – a neuropeptide involved in the control of GnRH release (15) and which is extensively coexpressed in kisspeptin neurons (19) – showed sex differences as well (16). Moreover, by analyzing the relationship between NKB-ir and kisspeptin-ir neurons in subjects that were included in both the previous (16) and the present study, we found that the number of kisspeptin-ir neurons was significantly and positively correlated with the number of NKB-ir neurons ($r = 0.649$; P 0.0001; $n = 37$).

Development and sex steroids regulation of the human infundibular kisspeptin system

Most of our knowledge about the developmental pattern in kisspeptin expression and its regulation by sex steroids is largely derived from studies in rodents. In the rodent brain, kisspeptin is predominantly expressed in the anteroventral periventricular nucleus and periventricular nucleus (AVPV/PeN) and the arcuate nucleus (ARC, the rodent homologue of the human INF). The AVPV/PeN population shows sex differences (female > male), is upregulated by sex steroids and presumably involved in the preovulatory GnRH/LH surge. By contrast, the ARC population – which shows no sex differences in adulthood – is down-regulated by sex steroids and has been implicated in the negative feedback action of gonadal steroids (see (26) for a review). In humans, in situ hybridization and immunohistochemical studies have failed to identify a homologue of the rodent AVPV/PeN kisspeptin population. Although some were found scattered within the medial preoptic area, the vast majority of kisspeptin neurons are located in the INF (18, 19). It is very likely that the INF represents the hypothalamic site that mediates the negative feedback action of estradiol in primates and humans as well since low kisspeptin mRNA expression is observed in estradiol-treated ovariectomized monkeys and in premenopausal women whereas kisspeptin mRNA expres-

sion is elevated in untreated ovariectomized macaques and postmenopausal women (18). Moreover, a similar estrogen regulation was observed for NKB (16, 27). The present data corroborate these previous findings: we observed that, in females, kisspeptin expression followed a convex U-shaped curve throughout life, with a greater number of kisspeptin-ir neurons in the infant/prepubertal and elderly periods compared to the adult period. A similar, albeit not statistically significant, effect of stage of life was observed on kisspeptin expression in males. Our observation of a U shaped curve in kisspeptin expression is rather unexpected compared with what is observed in animal studies. In most animal species analyzed so far, hypothalamic kisspeptin expression increased steadily during postnatal development with the highest expression around puberty (see (28) for a review). However, higher plasma levels of kisspeptin have been reported in children compared to adults (29). Although these concentrations were most significantly elevated in children at ages commonly associated with puberty (9–12 years), concentrations of plasma kisspeptin in children below 9 years of age (approx. 35 pmol/L) were still significantly higher compared to adults (approx. 10 pmol/L). Future studies are thus needed to further dissect kisspeptin expression during postnatal development and puberty in human.

Female-like number of kisspeptin-ir neurons in male-to-female transsexuals

One neurobiological theory of the origin of transsexuality is based on the fact that the sexual differentiation of the genitals (ie, in the first two months of pregnancy) takes place well before the sexual differentiation of the brain (second half of pregnancy). As the two processes are not simultaneous, they might go into opposite directions. If true, one might expect to find, in transsexuals, female sexual organs and male brain structures or vice versa. Indeed, postmortem studies investigating the brains of transsexual individuals have observed sex-reversal in the volume and number of neurons in the central subdivision of the sexually dimorphic bed nucleus of the stria terminalis and the third interstitial nucleus of the anterior hypothalamus (INAH3; [5–6, 30]). Recently, we have also observed that MTF transsexuals had a female-typical NKB expression in the INF (16). In addition, structural and functional neuroimaging studies have observed sex-atypical neuroanatomical features and sex-atypical hypothalamic activation in MTF transsexuals (13, 31–32).

In line with these previous studies, our data revealed a female-like number of kisspeptin-ir neurons in MTF transsexuals who had undergone estrogen treatment and sex-reassignment in adulthood. The sex-reversal of the kisspeptin neuronal population in the INF of MTF transsexuals might be explained either by the presence of higher estrogen concentrations in the blood due to estrogen treatment or the lack of androgens due to orchidectomy. However, based on the individual numerical data, it appears that the number of kisspeptin-ir neurons does not seem to be influenced by circulating hormone levels in adulthood. Indeed, although subject T4 and T5 were both estrogen and antiandrogen-treated for approximately 8 years, subject T5 (5458 neurons) showed a much greater number of kisspeptin-ir neurons compared to T4 (376.5 neurons). The same observation is true for subjects T6 and T8. After being treated for approximately 13 and 11 years respectively, subject T6 (5808 neurons) displayed a much greater number of kisspeptin-ir neurons than T8 (99.5 neurons). Also, subject T2, who was never orchidectomized but hormonally treated, had one of the greatest number of kisspeptin-ir neurons (15027.5 neurons) of the MTF transsexual group. This variability in kisspeptin expression might be explained in different but nonexclusive ways. First, it might be attributed to different grades of feminization over the neuronal network. Indeed, even though MTF transsexuals might have been exposed to atypical levels of sex hormones during development, they were still exposed to androgens throughout life, especially during puberty – a period that has been proposed as an additional period during which gonadal hormones might organize the nervous system (33). Alternatively, this variability might reflect the different mechanisms underlying gender dysphoria, in particularly regarding the onset age of gender dysphoric feelings (late vs early) as well as their sexual orientation (androphilic vs gynephilic) (34). Since the sex reversal observed in kisspeptin expression does not seem to be clearly influenced by circulating hormone levels in adulthood, our results may thus suggest a sex-atypical development in transsexual people.

Kisspeptin expression and sexual orientation

One theory about sexual orientation suggests that it results from low fetal exposure to testosterone, and the absence of organizational effects of testosterone in homosexual men is responsible for feminization of certain brain regions. Indeed, hypothalamic differences in relation to sexual orientation have been observed. The suprachiasmatic nucleus (35) and the anterior commissure (36) are larger in homosexual than in heterosexual men, whereas INAH3 is smaller in homosexual than in heterosexual subjects (37). In the present study, no difference was observed in the number of kisspeptin-ir neurons in HIV^+ homosexual compared to HIV^+ heterosexual men. The statistically significant greater number of kisspeptin-ir neurons observed in HIV^+ heterosexual men compared to HIV^- heterosexual men is likely to be related to their AIDS status,

as some of these patients have subnormal testosterone levels and associated hypogonadism (38), and thus decreased negative feedback as observed in the elderly period.

Concluding remarks

Our data are based on postmortem brain material derived from a heterogeneous patient population. The high variability in the number of kisspeptin-ir neurons, especially observed 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. Secondly, it should be noted that kisspeptin expression varies across the rat estrous cycle (39). Unfortunately, the stage of the menstrual cycle at death was not reported in the patient's medical folder. However, despite these limitations, our data showed that females have quantitatively more kisspeptin-ir neurons compared to age-matched males throughout life, suggesting that this sex difference might reflect organizational effects of sex steroids during early development. Furthermore, the human infundibular kisspeptin system appears to remain sensitive to gonadal hormones throughout life since kisspeptin expression is higher in the infant/prepubertal and elderly periods, which are both characterized by low levels of circulating gonadal hormones. Finally the sex-reversal observed in MTF transsexuals might reflect, at least partially, an atypical brain sexual differentiation.

Acknowledgments

This research was supported by a VICI grant of the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO; 453–08–003) to JB. We thank A. Sluiter, R. Balesar, U. Unmehopa, B. Fisser, J.J. van Heerikhuize for their technical assistance. Brain material was provided by the Netherlands Brain Bank (coordinator Dr. I. Huitinga). J. Bakker is a senior research associate at the Fonds National de la Recherche Scientifique (FNRS). M. Taziaux is a postdoctoral researcher at the FNRS.

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Disclosure Summary: the authors have nothing to disclose.

This work was supported by .

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