Neuron, Vol. 31, 661–668, August 30, 2001, Copyright ©2001 by Cell Press

Smelling of Odorous Sex Hormone-like Compounds Causes Sex-Differentiated Hypothalamic Activations in Humans

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Summary

The anatomical pathways for processing of odorous stimuli include the olfactory nerve projection to the olfactory bulb, the trigeminal nerve projection to somatosensory and insular cortex, and the projection from the accessory olfactory bulb to the hypothalamus. In the majority of tetrapods, the sex-specific effects of pheromones on reproductive behavior is mediated via the hypothalamic projection. However, the existence of this projection in humans has been regarded as improbable because humans lack a discernable accessory olfactory bulb. Here, we show that women smelling an androgen-like compound activate the hypothalamus, with the center of gravity in the preoptic and ventromedial nuclei. Men, in contrast, activate the hypothalamus (center of gravity in paraventricular and dorsomedial nuclei) when smelling an estrogen-like substance. This sex-dissociated hypothalamic activation suggests a potential physiological substrate for a sex-differentiated behavioral response in humans.

Introduction

The pheromones are, according to the original definition, volatile compounds secreted into the environment (in sweat, urine) by one individual of a species, perceived by another individual of the same species, in whom they trigger a behavioral response or physiological change (Karlson and Luscher, 1959). Pheromones are, in the majority of mammals, transduced in the vomeronasal organ (VNO) (situated in the nasal cavity) to signals which, via the accessory olfactory bulb, the medial amygdala, and stria terminalis, reach the anterior hypothalamus (Keverne, 1999). In ferrets and pigs, an alternative pathway via the main olfactory bulb has been suggested (Wersinger and Baum, 1997; Dorries et al., 1997). Via the hypothalamus and its connections, the pheromones influence sexual behavior and reproductive functions (Keverne, 1999; Wersinger and Baum, 1997) in a sex-specific way (Yokosuka et al., 1999). Although VNO is reported to exist in human adults (Moran et al., 1995), it is uncertain whether and how possible pheromone signals may be mediated, as the accessory olfactory bulb regresses after the fetal period (Keverne, 1999), and alternative neuronal connections from VNO to the brain have not been convincingly demonstrated (Roslinski et al., 2000; Trotier et al., 2000). Nevertheless, the possibility of pheromone-like effects in humans is discussed in the literature.

Female axillary extract applied to the upper lip can alter the timing of ovulation and menstruation of the recipient (Stern and McClintock, 1998). This phenomenon is suggested to underlie the menstrual synchrony among roommates and is assumed to be mediated by the hypothalamus (Stern and McClintock, 1998; McClintock, 1971). Exposure to male axillary secretion is reported to give more regular menstrual cycles (Cutler et al., 1986). The axillary and skin secretions contain compounds resembling sex hormones (Gower and Ruparelia, 1983; Smals and Weusten, 1991). Monti-Bloch et al. found that such compounds induce changes in body temperature, skin conductance, respiration, and heart rate and produce surface electrical potential recorded from the nasal epithelium of the vomeronasal pit in a sex-related way. The authors, therefore, suggested that the steroids they used had pheromone-like characteristics (Monti-Bloch and Grosser, 1991). One of them, oestra-1,3,5(10),16-tetraen-3yl acetate, was shown to elicit cerebral activation in males in nonodorous, undetectable concentrations (Sobel et al., 1999). This finding could reflect a cerebral effect which is unrelated to odor. Also, it has recently been reported that a putative pheromone receptor gene is expressed in human olfactory mucosa (Rodriguez et al., 2000). These data raise the question whether there are compounds that via the nasal mucosa activate the human hypothalamus in a sex-specific mode. If so, such compounds would fulfill one important criterium to qualify as candidates for putative pheromones in humans. Theoretically, the signals from such compounds could in humans, as in pigs and ferrets, be mediated by other sensory pathways than VNO.

In the present study, we therefore investigated possible sex differences in cerebral activation, using two different sex hormone-resembling substances—first in females, then in males. These compounds were 4,16-androstadien-3-one (AND), a derivative of testosterone produced in human axillary secrete in concentrations which are up to twenty times higher in men compared to women; and oestra-1,3,5(10),16-tetraen-3-ol (EST), a substance resembling naturally occurring oestrogenes (Gower and Ruparelia, 1993; Smals and Weusten, 1991; Monti-Bloch and Grosser, 1991; Sobel et al., 1999). Three specific issues were addressed. (1) Do AND and EST activate the human brain? (2) Are the activations sex specific, i.e., do AND and EST activate different regions in males and female? (3) Are the activations located in regions mediating reproductive behavior?

The cerebral activations were studied by measurements of regional cerebral blood flow (rCBF) with positron emission tomography (PET), during passive and birhinal smelling of AND, EST, and odorless air (AIR). Twenty-four healthy subjects (12 females) participated. Smelling of AND and EST was assumed to cause cere-

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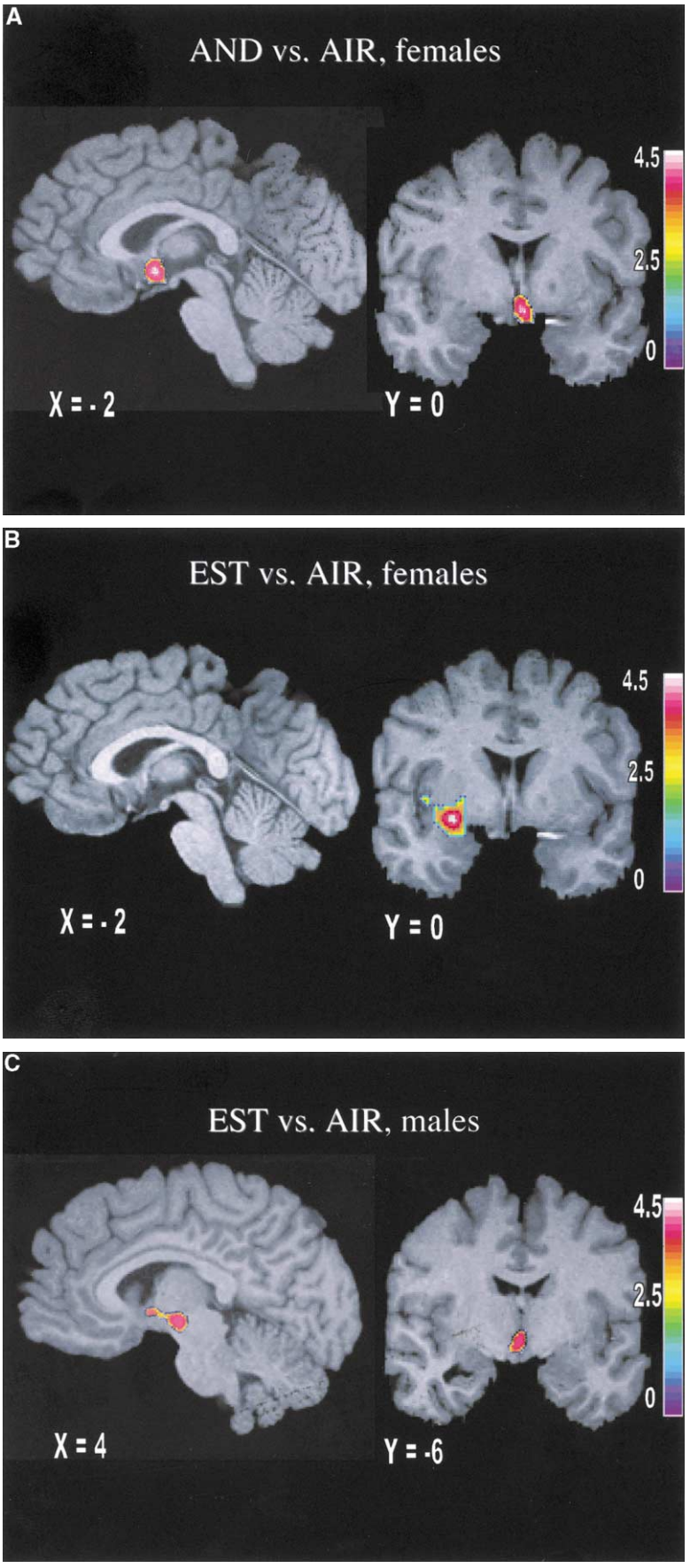

Figure 1. Activated Clusters Superimposed on a Standard Brain

The Sokoloff color scale illustrates z values (0.0–4.5). The clusters were thresholded at 3.1; thus, only regions with $z > 3.1$ and cluster size > 0.8 cm^3 are shown. (A) AND versus AIR in females. (B) EST versus AIR in females. (C) EST versus AIR in males. Subject's right side is to the left. The Talairach coordinates are given. The same brain sections are shown for the two contrasts within each sex group to illustrate the lack of hypothalamic activation with AND in males and EST in females. Only the significant clusters ($p < 0.05$) are shown. The AND versus AIR in males showed no clusters at $p < 0.05$ and is therefore not illustrated.

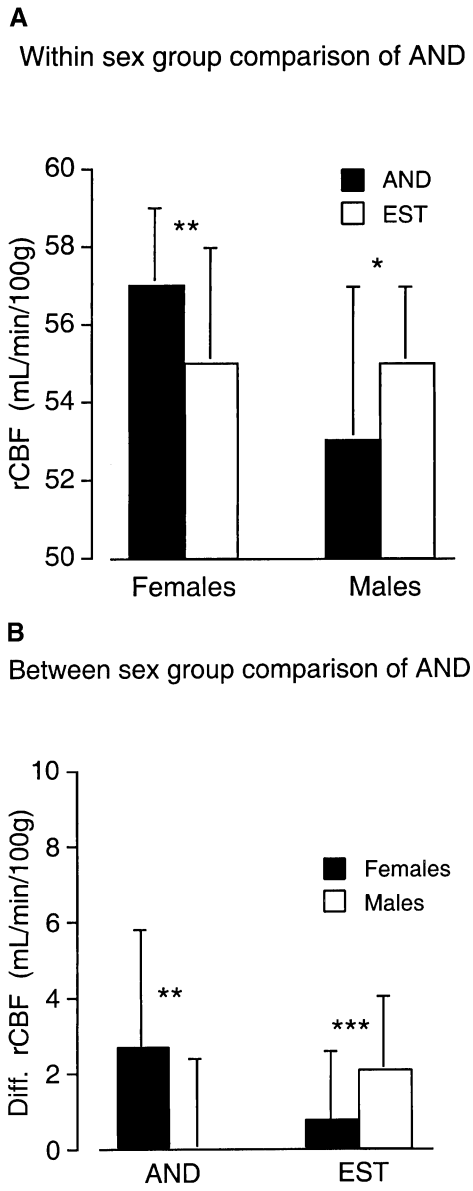

Figure 2. Gender Differences with Respect to AND and EST

(A) Normalized rCBF values in the hypothalamic VOI during smelling of AND and EST in females and males. Comparisons between AND and EST were carried out within each sex group. (B) Comparisons between males and females, based on the difference between the tested compound and AIR. There was a significant sex × VOI interaction ($F = 27.7$, $p < 0.001$). The AND versus AIR difference in males was zero. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. For further details, please see the Experimental Procedures and Results sections.

bral activation if rCBF was higher than during smelling AIR (see Experimental Procedures).

Results

In females, AND activated the anterior-ventral hypothalamus (Figures 1 and 2) but not the olfactory regions (amygdala, piriform, orbitofrontal, and insular cortex) (Zatorre et al., 1992; Savic et al., 2000b). These olfactory regions were activated when females smelled EST (Table 1). To the contrary, males activated the hypothalamus but not the olfactory regions when smelling EST (Figures 1 and 2; Table 1). When males smelled AND, no activations were found at a probability of $p < 0.05$. When lowering the threshold ($p < 0.1$), clusters appeared, however, in right amygdala + piriform cortex, right cerebellum, and right postcentral gyrus (Table 1).

At $p < 0.1$, the AND versus AIR contrast in females showed clusters not only in the hypothalamus but also in right amygdala + piriform cortex, anterior cingulate, and right lingual gyrus. Accordingly, in the EST versus AIR contrast in males, at $p < 0.1$, clusters appeared in right and left amygdala + piriform + insular cortex and in anterior cingulate in addition to the hypothalamus. No other clusters were observed in relation to AIR in males or females (Table 1). Thus, the pattern of activation with AND and EST clearly showed reciprocal features in the two sexes.

We used the atlas of Schaltenbrand (1959) to specifically localize the hypothalamic nuclei (see Experimental Procedures). According to Schaltenbrand, the hypothalamic activation in females covered the preoptic area and ventromedial nucleus with a center of gravity corresponding to the preoptic nucleus. The hypothalamic activation by EST in males covered the dorsomedial and paraventricular nucleus extending to lower fornix, with a center of gravity corresponding to the dorsomedial hypothalamic nucleus (Schaltenbrand, 1959). Coregistration and superpositioning of the two hypothalamic clusters on individual MR images showed a minor overlapping, with centers of gravity distanced by about 10 mm.

Next, we conducted a volume of interest (VOI) analysis (Savic et al., 2000a, 2000b) to examine whether the observed hypothalamic activations were truly sexually dimorphic. The respective hypothalamic clusters (obtained during AND versus AIR in females and EST versus AIR in males) were used as VOIs. The mean normalized rCBF was calculated in each subject during smelling of AND and EST. The values from the two conditions (six values per subject) were then compared within and between the two groups of subjects. In the between sexes comparisons, the difference between the respective compound and AIR in the same VOI were entered in the ANOVA model. The comparisons between AND and EST in the hypothalamic VOIs showed the following. When females smelled AND, the rCBF in the hypothalamic VOI (covering the preoptic and the ventromedial nucleus) was higher than when they smelled EST (57 ± 2 ml/min/100 g versus 55 ± 3 ml/min/100 g; $F = 9.3$, $p = 0.01$, one-way repeated measure ANOVA). The corresponding value in males smelling AND was 53 ± 4 ml/min/100 g. The calculated sex difference was significant: $F = 10.5$, $p = 0.004$, two-way repeated measure ANOVA. Conversely, when men smelled EST, the rCBF in the hypothalamic VOI (covering the dorsomedial and paraventricular nucleus) was higher than when they smelled AND (55 ± 2 ml/min/100 g versus 53 ± 4 ml/min/100 g; $F = 5.5$, $p = 0.04$, one-way repeated measure ANOVA). The corresponding value in females smelling EST was 53 ± 4 ml/min/100 g. The calculated sex difference was significant: $F = 15.5$, $p < 0.001$, two-way repeated measure ANOVA. There was a significant sex × VOI interaction of AND and EST activation in AND- and EST-defined VOIs ($F = 28.7$, $p < 0.001$, two-way repeated measure ANOVA).

Table 1. Activations

	Male			Female		
Contrast	Region Talairach Coordinates (*x*, *y*, *z*)	Size (cm^3)	Z Value (Mean)	Region Talairach Coordinates (*x*, *y*, *z*)	Size (cm^3)	Z Value (Mean)
EST versus AIR	**hypothalamus, dorsomedial, and paraventricular nucleus (4, −10, 0)**	**0.8**	**3.3**	**right piriform + insular cortex (28, 1, −6)**	**0.9**	**3.2**
				right amygdala + piriform cortex (20, −15, −18)	**1.3**	**3.3**
	right (35, −8, −9)	0.6	3.1			
	and left (−22, 2, −15) amygdala + piriform + insular cortex	0.6	3.1			
	anterior cingulate (10, 7, 32)	0.5	3.0			
AND versus AIR	right amygdala + piriform cortex (11, −14, −14)	0.5	3.2	**hypothalamus, preoptic, and ventromedial nucleus (−5, 2, −7)**	**0.8**	**3.2**
	right cerebellum (17, −51, −21)	0.6	3.1			
	right postcentral gyrus (46, −19, 39)	0.6	3.0			
				right amygdala + piriform cortex (17, −12, −19)	0.5	3.1
				anterior cingulate (1, 22, 15)	0.5	2.9
				right lingual gyrus (29, −77, −4)	0.7	2.9
EST versus AND	**right fusiform and lingual gyrus (46, −68, −6)**	**1.0**	**3.6**	no significant cluster		
AND versus EST	no significant cluster			**right fusiform gyrus (31, −46, −16)**	**0.9**	**3.2**
				right lingual gyrus (27, −78, 4)	**1.3**	**3.3**
				hypothalamus (−5, 2, −7)	0.5	2.9

Boldface type shows clusters calculated at $p < 0.05$ ($z > 3.1$ cluster size > 0.8 cm^3), whereas the nonboldface type shows clusters calculated at $p < 0.1$ ($z > 2.8$ cluster size > 0.4 cm^3). No other activations were observed. Thus, there was no hypothalamic activation in men with AND.

Comparisons between the two sexes were finally conducted with the random effect analysis (SPM 99, Wellcome Foundation, London), using a rectangular mask delineated on the standard brain MR image, covering the entire hypothalamus. Also, this calculation showed that the hypothalamic activation with AND was significantly higher in females (corrected $p = 0.001$; peak coordinate: 6, −6, 2), whereas the activation with EST was significantly more pronounced in males (corrected $p = 0.002$; peak coordinate: −8, 6, −12).

To evaluate whether AND and EST differently activated other regions than the hypothalamus, AND and EST were compared to each other with explorative statistics (see Experimental Procedures). In females, AND activated right fusiform and lingual gyrus compared to EST, whereas, in the males, EST activated these regions in relation to AND (Table 1). These areas have been attributed to visual imagery of faces (Ishai et al., 1999), and, although the activation in the sex opposite to the given compound is of note, the functional significance of this finding is presently unclear. No other significant activations were observed, but, in females, a cluster emerged in the anterior hypothalamus during the AND versus EST contrast when lowering the threshold ($p < 0.1$).

Women and men rated AND and EST similarly with respect to odor pleasantness, familiarity, irritability, and intensity (Figure 3). There were no differences in the adaptation rate to the odor of the respective compound, i.e., the time from the presentation to subjective loss of perception of odor (in females, 104 ± 24 s and 104 ± 30 s for AND and EST, respectively, versus 96 ± 36 s and 108 ± 36 s in males). No significant sex- or compound-related difference was found in breathing frequency or breathing amplitude (Figure 4). The olfactory thresholds for AND and EST were within the normal range (10^{-5} M to 3×10^{-4} M). Thus, all the measured psychophysical parameters were similar in males and females.

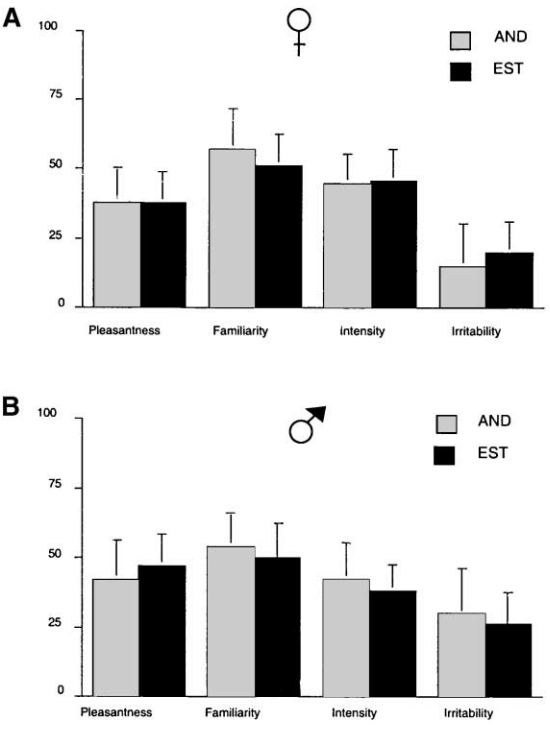

Figure 3. Odor Ratings

The vertical axis indicates a visual analog scale in millimeters. The bars show mean and SEM. (A) Females. (B) Males.

Discussion

The purpose of the present study was to investigate whether there are chemical compounds which when smelled produce sex-differentiated activation in the human brain similar to the brain activation associated with pheromones in other species. Two substances related to male and female sex hormones were used: AND, a compound suggested to have some pheromone-like properties, repeatedly found in human axillary sweat and in urine, with concentrations that are up to twenty times higher in men than women (Gower et al., 1988); and EST, which resembles naturally occurring estrogens (Monti-Bloch and Grosser, 1991). EST is less documented than AND. Nevertheless, it was found to best fulfill the inclusion criteria, as its odor characteristics have in our previous studies as in the present (Figure 3) been rated similarly by males and females, which was the prerequisite to allow any conclusions about possible sex differences not related to the mere odor stimulus. Also, like AND, EST is reported to cause change in mood and in skin conductance and heart and respiratory frequency in a sexually differentiated manner (Monti-Bloch and Grosser, 1991; Jacob and McClintock, 2000). Furthermore, Sobel et al. (1999) reported in a functional MR study that oestra-1,3,5(10),16-tetraen-3yl acetate, a compound very similar to EST, activates the brain in nonodorous concentrations, suggesting a stimulus other than the odor. Despite the fact that functional MR in limbic regions due to susceptibility artifacts may give slightly different results than PET, activation was found in anterior mesial thalamus-fornix-hypothalamus, thus, an area covering portions of our EST versus AIR cluster in males. At variance from our results, they also found a cluster in the right inferior frontal and cingulate cortex. This difference may, however, be attributed to different experimental procedures. The subjects in Sobel's study were instructed to judge whether an odor or nonodor was presented, whereas our subjects had to avoid all judgements of the presented items. The conscious judgement of odors is known to engage frontal lobes, including the anterior cingulate and inferior frontal gyrus (Royet et al., 1999).

Using a validated and established method (Savic et al., 2000a, 2000b; Savic and Gulyás, 2000), we found that AND in females activated the hypothalamus, whereas, in males, a closely situated hypothalamic region was activated with EST (Figures 1 and 2). This double-dissociated hypothalamic activation with respect to sex and compound is completely different from what is reported from activation studies with common odors (Zatorre et al., 1992; Zald and Pardo, 1997; Levy et al., 1997; Yousem et al., 1997; Royet et al., 1999; Sobel et al., 1998, 2000; Savic and Gulyás, 2000; Savic et al., 2000a, 2000b; Bengtsson et al., 2001; Savic, 2001). First of all, hypothalamic activation has to the best of our knowledge not been shown in response to passive perception of odors, despite the high number of odors tested (about 60); second, no sex difference in the cerebral pattern of activation has been found by others (Levy et al., 1997; Yousem et al., 1997) nor by us when applying five other odorants to the same subjects as those participating in the present study (Bengtsson et al., 2001). This implies that the presently observed hypothalamic involvement

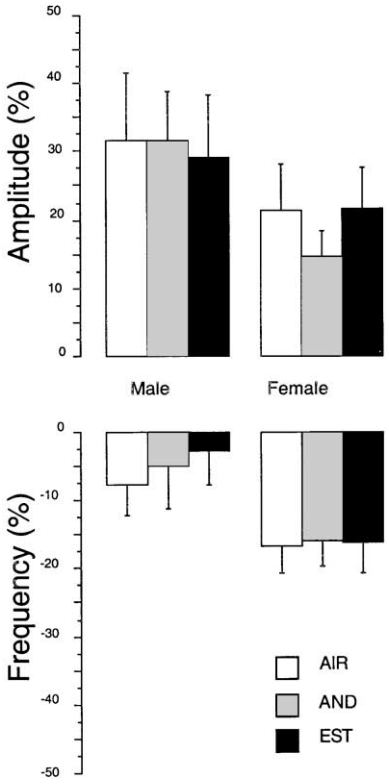

Figure 4. Respiratory Movements
The vertical axis shows percent change from baseline. The baseline values were measured during the 120 s immediately preceding the respective scan. Data are expressed as mean and SEM.

cannot be primarily attributed to the odor component of AND and EST (see Experimental Procedures). Neither can it be attributed to a trigeminal stimulation, which engages large portions of the insular, cingular, and somatosensory cortex (Cain and Murphy, 1980; Yousem et al., 1997; Savic et al., 2000a).

The hypothalamic activation seems, however, congruent with pheromone effects as they are described in animals. Pheromone stimuli from male ferrets augment Fos immunoreactivity (IR) in the main olfactory bulb, preoptic, and ventromedial nuclei of female ferrets. Soiled bedding from female mouse induces IR in the ventral premamillary nucleus in their male conspecifics (Wersinger and Baum, 1997; Yokosuka et al., 1999). That these findings are relevant for the changes in sexual behavior is suggested by electrophysiological experiments: the electric stimulation of the ventromedial nucleus in female monkey is shown to elicit the copulatory act, whereas the stimulation of the dorsomedial hypothalamic nucleus in male monkey elicits penile erection and yawning (Oomura et al., 1988). Notwithstanding that spatial restrictions by the resolution of PET scanner and the 10 mm filtering hamper a separation between our two hypothalamic activations, it is of note that the respective center of gravity in our male and female subjects was remarkably congruent with the cited animal data.

The routes by which AND and EST reached hypothalamus are not apparent from the present experiments, nor has the study been designed to address this issue. Theoretically, the signals could have been transduced

via the nasal mucosa or via absorption of the steroids into the nasal vasculature to have direct effects upon the central nervous system. Humoral transport of the bore pheromone androstenol has been shown in an in vivo study in gilts (Stefanczyk-Krzymowska et al., 2000) and of 3H-oestradiol in monkeys (Anand Kumar et al., 1970). Androstenol was measured in the carotid rete after 2 min, with a maximum between 40 and 50 min after application, and 3H-oestradiol was measured in cerebral spinal fluid 1 min after application, with a maximum after 3 min. However, because the blood flow information is obtained during the first 30 s of the PET scan, it is unlikely that the steroid distribution to the vasculature, CSF and brain, is rapid enough to allow recording of its possible accumulation in the hypothalamus and the presumptive subsequent effect on rCBF (Hurtig et al., 1994). Second, both steroids were detected in the hypothalamus after 60 min, which, if the same route of distribution was operating in our study, implies that the steroid effect on rCBF could persist throughout several scans (10 min elapsed between the different scans) and thus include the baseline condition. Third, the measured concentration of oestradiol in hypothalamus was in the cited studies not higher than in the cortex, suggesting a regional distribution incompatible with the present observations. Such activation would be expected if the observed effects were due to a humoral transport to the respective hypothalamic nuclei. However, in the absence of specific binding data from humans, the possibility of such a pathway cannot be definitively excluded. It is important to emphasize that even if the rCBF effects of AND and EST were mediated humorally, the observations still provide support for the principal hypothesis, namely, that these compounds would activate the sexually dirmorphic hypothalamic areas in a sex-related way.

As for the alternative explanation of a neuronal response, one must assume that AND and EST activate different peripheral chemosensory sites within the nasal mucosa and that, in females, the hypothalamus primarily receives signals from the AND-responding chemosensors and, in males, those from EST-responding chemosensors. Simply that the chemosensory sites and therefore the routes to the hypothalamus are different is, however, not sufficient to explain the observed sex-different activations. Neither can the different targets in the two sexes be explained from differences in odor perception, adaptation, respiratory pattern, or behavior (Figures 3 and 4). Thus, there must be a sex factor/s that influences either the neuronal network transferring the signals to the appropriate targets in the hypothalamus or sex factors which are expressed in the anterior hypothalamus or both. One factor could be the male and female sex hormones, which can influence the possible peripheral chemosensors, the pathways to hypothalamus, as well as the hypothalamic target nuclei in a sex-differentiated manner. Another is the inherent sex difference in morphology and/or neurochemistry of the hypothalamic nuclei. For example, the interstitial nucleus 3 of the anterior hypothalamus (INAH3) is larger in men (Swaab and Fliers, 1985; Byne et al., 2000). The preoptic and ventromedial nuclei contain higher concentrations of estrogen receptors in females (Scott et al., 2000), whereas, in the dorsomedial nucleus, the androgen receptors are more abundant in males (Fernandez-Gausti et al., 2000). Notably, both types of sex hormones and receptors are found in males as well as in females, and the sex differences regard the respective concentrations. This may explain why the sex difference in hypothalamic rCBF during AND and EST was significant only when the more sensitive VOI analysis was applied but subsignificant with the GLM analysis (Experimental Procedures).

Somewhat unexpectedly, during the conditions activating the hypothalamus, i.e., during smelling of AND in females and EST in males, clusters appeared in olfactory regions only when lowering the threshold for significance ($p < 0.1$). This was despite indifferent ratings of subjective perception of both compounds' odor component by both sexes (Figure 3). A similar phenomenon has, however, been reported with the combined trigeminal and olfactory odorants, which activate the olfactory areas to a minor extent even when the odor component is strong (Cain and Murphy, 1980; Yousem et al., 1997). We recently found, for example, that the odorant acetone strongly activates cortical regions related to the trigeminal stimulus but only to a minor extent the amygdala and no other classical olfactory regions (Savic et al., 2000a). A similar phenomenon was also detected with the combined trigeminal and olfactory odorant butanol (I.S., unpublished data). The underlying mechanism could be an inhibition of the olfactory stimulus by the trigeminal stimulus (Cain and Murphy, 1980). It is possible that such an interaction represents a more general phenomenon when a volatile odorous compound elicits two different chemosensory stimuli (pheromone and odorous, trigeminal and odorous). It is worth mentioning that a similar bimodal interaction has been reported during the perception of flavor (Small et al., 1997). We are, therefore, putting forward the hypothesis that, depending on the sex of the recipient, AND and EST are transmitted primarily as pheromone-like signals (to anterior hypothalamus) or odor signals (to the olfactory brain). When the hypothalamic pathway dominates (i.e., in females smelling AND and males smelling EST), the olfactory signals may be suppressed but still perceived and the olfactory regions engaged only to a lesser degree. The hypothalamic pathway may be direct from the olfactory bulb and separate from the bulbo-olfactory pathway, as shown in the old world monkey, which, like humans, lack the accessory olfactory nerve (Takagi, 1984).

Pheromones are (1) bodily secreted, (2) detected by another individual, (3) affect the neuroendocrine brain, and/or (4) behavior. According to previous studies, the first criterion is definitively fulfilled by AND (Gower and Ruparelia, 1993; Gower et al., 1988; Smals and Weusten, 1991). The present study shows that both AND and EST sex-specifically activate relevant hypothalamic regions. We have, however, not examined whether the short exposure to AND and EST altered the subjects reproductive/behavior functions and thus have not tested the last criterion. Also, to avoid activations due to a dissolvent or its interaction with the respective steroid (Gower et al., 1988), both AND (200 mg) and EST (200 mg) were used in pure, crystalline form. The air concentration reaching the subjects in the scanner (which had a continuos air suction device) was considerably lower but could still be higher than the physiological. However, it has recently been shown that the magnitude of neuronal response to pheromones does not change with phero-

mone concentration (Leinders-Zufall et al., 2000). These findings indicate that the present data may well be relevant also for the physiological conditions.

Thus, notwithstanding that the existence of human pheromones is still an open question, we suggest that the present observations of a sex-differentiated hypothalamic activation in humans provide a fundament for further, extensive research of chemosensory signals in humans.

Experimental Procedures

Two months prior to the PET experiments, the potential participants were tested for the olfactory thresholds to several different odorants, including AND and EST, which were solved in mineral oil (Eichenbaum et al., 1983). They were unaware of the identity of the presented items at the time of testing. Three subjects were excluded because they were anosmic to AND. The remaining 24 subjects which were finally included had normal olfactory thresholds for AND and EST. They also had normal thresholds for butanol. The subjects were healthy, nonsmoking, heterosexual, right handed, and divided into two groups: 12 women (20–28 years, investigated during second to third week of the menstrual cycle) and 12 men (23–28 years). The PET tracer was ^{15}O-H_2O (Fox et al., 1984).

AND and EST (Steraloids, Inc., Newport, RI) were used in crystalline form (200 mg) and were odorous. The purity of both compounds was confirmed by the doping laboratory, Department of Pharmacology, Huddinge University Hospital, in Stockholm. AIR, EST, and AND (Steraloids, Inc., confirmed for purity by the doping laboratory) were presented birhinally during separate scans. Both compounds were first tested with a 100 mm bipolar visual-analog scale (VAS) (Savic et al., 2000b) for the subjectively perceived odor characteristics (familiarity, pleasantness, irritability, and intensity) in 15 male and 15 female subject which did not participate in the present PET study.

During PET experiments, the items were given at 10 mm distance from the nose, in a just-opened glass bottle during separate, randomly interleaved scans. Each condition was scanned three times, according to a procedure described in detail elsewhere (Savic et al., 2000b). During each scan, the same item was given four times during 15 s, with 5 s in between. Subjects were instructed to breathe normally and that they would smell odor or odorless air, without knowing the type or order of items. Respiratory movements were recorded with a strain gauge around the lower thorax connected to a graph (Comair Ltd., Stockholm). Percent change in respiratory amplitude and frequency in relation to the respective prescan baseline (aquired during 2 min before each respective scan) was compared between males and females during smelling of AIR, AND, and EST, using a two-way ANOVA ($p < 0.05$). The parameters of variance were sex and type of stimulus. After PET scans, the participating subjects rated AND and EST for pleasantness, irritability, intensity, and familiarity, using a 100 mm bipolar visual-analog scale (Savic et al., 2000b). Ratings were compared with a two-way ANOVA, using odor (AND or EST) and sex as parameters of variance; the significance level was 0.05 before Bonferroni correction for the four comparisons (Figure 3).

Each subject was investigated with nuclear magnetic resonance (1.5 tesla GE scanner; 3D SPGR; TE = 5 ms, TR = 21 ms; Q = 50°, FOV = 256 mm), which always preceded the PET scan. The individual MR images were used for coregistration and careful stereotactic transformation of the individual PET images into a common space, by means of the Computerized Brain Atlas (Roland et al., 1994). PET images were filtered with 10 mm Gaussian filter, coregistered with the AIR program (Woods et al., 1992), and normalized to a global CBF of 50 ml/100 g/min (Fox et al., 1984; Savic et al., 2000a, 2000b). The contrasts were tested with the general linear model (Siearle, 1971) and the differences calculated pixel-by-pixel, generating t maps, later transformed into units of normal distribution (z maps). The number of df (calculated from the general linear model) was 90 for females and 88 for males. Significant activations ($p < 0.05$) were determined with the cluster statistics of Ledberg et al. (1998), using z threshold of 3.1 and the minimum cluster size 0.8 cm^3. Talairach coordinates were used for localization in relation to the computerized brain atlas and the reformatted mean MRI of participating subjects. For a precise location of hypothalamic activations, we also used the Schaltenbrand atlas (Schaltenbrand, 1959) after having translated the Talairach coordinates.

The VOI analysis was conducted to compare the hypothalamic rCBF during EST and AND in males and females. The respective hypothalamic VOI was determined by the AND versus AIR and EST versus AIR clusters. The mean normalized hypothalamic rCBF was then calculated from each scan with EST and AND and used for within group comparisons between AND and EST (one-way repeated measure ANOVA, $p < 0.05$) and between group comparisons (two-way repeated measure ANOVA, factoring for sex group and type of steroid, $p < 0.05$) (Figure 2A).

In the comparisons between sexes, the calculated differences between the respective compound and baseline (AND versus AIR and EST versus AIR) were entered in the two-way repeated measure ANOVA model (Figure 2B). Since the order of the scans was always randomized and balanced between the subjects, the differences were calculated as follows: the first AND scan with the first AIR scan, the second AND scan with the second AIR scan, and the third AND scan with the third AIR scan. The same order was used for EST. The sex × VOI interaction was also specifically tested in the two-way repeated measure ANOVA model including the AND and EST activation data from both the AND and the EST defined VOIs.

The comparisons between males and females were finally calculated with random effect analysis (SPM 99, Wellcome Foundation, London), using a significance level of $p < 0.01$ and a separate rectangular volume of interest, which covered the entire hypothalamus, and included both hypothalamic clusters. The outer borders of this mask had Talairach coordinates −8 and 8 for *x*; 8 and −18 for *y*; and −10 and 7 for *z*.

Acknowledgments

The Swedish Medical Research Council, Karolinska Institute, the Swedish Royal Academy of Sciences, the Captain Ericsson's, and the Åke Wiberg Foundation for financial support; and Hanna Lund, Eleonor Cohen, and Julio Gabriel for technical assistance.

Received May 22, 2001; revised July 31, 2001.

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