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

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Abstract (word count: 249 / max 250)

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 o 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 44 control subjects were categorized into 3 periods: infant/prepubertal (6 girls/7 boys), adult (11 women/7 men) and elderly (6 aged women/7 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 both early organizing and activating actions of sex steroids and 2) that the sex-reversal observed in MTF transsexuals probably reflects, at least partially, an atypical brain sexual differentiation.

Introduction

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; [2]). In the human hypothalamus, post-mortem and neuroimaging studies have identified several structural [3-8] and functional [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 steroid levels.

Materials and methods

Human brain tissue

Hypothalami of 64 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 non-treated 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. The subjects were matched for age, post-mortem 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, 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 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 h) 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 anti-rabbit 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 min, 700W). Next, sections were incubated sequentially in 5% normal donkey serum (NDS; 1 h) in TBST, and 2x overnight at 4°C in a sheep polyclonal anti-kisspeptin 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 anti-sheep 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. 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.

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

INF volume was calculated as follow: in every 50th section, the INF was encircled based on NPY staining and the Mercator image analysis software was used to calculate the area. The distance between two sections multiplied by their calculated area yielded the volume. The consecutive volumes were summed to give the whole INF volume for each subject.

Statistical analysis. 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 as well as possible confounding factors. One-way ANOVAs were performed to assess the effect of gender identity (adult men vs. adult female vs. MTF transsexuals) and sexual orientation (HIV^- heterosexual men vs. HIV^- heterosexual women vs. HIV^+ heterosexual men vs. HIV^+ homosexual men) on the number of kisspeptin-ir neurons. 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 general regression model for each sex separately. Differences were considered significant for $P < 0.05$.

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). Two-way ANOVA on the number of kisspeptin-ir neurons confirmed a significant effect of sex ($F_{1,37}$ = 4.890; P = 0.033), a significant effect of the stage of life ($F_{2,37}$ = 3.512; P = 0.040), but no significant interaction effect between the two factors ($F_{2,37}$ = 1.084, P = 0.349). *Post hoc* test revealed that the number of kisspeptin-ir neurons is significantly lower in the adult period compared to the infant/prepubertal (P = 0.040) and the elderly (P = 0.035) periods. No difference was found in the number of kisspeptin-ir cells between the infant/prepubertal and the elderly period (P = 0.922).

The developmental pattern of infundibular kisspeptin expression throughout life seemed to be quite similar between men and women, i.e. 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. This pattern of expression is, however, more pronounced in females compared to males (see Figure 3B and 3C for comparison). Quadratic polynomial regression analysis showed that the number of kisspeptin-ir neurons is significantly correlated with age in females (r = 0.641226; P = 0.0050, n = 23), but not in males (r = 0.5136; P = 0.0741, n = 20).

In a separate analysis, we compared the number of kisspeptin-ir neurons among adult men, adult women and MTF transsexuals (Figure 3D). One-way ANOVA indicated that the number of kisspeptin-ir neurons is significantly different between the three groups ($F_{2,23}$ = 3.642; P = 0.0422). *Post hoc* tests showed that the number of kisspeptin-ir neurons of men (373.28 ± 149.68 [mean ± SEM]) is

significantly lower compared to MTF transsexuals (6062.00 ± 2339.12 [mean ± SEM]; $P = 0.0148$) and slightly, although not significantly, lower compared to women (2440.45 ± 918.60 [mean ± SEM]; $P = 0.074$). The number of kisspeptin-ir neurons of women and MTF transsexuals did not differ ($P = 0.3160$). Among MTF transsexuals, linear regression analyses indicated that the number of kisspeptin-ir neurons is not correlated with age ($r = -0.5523$; $P = 0.1558$; $n = 8$) but is negatively, albeit not significantly, correlated with the duration of hormonal treatment ($r = -0.7056$; $P = 0.0505$; $n = 8$; Figure 3E), such that long-term estrogen treatment is associated with lower number of kisspeptin-ir neurons. Finally, it is interesting to note that the female-to-male 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).

In contrast to the sex-reversal observed in MTF transsexuals, it appears that kisspeptin expression is not related to sexual orientation of the subjects but rather to their AIDS status (Figure 3F). Indeed, both heterosexual and homosexual men HIV^+ had quantitatively more kisspeptin-ir neurons compared to heterosexual men HIV^-. However, no significant difference was found between the four groups (one-way ANOVA: $F_{3,22} = 2.495$, $P = 0.086$).

Confounding factors

As expected, two-way ANOVA on brain weights revealed a significant effect of sex ($F_{1,37} = 10.396$; $P = 0.0026$), a significant effect of stage of life ($F_{2,37} = 4.227$; $P = 0.0222$) but no interaction between the two factors ($F_{2,37} = 1.944$; $P = 0.1575$). *Post hoc* analysis showed that the brain weights of infant/prepubertal subjects is lower compared to adult subjects ($P = 0.009$) but not compared to elderly subjects. Although a sex difference and a stage of life difference in brain weights were detected, such an effect was not observed in the volume of the INF, suggesting that the sex difference observed in the number of kisspeptin-ir neurons was not consecutive to a structural sex difference of the brain area analyzed. Indeed, a two-way ANOVA showed that the volume of the INF (data not shown) is not statistically different at different stages of life ($F_{2,37} = 3.1510$; $P = 0.0545$), nor between males and females ($F_{1,37} = 0.0359$; $P = 0.8507$) and no interaction between the two factors was found ($F_{2,37} =$

0.9934; $P = 0.3799$). To further test whether the sex and the stage of life differences in the number of kisspeptin-ir neurons were affected by possible confounding factors such as post-mortem delay and fixation time, analyses of covariance were carried out. These factors seemed to have no significant effect on number of kisspeptin-ir neurons (post-mortem delay: $P = 0.18$; fixation time: $P = 0.97$). Moreover no difference was found in brain weights between adult men, women, MTF transsexuals and homosexual subjects (all $P > 0.1$).

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). Moreover, MTF transsexuals, but not homosexual men, had a female-typical number of kisspeptin-ir neurons. These results suggest that 1) infundibular kisspeptin neurons are sensitive to activating actions of sex steroids throughout life, 2) the sex difference in the number of kisspeptin-ir neurons might reflect organizational effects of sex steroids during early development and 3) that the sex-reversal observed in MTF transsexuals may reflect, at least in part, an atypical sexual differentiation of the hypothalamus.

Female-dominant sex difference in infundibular kisspeptin expression

The majority of 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 to mention that the expression of NKB in the INF – a neuropeptide involved in the control of GnRH release [15] and which is extensively co-expressed 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 [25] 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 expression is elevated in untreated ovariectomized macaques and postmenopausal women [18]. Moreover, a similar estrogen regulation was observed for NKB [16, 26]. 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. As illustrated in Figure 4, in females, these changes in kisspeptin expression are likely to reflect the loss

(elderly period) or the absence (infant/prepubertal period) of ovarian estrogen, whereas – in males - these changes probably reflect the decline (elderly period) or the absence (infant/prepubertal period) of testicular hormones. This steroid regulation of kisspeptin expression is also in agreement with the observation that long-term estrogen treatment is associated with lower number of kisspeptin-ir neurons in MTF transsexuals.

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

The neurobiological theory of the origin of transsexuality is based on the fact that the sexual differentiation of the genitals (i.e. 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, 27]). 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, 28-29].

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 by 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 recognized as another period of development during which gonadal hormones organize the nervous system [30]. 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 [31] and the anterior commissure [32] are larger in homosexual than in heterosexual men, whereas INAH3 is smaller in homosexual than in heterosexual subjects [33]. In the present study, no difference was observed in the number of kisspeptin-ir neurons in HIV^+ homosexual compared to HIV^+ heterosexual men. The greater number of kisspeptin-ir neurons observed in both HIV^+ heterosexual and HIV^+ homosexual 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 [34], and thus decreased negative feedback as observed in the elderly period.

Conclusions

Taken together, our data showed that the human infundibular kisspeptin system remains sensitive to gonadal hormones throughout life. Our finding of higher kisspeptin expression in the infant/prepubertal and elderly periods, which are both characterized by low levels of circulating gonadal hormones,

suggest that the human infundibular kisspeptin system might be involved in the negative feedback actions of gonadal hormones on GnRH secretion. Furthermore, the overall greater number of kisspeptin neurons in females compared to males throughout life suggests that the infundibular kisspeptin system might also be involved in the positive feedback actions of estrogens on GnRH secretion important for triggering ovulation, which is absent in men.

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References

1. Bao AM, Swaab DF. Sexual differentiation of the human brain: relation to gender identity, sexual orientation and neuropsychiatric disorders. Front Neuroendocrinol 2011; 32:214-226.
2. Simerly RB. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu Rev Neurosci 2002; 25:507-36.
3. Swaab DF, Fliers E. A sexually dimorphic nucleus in the human brain. Science 1985; 228:1112-1115.
4. Allen LS, Hines M, Shryne JE, Gorski RA. Two sexually dimorphic cell groups in the human brain. J Neurosci 1989; 9: 497-506.
5. Zhou JN, Hofman MA, Gooren LJ, Swaab DF. A sex difference in the human brain and its relation to transsexuality. Nature 1995; 378: 68-70.
6. Garcia-Falgueras A, Swaab DF. A sex difference in the hypothalamic uncinate nucleus: relationship to gender identity. Brain 2008; 131:3132-3146.
7. Goldstein JM, Seidman LJ, Horton NJ, Makris N, Kennedy DN, Caviness VS, Jr., Faraone SV, Tsuang MT. Normal sexual dimorphism of the adult human brain assessed by in vivo magnetic

resonance imaging. Cereb Cortex 2001; 11:490-497.

8. Lombardo MV, Ashwin E, Auyeung B, Chakrabarti B, Taylor K, Hackett G, Bullmore ET, Baron-Cohen S. Fetal testosterone influences sexually dimorphic gray matter in the human brain. J Neurosci 2012; 32:674-680.
9. Ishunina TA, Salehi A, Hofman MA, Swaab DF. Activity of vasopressinergic neurones of the human supraoptic nucleus is age- and sex-dependent. J Neuroendocrinol 1999 ; 11:251-8.
10. Savic I, Berglund H, Gulyas B, Roland P. Smelling of odorous sex hormone-like compounds causes sex-differentiated hypothalamic activations in humans. Neuron 2001; 31:661-8.
11. Savic I, Berglund H, Lindström P. Brain response to putative pheromones in homosexual men. Proc Natl Acad Sci U S A 2005; 102:7356-61.
12. Berglund H, Lindström P, Savic I. Brain response to putative pheromones in lesbian women. Proc Natl Acad Sci U S A 2006; 103:8269-74.
13. Berglund H, Lindström P, Dhejne-Helmy C, Savic I. Male-to-female transsexuals show sex-atypical hypothalamus activation when smelling odorous steroids. Cereb Cortex 2008; 18:1900-8.
14. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A 2003; 100: 10972-10976.
15. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN, Imamoglu S, Akalin NS, Yuksel B, O'Rahilly S, Semple RK. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. Nat Genet 2009; 41(3): 354-8.
16. Taziaux M, Swaab DF, Bakker J. Sex differences in the neurokinin B system in the human infundibular nucleus. J Clin Endocrinol Metab 2012; 97:E2210-E2220.
17. Guimiot F, Chevrier L, Dreux S, Chevenne D, Caraty A, Delezoide AL, de Roux N. Negative fetal FSH/LH regulation in late pregnancy is associated with declined kisspeptin/KISS1R expression in the tuberal hypothalamus. J Clin Endocrinol Metab 2012; 97(12):E2221-E2229.
18. Rometo AM, Krajewski SJ, Voytko ML, Rance NE. Hypertrophy and increased kisspeptin gene

expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab 2007; 92(7):2744-50.

19. Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Kallo I. The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. Eur J Neurosci 2010; 31(11):1984-98.
20. Hrabovszky E, Molnár CS, Sipos MT, Vida B, Ciofi P, Borsay BA, Sarkadi L, Herczeg L, Bloom SR, Ghatei MA, Dhillo WS, Kalló I, Liposits Z. Sexual dimorphism of kisspeptin and neurokinin B immunoreactive neurons in the infundibular nucleus of aged men and women. Front Endocrinol (Lausanne) 2011; 2:80.
21. Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab 2005; 90:6609-15.
22. Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM. Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (Macaca mulatta) as revealed by double immunofluorescence and confocal microscopy. Endocrinology 2008; 149:4387-95.
23. van der Beek EM, Pool CW, van Eerdenburg FJ, Sluiter AA, van der Donk HA, van den Hurk R, Wiegant VM. Fc-mediated nonspecific staining of the porcine brain with rabbit antisera in immunocytochemistry is prevented by pre-incubation of the sera with proteins A and G. J Histochem Cytochem 1992; 40:1731–1739.
24. Goldstone AP, Unmehopa UA, Bloom SR, Swaab DF. Hypothalamic NPY and Agouti-Related Protein Are Increased in Human Illness But Not in Prader-Willi Syndrome and Other Obese Subjects. J Clin Endocrinol Metab 2002; 87: 927-937.
25. Semaan SJ, Tolson KP, Kauffman AS. The Development of Kisspeptin Circuits in the Mammalian Brain. Adv Exp Med Biol 2013; 784:221-52.
26. Rance NE, Young WS 3rd. Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of

postmenopausal women. Endocrinology 1991; 128:2239-47.

27. Kruijver FP, Zhou JN, Pool CW, Hofman MA, Gooren LJ, Swaab DF. Male-to-female transsexuals have female neuron numbers in a limbic nucleus. J Clin Endo Met 2002; 85:2034-2041.
28. Burke SM, Cohen-Kettenis PT, Veltman DJ, Klink DT, Bakker J. Hypothalamic response to the chemo-signal androstadienone in gender dysphoric children and adolescents. Front Endocrinol (Lausanne) 2014; 5:60.
29. Hoekzema E, Schagen SE, Kreukels BP, Veltman DJ, Cohen-Kettenis PT, Delemarre-van de Waal H, Bakker J. Regional volumes and spatial volumetric distribution of gray matter in the gender dysphoric brain. Psychoneuroendocrinology 2015; 55:59-71.
30. Sisk CL, Zehr JL. Pubertal hormones organize the adolescentbrain and behavior. Front Neuroendocrinol 2005; 26:163–174.
31. Swaab DF, Hofman MA. An enlarged suprachiasmatic nucleus in homosexual men. Brain Res 1990 ; 537:141-8.
32. Allen LS, Gorski RA. Sexual orientation and the size of the anterior commissure in the human brain. Proc Natl Acad Sci U S A 1992 ; 89:7199-202.
33. LeVay S. A difference in hypothalamic structure between heterosexual and homosexual men. Science 1991; 253: 1034-1037.
34. Sellmeyer DE, Grunfeld C. Endocrine and metabolic disturbances in human immunodeficiency virus infection and the acquired immune deficiency syndrome. Endocr Rev 1996; 17:518-32.

Legends

Figure1. Distribution of kisspeptin-ir neurons throughout the rostro-caudal 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.

Figure 3. (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 MTF transsexuals. (E) Relationship between the total number of kisspeptin-ir neurons in the INF and duration of the hormonal treatment in MTF transsexuals. (D) 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.

Figure 4. Schematic representation of sex steroids levels (dotted line) and kisspeptin expression (solid line) in the INF throughout life in females (A) and males (B).