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Male-to-Female Transsexuals Show Sex-Atypical Hypothalamus Activation When Smelling Odorous Steroids

H. Berglund[1], P. Lindström[2], C. Dhejne-Helmy[3] and I. Savic[2]

[1]Department of Medicine, [2]Division of Neurology, Department of Clinical Neuroscience, Stockholm Brain Institute and [3]Department of Psychiatry, Karolinska University Hospital, Karolinska Institute, 171 76 Stockholm, Sweden

One working hypothesis behind transsexuality is that the normal sex differentiation of certain hypothalamic networks is altered. We tested this hypothesis by investigating the pattern of cerebral activation in 12 nonhomosexual male-to-female transsexuals (MFTRs) when smelling 4,16-androstadien-3-one (AND) and estra-1,3,5(10),16-tetraen-3-ol (EST). These steroids are reported to activate the hypothalamic networks in a sex-differentiated way. Like in female controls the hypothalamus in MFTRs activated with AND, whereas smelling of EST engaged the amygdala and piriform cortex. Male controls, on the other hand, activated the hypothalamus with EST. However, when restricting the volume of interest to the hypothalamus activation was detected in MFTR also with EST, and explorative conjunctional analysis revealed that MFTR shared a hypothalamic cluster with women when smelling AND, and with men when smelling EST. Because the EST effect was limited, MFTR differed significantly only from male controls, and only for EST-AIR and EST-AND. These data suggest a pattern of activation away from the biological sex, occupying an intermediate position with predominantly female-like features. Because our MFTRs were nonhomosexual, the results are unlikely to be an effect of sexual practice. Instead, the data implicate that transsexuality may be associated with sex-atypical physiological responses in specific hypothalamic circuits, possibly as a consequence of a variant neuronal differentiation.

Keywords: hypothalamus, odor, PET, pheromones, transsexuality

Introduction

Transsexuals have the strong feeling, often from childhood onward, of having been born the wrong sex. The possible etiology of transsexualism has been the subject of debate for many years (Benjamin 1967; van Goozen et al. 2002; Swaab 2004; Gooren 2006). Investigation of the genetics, hormone levels, gonads, and genitalia of transsexuals has not produced results that explain their status (van Goozen et al. 2002; Swaab 2004; Gooren 2006). In experimental animals, the gonadal hormones that prenatally determine the morphology of the genitalia are shown to also influence the morphology and function of the brain in a sexually dimorphic manner (Fels and Bosch 1971; Yalom et al. 1973; Baum 2003 2006). This led to the hypothesis that sexual differentiation of the brain in transsexuals might not have followed the line of sexual differentiation of the body as a whole and that transsexual persons may have sex-atypical cerebral programing (van Goozen et al. 2002). Such a scenario can be evaluated in humans by comparing transsexual and control subjects with respect to sexually differentiated cerebral functions.

In general, men tend to process verbal stimuli in a more lateralized way than women; they also show a weaker right-hand preference (McGlone 1978; Kimura 1996). Several studies of transsexual subjects have shown a sex-atypical performance in visuospatial (La Torre et al. 1976; van Goozen et al. 2002) and language functions (Cohen-Kettenis et al. 1998), although some were unable to reproduce these patterns (Haraldsen et al. 2005). A sex deviant performance has been described even prior to cross-sex hormone therapy (van Goozen et al. 2002), further emphasizing an impact of biologically determined factors, such as sex-atypical cerebral programing, in gender dysphoria. Of particular interest are reports that male-to-female transsexuals (MFTRs) have a less prominent cerebral lateralization compared with control males during dichotic listening, especially for nonverbal stimuli (Cohen and Forget 1995).

The idea that cerebral programing is atypical in transsexuals finds support in postmortem studies of the anterior hypothalamus and its primary connections, reported by Swaab and collaborators. This group found that the volume of the central subdivision of the bed nucleus stria terminalis (BNSTc) was sexually dimorphic (larger in men than women), showing a "female" size in MFTRs and a "male" size in female-to-male transsexuals (Zhou et al. 1995; Kruijver et al. 2000). Interestingly, its size in MFTRs was smaller also in comparison to male controls that had taken estrogens for medical reasons as well as in relation to those who were homosexual. Thus, not withstanding that the number of subjects and differences in estrogen doses limit the interpretation of these data, it seems improbable that hormone treatment and sexual orientation could be a major cause to the observed differences (Zhou et al. 1995). At variance to the BNSTc, no difference compared with control subjects was detected in other hypothalamic nuclei investigated—the paraventricular nucleus, the sexually dimorphic nucleus, and the suprachiasmatic nucleus—of which the latter 2 are reported to be sexually dimorphic in humans (Swaab and Fliers 1985; Allen et al. 1989; LeVay 1991). These intriguing postmortem data warrant for complementary investigations with in vivo brain imaging techniques. Anatomical investigations of the hypothalamic structures are, however, cumbersome with the current imaging tools because the separate nuclei are extremely small (in the order of mm^3) and difficult to separate with anatomical landmarks. In the quest for reliable in vivo methods to study sex differences in the neurobiology of the hypothalamus, we designed positron emission tomography (PET) activation experiments measuring changes in regional cerebral blood flow (rCBF) during smelling of 2 steroidal compounds: the progesterone derivative 4,16-androstadien-3-one (AND) and the estrogen-like compound estra-1,3,5(10), 16-tetraen-3-ol (EST) (Savic et al. 2001, 2005; Berglund et al.

2006). AND is present in human male secretions such as sweat, saliva, and semen (Grosser et al. 2000), whereas EST has been detected in the urine of pregnant women (Thysen et al. 1968). AND is reported to influence context-dependent mood, and physiological arousal in a sex-specific manner (Jacob et al. 2001, 2002; Savic et al. 2001; Lundstrom et al. 2003, 2006; Bensafi, Tsutsui, et al. 2004; Lundstrom and Olsson 2005), and to also influence endocrine balance by changing cortisol levels (Wyart et al. 2007). Some scientists, therefore, suggest that AND posses pheromone-like properties, although no direct role in sexual attraction has hitherto been demonstrated in humans. The psychophysical effects of EST are less documented but nevertheless reported in some studies (Jacob et al. 2001; Bensafi, Brown, et al. 2004). We found few years ago that the hypothalamus was activated by these 2 compounds in a sex-differentiated manner (Savic et al. 2001)—by AND in heterosexual women (HeW) and by EST in heterosexual men (HeM). In contrast, when men smelled AND and women EST, activations were observed only in the olfactory brain (the amygdala and piriform cortex and the anterior insular- and the anterior cingulate cortex [Zatorre et al. 1992; Sobel et al. 1998; Royet et al. 1999; Savic et al. 2000; Zald and Pardo 2000; Gottfried et al. 2002]). Using an identical experimental design, we subsequently reported a regionally differentiated pattern of activation both with respect to sex (Savic et al. 2005; Hillert et al. 2007) and sexual orientation (Savic et al. 2005; Berglund et al. 2006). Whereas HeW and HeM with chemical supersensitivity activated the hypothalamus with AND (Savic et al. 2005; Hillert et al. 2007), these activations were absent in HeM; they were absent also in lesbian women who recruited the hypothalamic networks with EST (Berglund et al. 2006). Together, these data suggest that the applied approach could be useful for investigations of sex-related neuronal processes in the hypothalamus.

We now report results from 12 nonhomosexual (gynaecophyl) MFTRs who were investigated with this approach. For more detailed information about their sexual orientation, please see Materials and Methods and Discussion. rCBF was measured with PET during smelling AND, EST, 4 common odors denoted as OO, and odorless air (AIR). AIR served as the baseline condition, and activations were defined as increases in rCBF during smelling of odorants compared with air (thus, using AND–AIR, EST–AIR, and OO–AIR as contrasts). The transsexual subjects were compared with 12 HeM and HeW, data from 22 of them presented in previous studies with identical design (Savic et al. 2005; Berglund et al. 2006). The working hypothesis was that the pattern of activation in transsexual subjects would differ from that of HeM. All the investigations were carried out before sex assignment surgery.

Materials and Methods

Subjects

Twelve healthy, unmedicated, right-handed, and nonsmoking MFTRs, who were osmic for both AND and EST and had normal magnetic resonance (MR) image of the brain, participated in the study. Their handedness was assessed according to Oldfield (1971). All the patients were recruited from the Department of Psychiatry, Karolinska University Hospital, Huddinge. None of the subjects had a history of neurological or psychiatric disorders or of brain injury. Before inclusion, a detailed history was obtained from each subject, including possible use of unregulated hormone supplements and synthetic steroids not prescribed by a physician. All the subjects declared that they had never received hormonal treatment, nor were they on any medication at the time of the study. Their statement was deemed reliable and confirmed by repeated tests of hormonal levels.

The diagnosis of gender identity disorder was made according to the International Classification of Disorders 10th edition A plus criteria for transsexualism (F64.0), as follows (American Psychiatric Association Task Force on DSM-IV 1994):

1. A desire to live and be accepted as a member of the opposite sex, usually accompanied by a sense of discomfort with the subject's anatomical sex, and a wish to have surgery and hormonal treatment to make the body as congruent as possible with the body of the preferred sex.
2. The transsexual identity has existed for at least 2 years.
3. The syndrome cannot be explained by any other psychiatric disorder or by chromosomal abnormality. Thus, any evidence of an abnormal male phenotype or genotype (i.e., hypospadias, cryptorchism, micropenis, and chromosome complement other than 46XY) excluded enrollment to the study. Homosexual MFTRs were excluded.

Adult transsexual persons commonly test sexual contacts with both men and women, and it is, therefore, difficult to select a group of nonhomosexual MFTRs who all score at the same end of the Kinsey Heterosexual/Homosexual scale (0 = maximally heterosexual, 6 = maximally homosexual) (Kinsey 1953) for the type of sexual contacts, sexual fantasies, and attraction. To avoid confusion, sexual orientation was here defined operationally, relating the biological sex of the transsexual subjects to the biological sex of their sexual partners. Nine of the recruited MFTRs conveyed at the time of the scan experience of only female sexual partners (they rated Kinsey 0–2), whereas 3 reported that they never had a sexual partner but stated attraction to women and not men. All reported a relatively early (before, or at puberty) awareness of gender dysphoria. Thus, the MFTRs participating in the present study were operationally defined as nonhomosexual MFTRs. All the controls rated Kinsey 0. Twelve right-handed HeM and HeW served as controls. The 3 groups were matched for age (HeM 26 ± 2, HeW 33 ± 6, and MFTRs 32 ± 8 years) and educational level (13 ± 2, 13 ± 2, and 12 ± 3 years). All the transsexual subjects had hormone levels within a normal range (Table S2, supplementary material online). The study was approved by the local human protection and the radiation safety committees of the Karolinska University Hospital.

The Odorous Compounds

Like in our previous studies the activation condition consisted of passive smelling (not sniffing) of AND, EST, and 4 different odors, denoted as OO (Savic et al. 2001, 2005; Berglund et al. 2006). The OO were lavender oil, cedar oil, eugenol, and butanol. Whereas the butanol was diluted in distilled water to a concentration of 10%, the other odors were undiluted. As previously, AND and EST were during the PET scans presented in crystalline and odorous form (200 mg, Steraloids Inc, Newport, RI). In contrast, for testing of the detection threshold to the odor of AND and EST, both compounds were solved in odorless mineral oil. The detection thresholds to the 2 putative pheromones were carried out using the forced choice paradigm (Jones-Gotman and Zatorre 1988), which was also applied for the test of olfactory thresholds, using different concentrations of n-butanol. The purity of AND and EST was repeatedly tested by our doping laboratory and assessed to be 98%.

PET Experiments and Image Data Analysis

All the subjects were investigated during an overlapping time period (2001–2006), in an identical way, and by the same experimenters. Thus, PET measurements were carried out at the same time of the day, with a standardized room temperature and air pressure (23 °C, 997 hPa). The experimental protocol and its justification have been described in detail in our previous publications (Savic et al. 2001, 2005; Berglund et al. 2006). In summary, it included MRI scans and PET (full width at half maximum 3.8 mm) measurements of rCBF with ^{15}O H_2O during 3 stimulus conditions (=smelling of AND, EST, and OO, respectively) and the base line condition (=smelling of room air which was kept odorless

by a suction devise in the scanner room). The duration of each scan was 60 s. All the stimuli (including room air) were presented in a glass bottle at a distance of 10 mm from the nose (Savic et al. 2000, 2001). There were 12 scans per person (3 scans/condition, balanced and randomly interleaved). During the scans, subjects were unaware of the identity of items and instructed to neither sniff nor judge the odorants.

Respiratory movements were recorded continuously 2 min before, and during each scan, by using a strain gauge around the lower thorax connected to a graph (Comair, Stockholm, Sweden) (Savic et al. 2000, 2001, 2005; Berglund et al. 2006). When all the PET scans were completed, the subjects were presented with each odorant again and asked to rate its pleasantness, irritability, intensity, and familiarity using a 100-mm visual analogue scale (Jones-Gotman and Zatorre 1988; Savic et al. 2000).

The individual MRI and PET images were reformatted into a common space (standard brain) and filtered with 10-mm Gaussian kernel (Savic et al. 2000, 2001). Significant activations were determined with the SPM statistics (SPM99, Wellcome Foundation, London, UK) (Friston et al. 1999; Frackowiak 2004), using the following contrasts: AND–AIR and vice versa; EST–AIR and vv; OO–AIR and vv; and AND–EST and vv. Activations were first evaluated in each separate group with 1-group random effect analysis. Next, group differences were tested using a 2-group random effect analysis. Finally, possible common activations between groups were investigated with conjunctional analysis. For the within-group analyses with AIR as baseline, the search space was the entire brain with the exception of the most caudal 16 mm of the cerebellum and the rostral 10 mm of the vertex, which were excluded in 2 patients because these portions of the brain were not covered during scanning. The within-group calculations of AND–AIR and EST–AIR and the between-group evaluations (the conjunctional and 2-group random effect analyses) relied on our previous findings showing that the AND- and EST-related activations were confined to the hypothalamus and the olfactory brain. Considering that the SPM statistics is rather conservative and to avoid type-II error, we used a rectangular mask that covered only the horizontal sections of the brain between $Z = +20$ and $Z = -20$ for the conjunctional and 2-group random effect analyses and for calculations of AND–EST and vv (Tables 1–3).

The single subject condition and covariance analysis was carried out using height threshold at $P = 0.001$ (SPM99, www.wellcome.foundation.uk). In the subsequent within-group analyses, the height threshold was first chosen at $P = 0.001$ (with corrected $P < 0.05$ for multiple comparisons according to the random fields theory). At this level, no cluster appeared in transsexual subjects in the EST–AIR contrast (see results). Because it was essential to evaluate which brain regions processed each type of odorous stimulus, the remaining statistical evaluations were carried out at $T = 0.01$ (corrected $P < 0.05$).

The location of hypothalamus activations was, like in our earlier studies, determined from the Schaltenbrant's atlas (Schaltenbrand and Bailey 1959), after translation of the Talairach's coordinates (Talairach and Tournoux 1988). The Schaltenbrand's atlas provides better information about the individual hypothalamic nuclei. For a more detailed description of the image analyses, please see Savic et al. (2001).

Comparisons of Psychophysical Parameters and Hormone Levels

The mean respiratory amplitude and frequency and a respiratory index (defined as scan frequency × amplitude) were first calculated during each prescan and scan period. Both the baseline and scanning amplitude and frequency were stable within the same subject throughout the study but tended to vary from subject to subject. Therefore, it was less suitable to carry out the group comparisons on the basis of absolute values during various conditions (however, we ascertained that no systematic shifts in overall, i.e., pre + during scan amplitude and frequency were present for any specific condition in any of the groups). Instead, we analyzed the relative change in respiratory amplitude and frequency during each presentation by calculating the mean percentage difference (index) between the scan- and immediate prescan values. MFTRs, HeM, and HeW were then compared for AIR, AND, EST, and OO, using a 2-way analysis of variance (ANOVA), factoring for subject group and stimulus type, as described previously (Zatorre et al. 1992; Sobel et al. 1998; Royet et al. 1999; Savic et al. 2000, 2001, 2005; Zald and Pardo 2000; Gottfried et al. 2002; Berglund et al. 2006; Hillert et al. 2006). A separate ANOVA was conducted for each odor quality (familiarity, irritability, intensity, and pleasantness), to test for group differences in odor ratings, but the stimuli included as the within factor were AND, EST, and OO because AIR was perceived as odorless. The significance level was 0.05 in all comparisons.

Blood samples were taken in each male subject before the scan to measure plasma levels of testosterone (by analyzing sexual hormone–binding globulin, both total and active testosterone levels were calculated), estrogen, prolactin, dehydroepiandrosterone sulfate, follicle-stimulating hormone, luteinizing hormone, and androstendione. Because estrogen levels were much higher than the other concentrations, comparisons between MFTRs and male controls were calculated with separate *t*-tests ($P < 0.007$ after Bonferroni correction).

Structural MR images were acquired on a GE 1.5 T scanner and used for evaluations of PET data according to a previously described protocol (Savic et al. 2000, 2001, 2005; Berglund et al. 2006).

Within-Group Activations

Activations were first evaluated in each separate group with 1-group random effect analysis. With the presently used group size, this method is regarded to provide data, which are representative for the entire group (Friston et al. 1999). When using *T*-threshold at $P = 0.001$ as significance level for each separate image element (pixel) and correction for the multiple comparisons at $P < 0.05$ (see Materials and Methods), HeW activated, as previously reported, the hypothalamus with AND, and HeM with EST. When HeW smelled EST and HeM smelled AND, the activated fields (clusters) were confined to the amygdala, the piriform cortex, and portions of the anterior insular and cingulate cortex. After lowering the threshold to $P = 0.01$ (corrected $P < 0.05$), clusters appeared also in the amygdala and piriform cortex in HeM when smelling EST (the corresponding cluster in women for AND–AIR appeared only at corrected $P < 0.1$) (Table 1 and Fig. 1). Furthermore, a cluster emerged in the middle temporal and lingular gyrus. These clusters had lower Z scores than the hypothalamus clusters. Their presence was, however, congruent with the fact that both compounds were odorous in the concentrations presented. No hypothalamic clusters were detected when HeW smelled EST and HeM AND, independently of whether the height threshold was at $P = 0.001$ or $P = 0.01$.

Like HeW, but unlike HeM, MFTRs activated the anterior hypothalamus with AND. In addition, a cluster was detected in the anterior cingulate, extending to the right amygdala and piriform cortex. When smelling EST, MFTRs recruited only the right amygdala and piriform cortex (Table 1 and Fig. 1). The "AND-related" hypothalamic activation was close to and partly overlapping with the "EST-related" activation in HeM, which was posterior to the corresponding AND-related cluster in HeW. Notably, the respective hypothalamus clusters were detected also when altering the type of contrast (thus, for AND–EST in MFTRs and HeW and for EST–AND in HeM [Table 1 and Fig. 1]). The coverage of the hypothalamic clusters with respect to the location of specific hypothalamic nuclei is given in the supplementary material online (Fig. S2).

At variance to the 2 steroid compounds, and in accordance with our previous studies, smelling of OO yielded similar clusters in all 3 groups of subjects. These clusters covered the amygdala, piriform and insular cortex, as well as minor portions of the anterior cingulate, and the dorsolateral orbitofrontal cortex (Table 1)—regions repeatedly shown to be involved in odor processing (Zatorre et al. 1992; Sobel et al. 1998; Royet et al. 1999; Savic et al. 2000, 2001, 2005; Zald and Pardo 2000; Gottfried et al. 2002; Berglund et al. 2006; Hillert et al. 2006). Coregistration and repositioning of PET clusters in each individual reformatted MRI revealed similar locations in all subjects, without any systematic shifts between the groups.

Within-Group Deactivations

Deactivations were defined as decreases in normalized rCBF when smelling odorants compared with air (AIR–AND, AIR–EST, and AIR–OO). All 3 groups of subjects showed odorant-related deactivations in the temporo-occipital regions; the controls also showed frontal lobe deactivations (Table S1), which in MFTRs were subsignificant (corrected $P < 0.1$).

Table 1
Activations

Region	Transsexual men			HeM			HeW		
	Z level	Size cm^3	Coordinates	*Z* level	Size cm^3	Coordinates	*Z* level	Size cm^3	Coordinates
EST−AIR									
Hypothalamus				5.1	6.4	6, −12, 2			
R amygdala + piriform cortex	3.5	2.0	24, 8, −24				5.1	4.8	34, −2, −12
L amygdala + piriform + insular + cingulate cortex				4.6	3.6	−20, −6, −20	4.5	6.4	−24, 0, −22
R middle temporal and lingular gyri				4.0	2.0	36, −66, 8			
AND−AIR									
Hypothalamus	3.7	3.6	−8 −6, 2				5.7	3.2	−6, 0, −12
R amygdala + piriform cortex				4.4	4.0	18, −10, −12			
						26, −6, 6			
Anterior cingulate + amygdala + piriform cortex	4.3	5.4	−2, 42, −6						
OO−AIR									
R amygdala + piriform + insular + anterior cingulate cortex[a]	3.4	6.4	20, 6, −6	4.9	5.3	24, 0, -8	4.9	6.8	24, −8, 0
L amygdala + piriform + insular cortex	3.8	5.6	−20, −4, −12	4.5	7.2	−30, 2, 6	4.5	7.2	−38, 2, 6
AND−EST									
Hypothalamus	4.0	1.9	−10, −10, -4				4.3	2.4	4, 0, 6
									−10, 0, −6
R middle frontal gyrus				4.3	1.6	20, 40, 6			
L fusiform gyrus							4.0	3.2	−22, −40, −14
R lingular gyrus							4.6	11.0	28, −68, 0
EST-AND									
Hypothalamus				4.5	2.0	−6, −2, 2			
L orbitofrontal + piriform cortex + R inferior frontal gyrus							5.0	5.4	−24, 26, −14
							4.3	1.3	38, 30, −10
R middle frontal gyrus							4.9	1.0	14, 40, −16
Lingular gyrus				5.1	2.9	16, −76, −2			
					4.0	−14, −76, −2			
R middle temporal + fusiform gyrus	3.9	2.1	46, −32, −12						
Cuneus				3.7	4.5	22, −92, 4			

Note: Clusters calculated with 1-group random effect analysis, SPM99, at height threshold $P = 0.01$, and a corrected $P < 0.05$. Talairach's coordinates show local maxima, and regions denote the coverage of the respective cluster. R = right; L = left. No specific side is indicated when the clusters presented in the same row were either on the left or right side. Because the AND−AIR and EST−AIR clusters covering the hypothalamus were large and to investigate whether center of gravity really was located in the hypothalamus, these specific clusters were evaluated also using height threshold at $P = 0.001$ (corrected $P < 0.05$). The Talairach's coordinates remained identical, but the clusters were considerably smaller (0.9 cm^3 for AND−AIR in HeW and 0.7 cm^3 in MFTRs; 0.9 cm^3 for EST−AIR in HeM).

[a]Covered portion of the right orbitofrontal cortex.

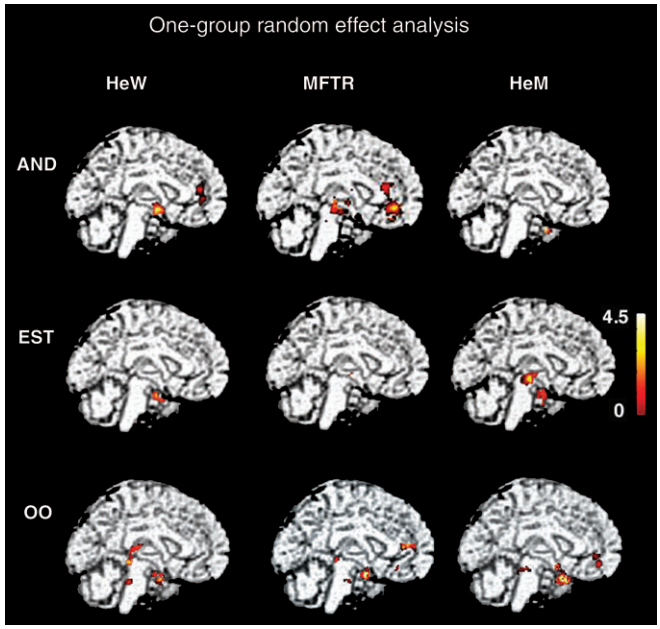

Figure 1. Illustration of group-specific activations with the putative pheromones and odors. The Sokoloff's color scale illustrates *Z* values reflecting the degree of activation. As the same brain section is chosen, the figures do not always illustrate maximal activation for each condition. Clusters of activated regions are superimposed on the standard brain MRI, midsagittal plane. The cingulate cluster in HeW and the left amygdala + piriform cluster in HeM for AND were not significant. Neither were the midbrain clusters in the bottom row.

Group Comparisons

The pattern of activation in MFTRs differed significantly only from HeM. Difference between HeM and MFTRs in the hypothalamic activation was also found when applying AND-EST and EST-AND contrasts, Table 2 and Figure 2. In contrast, no significant differences were observed between MFTRs and HeW (Table 2). Furthermore, as in our previous studies, no group differences were found with OO-AIR.

When using AIR as reference, the HeW and HeM differed, as shown previously, only in the hypothalamic activations. The local maximum for HeW-HeM with respect to AND-AIR corresponded to Talairach's coordinates 0, 0, -13, *Z* level = 3.2, cluster size 0.8 cm^3. HeM showed, instead, a significantly higher hypothalamus activation with respect to EST-AIR (the local maximum corresponded to Talairach's coordinates 6, -8, 2, *Z* level = 4.0, cluster size 1.4 cm^3, Fig. 2). Direct AND-EST and EST-AND comparisons revealed similar group differences (coordinates -8, 0, -6; *Z* level = 4.4, size 3.2 cm^3 for AND-EST in HeW-HeM and vv). In addition, as reported previously, clusters appeared also in the left lingular and fusiform gyri and the middle occipital gyrus (Talairach's coordinates 46, -70, -6, *Z*-level = 4.5, size 7.5 cm^3 for HeW-HeM in AND-EST and vv). These clusters corresponded to the deactivations by EST in HeW and by AND in HeM (threshold level at $P = 0.01$, and corrected $P < 0.05$ for all the calculations, Table S1).

To investigate whether and how the transsexual subjects shared activations with the respective control group, we also carried out conjunctional analysis. At variance from the results of random effect analyses, MFTRs were found to share hypothalamic activation not only with HeW when smelling AND but also with HeM when smelling EST. All the 3 groups of subjects also shared clusters in the olfactory networks, independently of the stimulus-compound (AND, EST, and OO) (Table 3 and Fig. 3).

The observation that MFTRs shared hypothalamus activations with both HeM and HeW motivated 2 separate post hoc analyses:

Table 2
Group differences

Region	HeM–transsexual men			Transsexual men–HeM		
	Z level	Size cm^3	Coordinates	*Z* level	Size cm^3	Coordinates
EST−AIR						
Hypothalamus	3.9	4.0	4, −8, 2			
EST–AND						
Hypothalamus	4.6	2.4	4, −7, −4			
Left putamen and insular cortex				3.3	1.2	−18, −8, 0

Note: Clusters calculated with 1-group random effect analysis, SPM99, height threshold $P = 0.01$, corrected $P < 0.05$. Talairach's coordinates indicate local maxima. No differences were found between MFTRs and HeW, and no group differences were observed for OO−AIR. Furthermore, at $P < 0.05$ corrected, AND−AIR showed no significant difference between transsexual men and HeM. When applying height threshold at $P = 0.01$ and $P < 01$ corrected, additional clusters appeared for MFTRs−HeM in the hypothalamus for AND−AIR ($Z = 3.4$, size 0.8 cm^3, coordinates −8, −10, −8) and AND−EST ($Z = 3.6$, size 1.2 cm^3; 4, −7, −4); contrasting HeM−MFTRs for AND−AIR, on the other hand, showed a cluster in right piriform and insular cortex ($Z = 3.8$, size 3.2 cm^3; 32, −4, 0). With respect to HeW, a cluster appeared only for HeW−MFTRs in AND−EST ($Z = 3.6$, size 0.4 cm^3; −2, 0, −10).

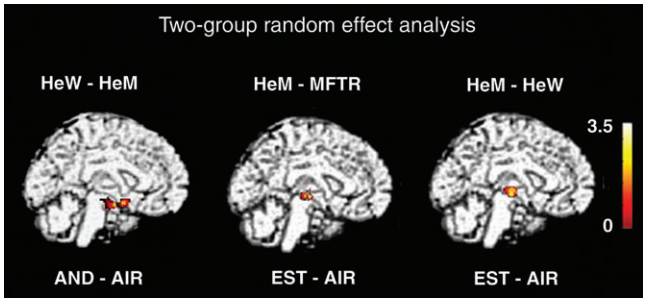

Figure 2. Significant differences between the groups. Shown are the clusters calculated with 2-group random effect analysis, superimposed on standard brain MRI. The Sokoloff's color scale illustrates *Z* values reflecting the degree of activation. The cingulate cluster in HeW and the left amygdala + piriform cluster in HeM for AND were not significant.

Table 3
Conjunctional analysis

Region	Transsexual men and HeM			Transsexual men and HeW		
	Z level	Size cm^3	Coordinates	*Z* level	Size cm^3	Coordinates
AND−AIR						
Hypothalamus				3.6	2.0	−6, −6, −4
R amygdala + piriform cortex	3.8	2.2	24, 6, −4	3.5	3.0	20, 2, 0
EST−AIR						
Hypothalamus	5.1	2.0	6, −6, 0			
R amygdala + piriform cortex	4.5	1.3	18, 4, −8[a]	4.3	4.2	20, −2, −4 14, 6, −10[a]
OO−AIR						
R amygdala + piriform cortex	6.2	5.6	18, 2, −12[a]	6.6	9.6	18, 2, −10[a]
L amygdala + piriform cortex	5.9	7.2	−20, −2, −12	5.9	9.6	−22, 0, −12

Note: Clusters calculated with conjunctional analysis (see Materials and Methods), SPM99, at height threshold $P = 0.01$, and a corrected $P < 0.05$. Talairach's coordinates indicate local maxima.
[a]Clusters which partly covered the caudate nucleus.

1. Volume of interest (VOI) analysis of the hypothalamus. The purpose was to evaluate whether the MFTRs activated this region in line with HeM or HeW. Because the anatomical boundaries of the anterior hypothalamus are difficult to determine with MRI, so-called functional VOIs were employed. They were generated in a previous

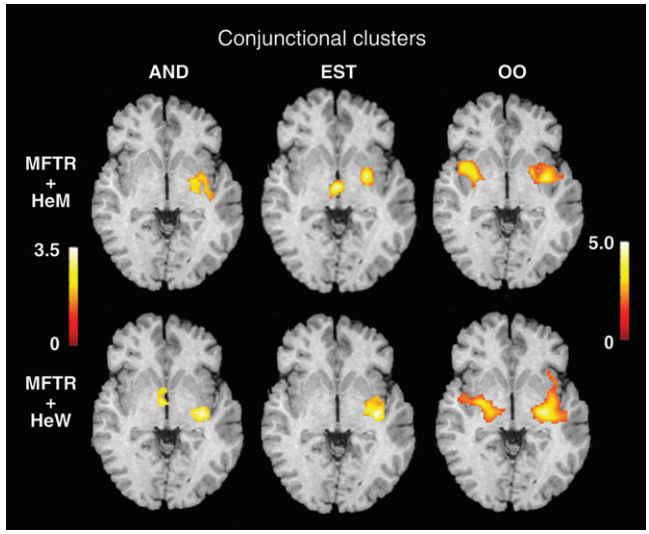

Figure 3. Common activations between the groups. Shown are conjuctional clusters in different groups of subjects, superimposed on the standard brain. All images show horizontal level at $Z = -5$ according to Talairach's atlas. The Sokoloff's color scale illustrates *Z* values (0.0–3.5 for AND and EST and 0.0–5.0 for OO). The OO clusters were large and covered several lower sections. Because the same brain section is chosen, figures do not always illustrate maximal activation for each condition. Subject's right side is to the right.

activation study of heterosexual controls using identical compounds (Savic et al. 2001). The VOIs were constituted by the hypothalamus cluster generated by AND–AIR in HeW (here denoted as the AND VOI) and a separate hypothalamus cluster generated by EST–AIR in HeM (here denoted as the EST VOI). Both VOIs were derived from PET images reformatted to the same standard brain and could, therefore, be directly transferred to the PET images from presently investigated controls and transsexuals. In these images, the rCBF was in all subjects first normalized to the global cerebral mean of 50 ml/min/100 g. The rCBF was extracted for each VOI, and the mean rCBF of the 3 scans per condition was calculated in each subject (Savic et al. 2001). The mean rCBF for AIR, EST, AND, and OO was then compared for each subject group in separate repeated-measures ANOVAs (1 for each VOI). The degree of freedom (df) was 3. If there was a significant interaction at this level, appropriate contrasts were calculated. In addition, we tested possible differences between MFTRs, HeW, and HeM for AND–AIR, EST–AIR, and OO–AIR in each predetermined VOI by means of 2-way repeated-measures ANOVA with subject group as the between factor and the type of odorant as the within factor. Because the variable of interest, the group by odor interaction, was significant in the AND VOI ($F = 3.2$; $P = 0.02$; df = 4) as well as in the EST VOI ($F = 2.9$; $P = 0.03$; df = 4), the results were further explored with contrasts (df = 1) to determine which specific subject group and type of odorant determined the observed interaction. *P* values were considered significant when <0.05.

In MFTRs, the VOI analysis of mean rCBF yielded a significant interaction between the 3 stimuli and air only in the EST VOI ($F = 4.4$; $P = 0.01$; df = 3). The interaction was constituted by activation with both AND ($F = 5.5$; $P = 0.02$; df = 1) and EST ($F = 8.7$; $P = 0.005$; df = 1). No significant interaction was found in the AND VOI ($F = 0.9$; $P = 0.7$; df = 3). In accordance with previous studies, HeM activated the EST VOI with EST ($F = 10.2$; $P = 0.002$; df = 1) but not AND ($F = 0.5$; $P = 0.5$; df = 1). This activation was significantly more pronounced than in HeW ($F = 9.0$; $P = 0.004$; df = 1) and MFTRs ($F = 4.5$; $P = 0.04$; df = 1). The HeW, however, increased the rCBF in the AND VOI but only when smelling AND ($F = 12.3$; $P = 0.001$; df = 1) and not EST ($F = 0.28$; $P = 0.6$; df = 1). The increase was significant in relation to HeM ($F = 13.9$; $P = 0.0004$; df = 1) and in relation to MFTRs ($F = 7.0$; $P = 0.01$; df = 1). In contrast to AND and EST, OO showed no significant activation in any of the groups ($F = 0.16$, df = 1; $P = 0.84$ and $F = 0.12$, df = 1; $P = 0.85$ for the AND and EST VOI, respectively).

2. The second post hoc analysis was conducted to further evaluate the more intermediate pattern in MFTRs suggested by the conjunctional and VOI analyses. Explorative group comparisons were, therefore, carried out also with $P < 0.1$ corrected, in order to evaluate the location of possible subsignificant clusters. Like in the first analysis ($P < 0.05$ corrected), MFTRs differed from HeM, but now a cluster appeared also for AND–AIR (MFTRs–HeM; $Z = 3.4$, size 0.8 cm^3, coordinate –8, –10, –8) and AND–EST ($Z = 3.6$, size 1.2 cm^3; 4, –7, –4). Thus, the difference was constituted by the AND-related hypothalamus activation in MFTRs (not observed in HeM), as well as by their significantly less pronounced EST-induced elevation of rCBF. Notably, the HeM–MFTRs contrast for EST–AIR only partly overlapped with the EST VOI described in the previous paragraph, suggesting that the predefined VOI did not depict the maximal group difference. The comparison with HeW, on the other hand, showed a subsignificant cluster only for AND–EST (HeW–MFTRs; $Z = 3.6$, size 0.4 cm^3; –2, 0, –10) but not for AND–AIR or EST–AIR (please also see Table 2). Together, all these analyses suggested a less pronounced dichotomy in MFTRs but with primarily female-like pattern of activation.

There were no significant group by odor interactions for any of the rated parameters (Fig. 4), neither did we find any group by stimulus interaction in respiratory variables (Fig. S1). No group differences were observed in odor thresholds or plasma concentrations of the tested hormones (Tables S2 and S3).

Discussion

Based on investigations showing that men and women process signals from putative pheromones in a sex-differentiated manner, we carried out experiments in 12 MFTRs and found a sex-atypical mode of activation. The generated data will be discussed in the perspective of sex differences in the measured activation pattern and their possible relationship to transsexualism.

Explorative analysis using the entire brain as search space showed that MFTRs activated the anterior hypothalamus with AND. In contrast, when they smelled the estrogen-like compound, EST, activation was observed in the amygdala and piriform cortex, as it did during exposition to common odors (OO). The picture was more complicated when restricting the analysis to the 2 predefined hypothalamus VOIs. MFTRs were found to increase their rCBF with both AND and EST and showed conjunctional clusters in the hypothalamus with both male and female controls. However, the increase with EST was less pronounced, and direct group comparisons in the explorative SPM analysis ($P < 0.05$ corrected) showed that MFTRs differed significantly only from the male controls and resembled the female controls, independently of whether the contrast used was EST–AIR or EST–AND. Thus, taken together, MFTRs occupied an in-between position between HeM and HeW but with overall predominantly female features. Notably, as opposed to putative pheromones, ordinary odorants activated only the common olfactory regions in all 3 groups, suggesting that the differentiated activations by AND and EST were of physiological relevance.

Methodological Issues

The data were analyzed with the user-independent SPM statistics. This method is known to be conservative and carries a certain risk that physiologically relevant changes in rCBF can remain undetected (Friston et al. 1996). Activations were, in the present study, regarded significant at height threshold $P = 0.01$ because no clusters were detected in MFTRs when contrasting EST to AIR at $P = 0.001$, despite normal odor detection thresholds and a normal perception of this odorant

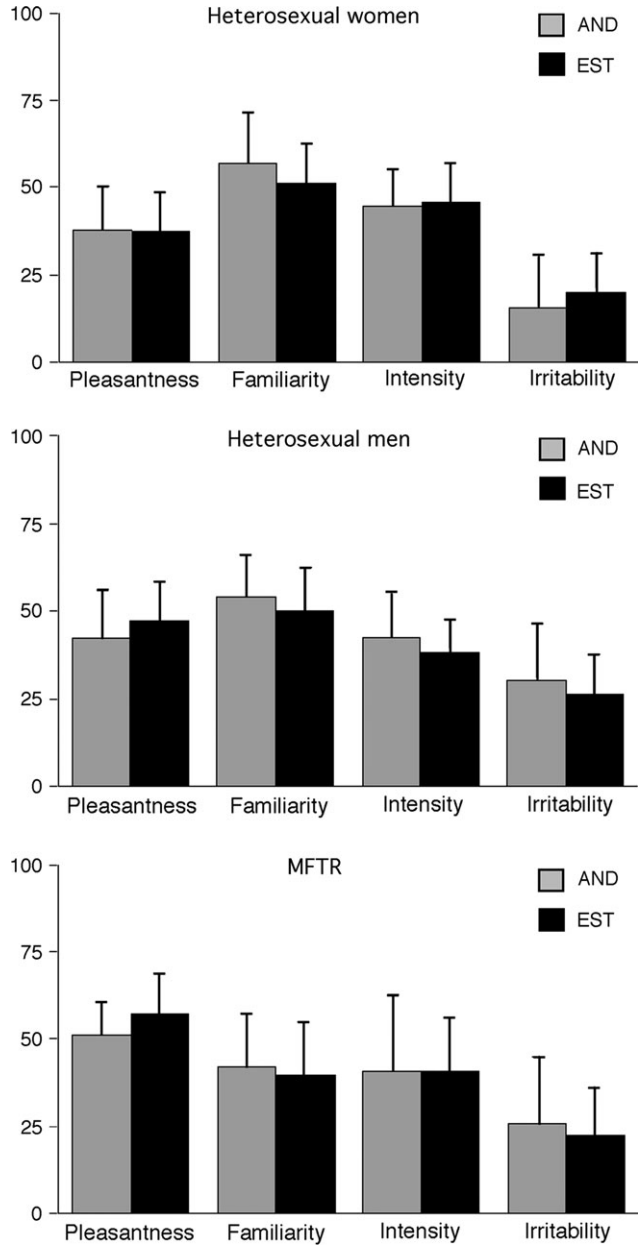

Figure 4. Odor ratings for AND and EST. The vertical axis indicates a visual analogue scale in millimeters (mean ± SEM). (Top) HeW. (Middle) HeM. (Bottom) MFTRs. None of the ratings differed between the 3 groups of subjects

(Fig. 4). This maneuver was regarded adequate, considering that the choice of significance level in SPM statistics is rather arbitrary (Friston et al. 1996), that we wanted to evaluate the pattern of activation exploratively, without lowering the corrected P level, and provide data comparable with those presented earlier (Savic et al. 2005; Berglund et al. 2006). The major portion of control data was reported in these previous publications. Because the inclusion of transsexual subjects with the restrictive criteria applied needed a long time period (from 2001 to 2006), 2 female controls had to be replaced by more recently investigated subjects to match the study period for patients and controls. Thus, the acquisition time overlapped between the groups. The time period for data acquisition did, however, not pose methodological problems as the

experimental conditions (including the experimenters, the scanner, time of the day, room temperature, and humidity) were identical during all experiments. Furthermore, the compounds were repurchased and tested for purity on several occasions.

According to the method applied, the material was sufficient to generate inference at group level (Friston et al. 1999). Furthermore, use of random effect analysis implies that each individual is representative for his/her designated group and that the results from within- and between-group analyses are deductible for the respective type of population (Friston et al. 1999). It should, however, be emphasized that the method is not informative about the separate individuals belonging to a group. A further issue deserving comment is that the precise cluster locations within the hypothalamus should be taken with precaution because of the 10-mm filtering and the scanner resolution. Finding of a local maximum with atlas coordinates indicates that an area of 10 mm around this coordinate was maximally involved.

The cluster locations in relation to the separate nuclei in the supplementary material online (Fig. S2) are shown only for general orientation; this schematic presentation does not imply that the activations were strictly confined to the respective nuclei but illustrates that AND activation in MFTRs was slightly rostro-posterior to that of HeW. This slight shift is unlikely to be an effect of image processing as each individual reformatted PET image was specifically controlled for its position in Talairach's space and because coregistration and repositioning of PET clusters in each individual reformatted MRI revealed similar locations in all subjects, without any systematic shifts between the groups.

The seemingly complex picture given by the combination of random effect, VOI, and conjuctional analyses deserves a comment. Obviously, the signals from AND and EST elicited a less differentiated response pattern in MFTRs than in controls. Because MFTRs elevated their CBF in the hypothalamus also when smelling EST, the conjunctional analysis (which is more sensitive when the responses are congruent) depicted a common EST cluster with HeM, although the increase was not strong enough to pass the significance limit in the random effect analyses. The results from the different analyses are, therefore, not incompatible.

The primary hypothesis with the present study was that the pattern of activation in MFTRs would be sex atypical and that hypothalamic clusters would be observed with AND but not EST. Notably, an AND-related hypothalamic activation was recently observed in homosexual men (Savic et al. 2005). Thus, a sex-atypical pattern in transsexuals could in the presence of homosexual orientation be attributed both to homosexual sexual practice and to transsexualism. Therefore, only nonhomosexual (gynaecophyl) transsexuals were included in the present study. Nonhomosexual transsexuals may be heterosexual, asexual, and bisexual (Blanchard 1989). Optimally, our transsexual subjects should have been strictly heterosexual according to the present operative definition (having only female sex partners), like our male controls. With the conservative inclusion criteria applied (no history of estrogen treatment, right-handedness, normal odor thresholds, and hormonal levels), it was difficult to fulfill this goal, and 3 MFTRs denied any sexual contacts although they expressed sexual attraction to women. They showed, however, almost identical hypothalamic increases of rCBF as the MFTRs who have had female partners, and the group inhomogeneity is unlikely to explain the present results (see further). Several centers currently recognize essentially 2 types of MFTRs who can be distinguished on the basis of their sexual orientation (Chivers and Bailey 2000; Smith et al. 2005). The first type is homosexual transsexuals, extremely gender-transposed (feminine) men, whose sexual object choice is toward men instead of women. The second type is men, whose sexual object choice is interpreted to be toward the image of themselves as women. For this group, the primary motivation for changing sex is to become the object of their own desire (Chivers and Bailey 2000; Smith et al. 2005). In this respect, all our patients can be regarded as nonhomosexual (gynaecophyl) transsexuals (Chivers and Bailey 2000), although we used a more operative approach in our classification, taking into consideration also the sexual partners, sexual fantasies, and expressed attractions (Kinsey 1953).

Possible Underlying Mechanisms

AND and EST are 2 synthetical steroids whose reported effects (see Introduction) have triggered a discussion as to whether they can be regarded as putative pheromones. Although this discussion is yet to be settled, the short (60 s) duration of the PET scans strongly suggests that the clusters detected were a result of localized neuronal activations and not an effect of binding to estrogen and androgen receptors in the brain (for a more detailed argumentation, please see Savic et al. 2001, 2005; Berglund et al. 2006).

As suggested in our previous publications, the presently found response to the 2 steroids could, theoretically, be explained by sexual arousal, by acquired sensitization to the specific compound shaped by sexual experience, and by the organization of neuronal circuits of the anterior hypothalamus. Sexual arousal seems to engage several cerebral structures outside the hypothalamus (Karama et al. 2002; Canli and Gabrieli 2004), which were not activated in the present study. Furthermore, none of our subjects reported sexual arousal. If existing, a sensitization/shaping of the neuronal response in relation to sex-related exposure to AND and EST would favor a "male-like" pattern of activation, as the majority of our patients had female partners; absence of such exposure (as in the 3 patients who were virgins) would, on the other hand, result in an undifferentiated response. Group analyses showed that our MFTRs differed only from the male controls and showed a predominantly "female" pattern of activation. By logical inference, it seems, therefore, unlikely that sexual experience had a major impact on the present results. An alternative explanation, which seems more congruent with the generated data and the current literature, is that transsexual persons could have variant organization in neuronal circuits of the anterior hypothalamus and, perhaps, also its primary connections. Such a mechanism could in MFTRs have led to the here observed female-like responses while maintaining certain male features. A similar mechanism has also been discussed in relation to AND activation in homosexual men (Savic et al. 2005). However, whereas the data from homosexuals could not disentangle between innate and learned effects, the present constellation (with no male sexual contacts) makes the first alternative more probable.

In homosexual men, like in heterosexual controls, either the hypothalamic or the olfactory pathway dominated during smelling of the 2 steroids. This distinction was ambiguous in our MFTRs who, for example, activated the anterior cingulate

with AND even more strongly than the anterior hypothalamus. Theoretically, both the poor pathway distinction and the fact that MFTRs shared hypothalamic clusters with both men and women could reflect a sex-atypical organization of the hypothalamic networks. The pattern of activation was found to be sex atypical also in homosexual men (Savic et al. 2005). However, the underlying mechanisms could be different. Sexual orientation and sexual identity are 2 different entities, even if homosexuals reportedly recall more cross-gender behavior in childhood than male and female heterosexuals (Slijper et al. 1998).

The major contribution of the present findings is that MFTRs are found to respond in a sex-atypical manner in areas of the hypothalamus, which are regarded to be involved in sexual and reproductive behavior and which are reported to harbor sexually dimorphic features. The study also suggests that the way smelling of AND and EST activates the human brain is not necessarily an effect of the exposure history. Thereby, it adds conceptually new information to the previous reports.

The generated data relate to reports by Swaab's group of a female size of BNSTc in MFTRs (Zhou et al. 1995; Kruijver et al. 2000). Although structural and functional dimorphism are not directly translatable and the BNST is too small to be detected with the imaging methods applied, it is of note that this nucleus in animals mediates pheromone signaling and that it in humans has reciprocal connections with the anterior hypothalamus (Eiden et al. 1985). Both Swaab's and our findings may, therefore, reflect a common organizational deviation of certain sexually dimorphic circuits involved in human reproduction. Whether and how this links to the perception of sexual identity remains unclear and awaits further investigations.

In summary, albeit the present study does not provide conclusions concerning the possible etiology, it suggests that in transsexuals the organization of certain sexually dimorphic circuits of the anterior hypothalamus could be sex atypical. It adds a new dimension to our previous reports by showing that the observed effects are not necessarily learned and that a sex-atypical activation by the 2 putative pheromones may reflect neuronal reorganization.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Notes

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Address correspondence to Ivanka Savic, Department of Clinical Neuroscience, Stockholm Brain Institute, Karolinska University Hospital, Karolinska Institute, MR centre, M8, 171 76 Stockholm, Sweden. Email: ivanka.savic-berglund@ki.se.

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