5

HYPOTHALAMIC RESPONSE TO THE CHEMO-SIGNAL ANDRO-STADIENONE IN GENDER DYSPHORIC CHILDREN AND ADOLESCENTS

Sarah Burke, Peggy Cohen-Kettenis, Dick Veltman, Daniel Klink, Julie Bakker

Published in Frontiers in Endocrinology, section Neuroendocrine Science, May 2014

Abstract

The odorous steroid androstadienone, a putative male chemo-signal, was previously reported to evoke sex differences in hypothalamic activation in adult heterosexual men and women. In order to investigate whether puberty modulated this sex difference in response to androstadienone we measured the hypothalamic responsiveness to this chemo-signal in 39 prepubertal and 41 adolescent boys and girls by means of functional magnetic resonance imaging. We then investigated whether 36 prepubertal children and 38 adolescents diagnosed with Gender Dysphoria (GD; DSM-5) exhibited sex-atypical (in accordance with their experienced gender), rather than sex-typical (in accordance with their natal sex) hypothalamic activations during olfactory stimulation with androstadienone. We found that the sex difference in responsiveness to androstadienone was already present in prepubertal control children and thus likely developed during early perinatal development instead of during sexual maturation. Adolescent girls and boys with GD both responded remarkably like their experienced gender, thus sex-atypical. In contrast, prepubertal girls with GD showed neither a typically male nor female hypothalamic activation pattern and prepubertal boys with GD had hypothalamic activations in response to androstadienone that were similar to control boys, thus sex-typical. We present here a unique data set of boys and girls diagnosed with GD at two different developmental stages, showing that these children possess certain sex-atypical functional brain characteristics and may have undergone atypical sexual differentiation of the brain.

In humans, the odorous steroid 4,16–androstadien-3–one (androstadienone) has been studied intensively as a putative male modulator chemo-signal. Androstadienone, probably synthesized in the gonads (Kwan et al. 1997), is secreted by the apocrine glands and can be found on the skin surface and axillary hairs (Nixon et al. 1988), as well as in several body fluids including sweat and semen (Brooksbank et al. 1969; Kwan et al. 1992). Higher concentrations of androstadienone in sweat have been found in men compared to women (Brooksbank et al., 1972; Gower and Ruparelia, 1993). Exposure to androstadienone has been shown to affect wom-

en's mental state (Jacob and McClintock 2000; Jacob, Hayreh, et al. 2001; Bensafi, Brown, et al. 2004; Villemure and Bushnell 2007), and to elicit physiological responses in a sex-dependent manner. (Grosser et al. 2000; Jacob, Hayreh, et al. 2001; Bensafi et al. 2003; Lundstöm et al. 2003; Bensafi, Brown, et al. 2004; Bensafi, Tsutsui, et al. 2004; Lundström and Olsson 2005). Moreover, smelling androstadienone had an impact on women's hormone levels (Wyart et al. 2007) and affected their behavioral responses dependent on the phase of the menstrual cycle (Parma et al. 2012).

In line with the effects androstadienone had on the autonomic nervous system and on behavior, studies conducted by Savic et al. (2001), using *positron emission tomography* (PET), showed that smelling androstadienone induced a response in the hypothalamus of heterosexual women but not in heterosexual men. The latter only showed an activation in brain areas belonging to the main olfactory system, such as the piriform cortex and amygdala (Savic et al. 2001). Thus, olfactory stimulation with androstadienone offers a relatively simple and objective experimental procedure for investigating functional sex differences in the human brain. Using *functional magnetic resonance imaging* (fMRI), we recently replicated the finding of women showing a stronger hypothalamic activation than men, after smelling the highest (10mM) out of three concentrations tested (Burke et al. 2012).

Secretion of androstadienone increases substantially during puberty. As a result, sex-related changes in olfactory sensitivity to androstenes have been reported during adolescence (Koelega and Köster 1974; Dorries et al. 1989; Hummel et al. 2005; Chopra et al. 2008) with male adolescents exhibiting more anosmia to androstadienone, and an increase of odor threshold with age, compared to female adolescents. It may be inferred that the decrease in sensitivity to odorous steroids in pubescent boys is related to their increased production of endogenous androgens during puberty, probably reflecting adaptation towards (male) body odors.

Sisk and colleagues (Sisk and Zehr 2005; Schulz, Molenda-Figueira, et al. 2009) proposed a two-stage model of sexual differentiation of brain and behavior, in which early perinatal organizational effects of steroid hormones are followed by a second steroid-dependent sensitive phase of neurodevelopment during adolescence. Thus, puberty may be considered an organizational period in itself, in which sex differences in brain morphology and function are established or consolidated. Therefore, our first aim was to investigate whether the

hypothalamic response to androstadienone develops during puberty, as part of sexual maturation, or whether it would already be present in prepubertal children and thus established during early brain development. Thus, we tested whether the response to androstadienone varied as a function of sex and pubertal status in four groups of prepubertal and adolescent boys and girls.

Of note, further studies by Savic et al. (Savic et al. 2005; Berglund et al. 2006, 2008) showed that hypothalamic activation upon exposure to androstadienone also depended on sexual orientation (Savic et al. 2005; Berglund et al. 2006) and gender identity. Adult men diagnosed with *Gender Dysphoria* (GD) (DSM-5, American Psychiatric Association, 2013), and thus having a female gender identity showed a sex-atypical hypothalamic response, which reflected more their experienced gender than their natal sex (Berglund et al. 2008). This is in line with the hypothesis that individuals, diagnosed with GD might have undergone a sex-atypical programming of the nervous system (van Goozen et al. 2002; Swaab and Garcia-Falgueras 2009). The etiology of GD is currently unknown, however, a multitude of factors, ranging from adverse psycho-dynamic parent-child interactions (Marantz and Coates 1991), an anxious personality predisposition (Zucker and Bradley 1995), genetic risk factors (Coolidge et al. 2002), and exposure to atypical levels of perinatal sex steroids during a critical period of sexual differentiation of the brain (Gooren 2006) have all been proposed to facilitate the development of GD.

At the *Center of Expertise on Gender Dysphoria* in Amsterdam, the current treatment protocol allows adolescents diagnosed with GD that persisted from childhood into adolescence to start treatment with *gonadotropin-releasing hormone analogues* (GnRHa) from the age of 12 years, to suppress endogenous gonadal stimulation and thus the development of irreversible sex-characteristics of the natal sex (Kreukels and Cohen-Kettenis 2011). From the age of 16 years on, as a first step in sex reassignment they receive cross-sex hormone treatment, i.e. biological boys receive estrogens and biological girls receive androgens (Delemarre-van de Waal and Cohen-Kettenis 2006; Hembree et al. 2009).

A second aim of the current study was therefore to explore whether children and adolescents, diagnosed with GD, would show brain responses that reflect their expressed/experienced gender rather than their natal sex, and whether these would vary as a function of their developmental phase. Four groups of subjects, all diagnosed with GD, participated in the current study: girls and boys that were prepubertal and treatment-naïve, and girls and boys

that were adolescent in age (though in hypogonadal state due to GnRHa treatment). None of our participants received cross-sex hormones at the time of data acquisition.

Material and Methods

SUBJECTS

THE INITIAL STUDY SAMPLE consisted of a total of 158 participants. Four subjects were excluded from further analysis, because of anatomical anomalies (1 adolescent), technical errors during data collection (1 child), or because the diagnosis GD had been revised since their participation in the study (2 boys with GD in remission). All participants diagnosed with GD, were recruited via the *Center of Expertise on Gender Dysphoria* at the VU University Medical Center in Amsterdam. The control participants were recruited via several primary and secondary schools in the Netherlands and by inviting friends and relatives of the participants with GD.

The children sample consisted of 19 control girls (mean years of age M = 9.7, standard deviation SD = 0.9), 20 control boys (M = 9.5, SD = 1.1), 17 girls with GD (M = 9.6, SD = 1.1), and 19 boys with GD (M = 10.4, SD = 0.9). All children underwent a short physical examination by a pediatric endocrinologist (DTK) in order to ascertain their prepubertal status (Tanner stage 1) (Marshall and Tanner 1969, 1970).

The adolescent groups consisted of 21 control girls (M = 16.3, SD = 0.9), 20 control boys (M = 15.0, SD = 0.6), 21 girls with GD (M = 16.1, SD = 0.8), and 17 boys with GD (M = 15.3, SD = 1.2). The adolescent participants, diagnosed with GD, had been treated monthly with 3.75 mg of Triptorelin (Decapeptyl-CR®, Ferring, Hoofddorp, the Netherlands) by injection for on average 24 months (range 2 – 48 months), resulting in complete suppression of gonadal hormone production. Female adolescent controls were tested randomly according to their menstrual cycle and 11 out of 21 control girls reported using hormonal contraception.

ASSESSMENTS AND SUBJECT CHARACTERISTIC

SEXUAL ORIENTATION WAS difficult to assess, especially in the prepubertal sample, because most children were simply too young to be able to report their sexual orientation. Therefore, current or presumed future sexual attraction was assessed by asking whether the participant had ever been in love with somebody, and if yes, whether that person was a boy or a girl. Normal olfactory function was ascertained by means of an extended version of the *Sniffin' Sticks* test battery (32 – item odor identification test and olfactory threshold measurement) (Kobal et al. 1996, 2000; Hummel et al. 2007). Furthermore, participants were asked to report the perceived intensity of a 10 mM androstadienone solution (on a scale from 0 to 10). Separately, for the adolescent and the children samples, one-way analyses of variance (ANOVA) were conducted, using the Statistical Package for the Social Sciences, version 20 (SPSS Inc., Chicago, IL, USA), testing whether the four gender groups differed on any of these measures. Bonferroni correction for multiple comparisons was applied for post-hoc tests, considering a threshold of $p < 0.05$ as statistically significant. All subjects and their legal guardians gave their informed consent according to the Declaration of Helsinki, and the study was approved by the Ethics Committee of the VU University Medical Center Amsterdam (application number NL31283.029.10).

OLFACTORY STIMULATION

ANDROSTADIENONE (Steraloids Inc., Newport, RI 02840, US) was diluted in propylene glycol (Sigma) to a concentration of 10 mM, according to the *high* concentration used in our previous study (Burke et al. 2012). The volume of the solution used during the fMRI experiments was 20 ml. Olfactory stimuli were delivered through a tubing system to the subjects' nostrils by means of a custom-built air-dilution olfactometer (for details of the olfactometer set-up and procedure see (Burke et al. 2012). With a total air flow of about 1 L per minute, during ON periods every 2 seconds the odor was delivered during 1 second, while during OFF periods subjects received odorless air.

IMAGE ACQUISITION

SCANS WERE PERFORMED ON a 3.0 Tesla GE Signa HDxt scanner (General Electric, Milwaukee, WI, USA). A gradient echo echoplanar imaging sequence was used

for functional imaging (19.2 cm² field of view, TR of 1950 ms, TE of 25 ms, an 80° flip angle, isotropic voxels of 3 mm, and 36 slices). Before each imaging session a local high-order shimming technique was used to reduce susceptibility artifacts. A scanning session consisted of 6 alternating ON-OFF cycles over 108 volumes in a classical block design (one block consisted of 9 volumes), lasting 3.6 min. For co-registration with the functional images a T1 – weighted scan was obtained (3D FSPGR sequence, 25cm² field of view, TR of 7.8 ms, TE of 3.0 ms; slice thickness of 1 mm, and 176 slices).

IMAGE PROCESSING

DATA ANALYSIS WAS PERFORMED with SPM8 software (*Statistical Parametric Mapping*; Wellcome Department of Imaging Neuroscience, Institute of Neurology at the University College London, UK) implemented in Matlab R2009b (Math Works Inc., Natick, MA, USA). Functional images were slice-timed and realigned to the mean image, followed by unwarp. Applying the *New Segment* and *Create Template* options of the DARTEL (*Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra*) toolbox, structural images were segmented. Then, gray matter and white matter images were used for creating age-group specific templates (one for the children and the adolescents sample each), registered in Montreal Neurological Institute (MNI) space. Functional images were spatially normalized to their respective group-template, applying each individual's DARTEL flow field, and finally, images were smoothed by means of a 5 mm *full width half maximum* (FWHM) isotropic Gaussian kernel.

Individual image data were analyzed using boxcar regressors convolved with a synthetic haemodynamic response function and a *first-order time modulation* (TM) regressor to test for possible effects of adaptation/sensitization to androstadienone. In order to account for assumed late *wash-out* effects during OFF blocks and an early peak response to the odor stimulation during ON blocks, first-level contrast images were built by subtracting the second half b) of the OFF blocks (4 volumes) from the first part a) of the ON blocks (4 volumes). Accordingly, this was done with the associated TM regressor blocks. Further, based on the image realignment process, individual head jerks were identified (› 1mm displacement) (Lemieux et al. 2007). Together with the six motion parameters, these so-called scan nulling regressors were included in every first-level design matrix to account for the effects of excessive head motion.

FIRST, IN ORDER TO TEST whether the sex difference in response to androstadienone was present in both developmental control groups, and whether that sex difference in responsiveness varied as a function of adaptation/sensitization to the odor, we conducted a sex (control boys, control girls) by odor stimulation ANOVA, for both the prepubertal and the adolescent control groups. The factor odor stimulation consisted of two levels, a regressor modeling the condition ONa - OFFb effect, thus the hypothesized hypothalamic response to the odor, and a first order parametric modulation TM ONa – TM OFFb regressor, which signifies how well the hypothalamic response correlates with changes over time, thus modeling possible effects of adaptation or sensitization.

Second, by means of four gender by odor stimulation ANOVAs separately for the prepubertal and the adolescent groups, we tested whether boys and girls diagnosed with GD differed significantly in response to the steroid odor in comparison to their respective natal sex or their experienced gender control group. Analyses were restricted to the hypothalamus area as *region of interest* (ROI), defined (with *Marsbar* (Brett et al. 2002)) as a sphere (centered at MNI coordinates x = 0, y = -10, z = -7; with a 7mm radius), and based on anatomical demarcations following (Makris et al. 2013) and (Baroncini et al. 2012). The threshold for statistical significance was set at $p < 0.05$ family-wise error (FWE)-corrected for the extent of the hypothalamus ROI.

Results

PSYCHOPHYSICS & SUBJECT CHARACTERISTICS

DEMOGRAPHIC, SELF-REPORT and subject characteristics are presented in Table 1. The adolescent boys with GD were significantly younger and less physically mature than the three other adolescent groups. The prepubertal boys with GD were significantly older than the prepubertal control boys. Therefore, in all group comparisons involving boys with GD, age was included as covariate. As expected, both adolescent control groups differed significantly from the boys and girls diagnosed with GD with respect to reported sexual orientation. There were no group differences in olfactory performance and on ratings of the perceived intensity of androstadienone.

Table 1 Subject characteristics & psychophysiological data

			Prepubertal children					
			CTRL GIRLS	CTRL BOYS	GIRLS WITH GD	BOYS WITH GD	*F*(DF)	*P* VALUE
Group size	N		19	20	17	19		
Age in years	(mean (SD))		9.7 (0.9)	9.5 (1.1)	9.6 (1.1)	10.4 (0.9)	3.1 (3, 71)	**.033**
Pubertal stage	(mean (SD))	P	1	1	1	1	-	-
		G/ M*	1	1	1	1	-	-
Sexual orientation	% (N)	gynephilic	-	66.0 (13)	17.6 (5)	21.1 (4)		
		androphilic	89.5 (17)	-	29.4 (3)	42.1 (8)		
		ambiphilic	-	-	5.9 (1)	-	1.1 (3, 71)	.364
		don't know	10.5 (2)	35.0 (7)	47.1 (8)	36.8 (7)		
		missing	-	-	-	-		
Androstadienone**	(mean (SD))		6.1 (2.4)	6.9 (1.5)	6.2 (2.7)	5.7 (2.6)	0.7 (3, 55)	.548
Sniffin' Sticks	(mean (SD))	threshold	7.3 (3.9)	6.2 (4.1)	8.5 (3.4)	8.6 (3.4)	1.6 (3, 58)	.212
		identification	21.5 (3.6)	18.5 (3.8)	19.1 (4.0)	19.9 (5.3)	1.8 (3, 66)	.162

Ctrl = control; GD = Gender Dysphoria; N = group size; SD = standard deviation; P = pubic hair growth; G = genital development; M = breast development; *G applies for natal boys and M for natal girls; pubertal stages were assessed by means of the five-point (1 = prepubertal, 5 = postpubertal) Tanner Maturation Scale; **androstadienone was rated for perceived intensity on a scale from 0 to 10; scores on the Sniffin' Sticks threshold test range from 1 (low sensitivity) to 16 (high sensitivity); scores on the odor identification test range from 1 to 32.

SEX DIFFERENCES IN HYPOTHALAMIC ACTIVATION – EFFECTS OF PUBERTY

IN ORDER TO DETERMINE whether hypothalamic activation upon smelling androstadienone is dependent on puberty, we conducted separate ANOVAs in the prepubertal and the adolescent groups, and compared hypothalamic activation in control girls to that of control boys during exposure to androstadienone. Results are displayed in Table 2.

Prepubertal girls and boys did not differ in terms of general hypothalamic responsiveness (condition effect ON > OFF) to androstadienone, but directional t-tests showed that the sex difference (girls > boys) was significantly modulated by the effect of the TM regressor ($t = 3.3$; $p = 0.033$), indicating that hypothalamic

Table 1 (continued)

Adolescents							
CTRL GIRLS	CTRL BOYS	GIRLS WITH GD	BOYS WITH GD	*F*(DF)	*P* VALUE		
21	20	21	17			N	**Group size**
16.3 (0.9)	15.9 (0.6)	16.1 (0.8)	15.3 (1.2)	4.6 (3,75)	**.005**	(mean (SD))	**Age in years**
4.2 (0.7)	4.7 (0.7)	4.7 (0.6)	3.1 (1.1)	17.4 (3,74)	**< .001**	(mean (SD))	**Pubertal stage**
4.1 (0.8)	4.1 (0.8)	4.1 (1.1)	3.1 (0.8)	5.1 (3,74)	**.003**		
-	100 (20)	100 (21)	-			% (N)	**Sexual orientation**
100 (21)	-	-	70.6 (12)				
-	-	-	5.9 (1)	33.3 (3,74)	**< .001**		
-	-	-	17.6 (3)				
-	-	-	5.9 (1)				
5.7 (2.3)	4.8 (1.9)	5.5 (1.9)	4.6 (2.0)	1.1 (3,63)	.351	(mean (SD))	**Androstadienone****
9.9 (3.3)	8.5 (3.4)	8.1 (2.9)	9.1 (3.4)	1.1 (3,68)	.356	(mean (SD))	**Sniffin' Sticks**
25.2 (3.8)	23.9 (2.8)	23.5 (3.9)	23.7 (2.8)	1.0 (3,71)	.419		

activation in boys differed from that of the girls during the course of the scanning session, thus suggesting differences in adaptation and/or sensitization to the odor between groups (see Figure 5.1A and Figure 5.2A and 5.2C).

< FIGURE 5.2
page 102-103

Accordingly, similar comparisons were done in the adolescent groups. Again, the sex difference in hypothalamic activation (girls › boys) was significantly dependent on the factor time ($t = 3.5$; $p = 0.019$) (see Figure 5.1B and 5.3A and 5.3D). Visual inspection of the data (see Figure 5.1) revealed that control girls' activation increased with repeated exposure to androstadienone (sensitization), particularly towards the end of the session, whereas control boys' responsiveness to the steroid odor seemed relatively stable, showing a slight decrease in activation during the course of the stimulation session.

< FIGURE 5.3
page 102-103

GENDER IDENTITY & HYPOTHALAMIC ACTIVATION

IN ORDER TO TEST WHETHER individuals diagnosed with GD would show a hypothalamic activation that reflected their experienced gender, and whether this

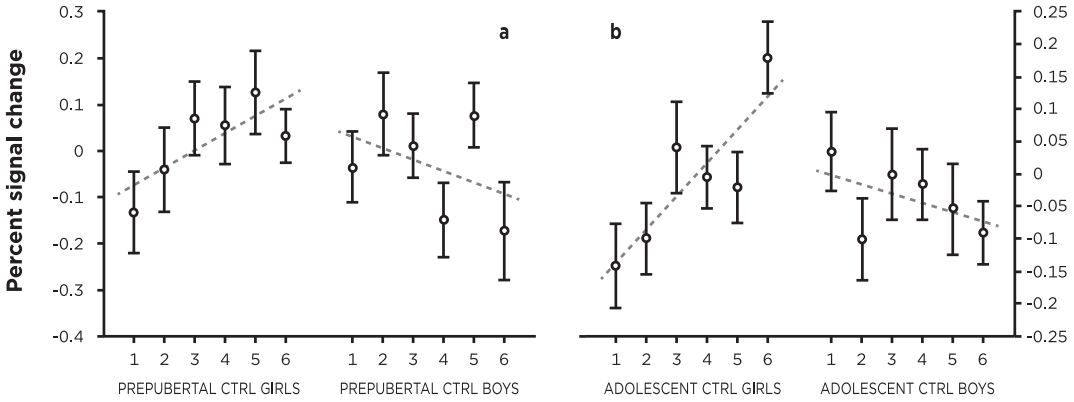

Figure 5.1 The mean percent signal change (error bars indicate SE) for the hypothalamic region of interest during the course of the scanning session in the control groups. Panel **a** shows the responses in the prepubertal boys and girls; panel **b** shows the changes in activation over time in the adolescent control groups. Numbers 1 – 6 indicate the six time bins of the odor stimulation block design. Irrespective of pubertal status, both female age groups showed a significant increase ($p = .033$ in the prepubertal girls; $p = .019$ in the adolescent girls) in activation with repeated exposure to androstadienone, whereas hypothalamic activations tended to decrease in boys towards the end of the odor stimulation. The data for this figure was visualized using the toolbox rfxplot by Gläscher, 2009.

brain response would be different for prepubertal and adolescent subjects, four ANOVAs were conducted, in which control girls were compared to girls with GD, and control boys were compared to boys with GD (see Table 2). In addition, we investigated the reverse effects, i.e. whether individuals with GD showed sex-typical activations, and thus deviated from their experienced gender by comparing girls with GD to control boys, and boys with GD to control girls (see Table 3). Comparing girls with GD to boys with GD revealed no significant differences, thus the sex difference in response to androstadienone observed in the control groups was absent in the groups diagnosed with GD.

GIRLS DIAGNOSED WITH GD

NONE OF THE COMPARISONS between prepubertal control girls and prepubertal girls with GD revealed any differences in hypothalamic activation upon smelling androstadienone. However, no sex-typical effects (i.e. a female-typical hypothalamus response), when compared to control boys, could be confirmed. Thus, prepubertal girls with GD neither differed significantly from their

Table 2 ANOVA results showing sex differences in hypothalamus activations in the control groups, and sex-atypical hypothalamic activations, reflecting their experienced gender, in individuals with GD.

	EFFECT	Z_{MAX}	X	Y	Z	N	P_{FWE}
	Ctrl Girls > Ctrl boys						
children	Gender	2,5	-6	-12	-5	30	.202
	Odor	2,4	-2	-15	-2	39	.244
	ON - OFF	1,8	0	-10	-14	1	.456
	ON_{TM} - OFF_{TM}	3,2	-6	-12	-5	92	**.033**
		2,9	6	-7	-8		
adolescents	Gender	1,7	-3	-7	-2	17	.091
	Odor	1,7	6	-7	-6	8	.655
	ON - OFF	3,4	6	-7	-8	1	.522
	ON_{TM} - OFF_{TM}	2,5	-6	-12	-5	115	**.019**
	Ctrl Girls > Girls with GD						
children	No effects						
adolescents	Gender	3,5	6	-7	-9	76	**.016**
	Odor	1,7	-6	7	-6	1	.627
	ON - OFF	--	-	-	-	-	
	ON_{TM} -OFF_{TM}	4,1	6	-7	-9	214	**.002**
	Boys with GD > Ctrl Boys						
children	Gender	1,8	2	-14	-2	4	.447
	Odor	1,9	0	-10	0	3	.436
	ON - OFF	1,7	-4	-6	-4	12	.437
	ON_{TM} - OFF_{TM}	1,9	0	-10	-12	31	.329
		2,4	0	-14	-12		
adolescents	Gender	2,2	6	-8	-6	11	.237
	Odor	3	0	-14	-12	1	.341
	ON - OFF	1,8	2	-14	-2	36	**.046**
	ON_{TM} - OFF_{TM}	--	-	-	-	-	
	Boys with GD > Girls with GD						
children	No effects						
adolescents	Gender	--	-	-	-	-	
	Odor	1,9	3	-12	-9	12	.535
	ON - OFF	--	-	-	-	-	
	ON_{TM} - OFF_{TM}	2,1	3	-10	-2	26	.323

Ctrl = control; GD = Gender Disphoria; Zmax = z-value of peak activation voxel; x y z = coordinates in Montreal Neurological Institute (MNI) space; N = number of clustering voxels (cluster size); FWE = family-wise error; the effects "Gender" and "Odor" indicate omnibus F-contrasts for the factors gender and odor stimulation; "ON - OFF" and "ON_{TM} - OFF_{TM}" indicate directional t-contrasts for the condition effect and the first order time-modulation regressor, respectively.

TABLE 3 ANOVA results showing sex-typical hypothalamic activations, reflecting their natal sex, in individuals with GD.

	EFFECT	Z_{MAX}	X	Y	Z	N	P_{FWE}
Girls with GD > Ctrl boys							
children	No effects						
adolescents	Gender	-	-	-	-	-	-
	Odor	2.5	6	-9	-5	25	.203
	ON - OFF	1.7	-6	-7	-5	2	.512
	ON_{TM} - OFF_{TM}	2.3	-6	-9	-8	25	.258
Ctrl Girls > Boys with GD							
children	Gender	3.0	-6	-10	-6	35	.065
	Odor	2.6	3	-16	-9	27	.168
	ON - OFF	1.6	-2	-16	-6	4	.550
	ON_{TM} - OFF_{TM}	3.5	-6	-10	-6	126	**.013**
adolescents	Gender	2.2	-2	-4	-10	5	.336
	Odor	1.5	2	-14	-12	4	.699
	ON - OFF	-	-	-	-	-	-
	ON_{TM} - OFF_{TM}	1.7	6	-8	-4	7	.504
		1.6	-6	-12	-8	16	.553
Girls with GD > Boys with GD							
children	No effects						
adolescents	Gender	-	-	-	-	-	-
	Odor	1.9	3	-12	-9	12	.535
	ON - OFF	-	-	-	-	-	-
	ON_{TM} - OFF_{TM}	1.9	-6	-7	-11	1	.459

Ctrl = control; GD = Gender Disphoria; Zmax = z-value of peak activation voxel; x y z = coordinates in Montreal Neurological Institute (MNI) space; N = number of clustering voxels (cluster size); FWE = family-wise error; the effects "Gender" and "Odor" indicate omnibus F-contrasts for the factors gender and odor stimulation; "ON - OFF" and "ON_{TM} - OFF_{TM}" indicate directional t-contrasts for the condition effect and the first order time-modulation regressor, respectively.

experienced (control boys) nor from their natal sex (control girls) in terms of hypothalamic activation when smelling androstadienone.

In contrast, the comparison of adolescent control girls to girls diagnosed with GD revealed a significant effect of gender (control girls › girls with GD), which was mainly explained by the effect of the TM regressor ($t = 4.3$; $p = 0.002$) (see Table 2 and Figure 5.3B and 5.3E). Thus, control girls showed a significantly stronger hypothalamic response to androstadienone over time as described

FIGURE 5.3 >
page 102-103

earlier, whereas the activation in girls with GD, similar to adolescent control boys, remained stable throughout the scanning session. The reverse group comparisons (girls with GD > control boys) revealed no significant effects (see Figure 5.3F), indicating that adolescent girls with GD showed no hypothalamic activation upon smelling androstadienone as was observed in adolescent control boys.

< FIGURE 5.3
page 102-103

BOYS DIAGNOSED WITH GD

NO SIGNIFICANT EFFECTS WERE revealed when comparing prepubertal boys with GD with control boys. In contrast, a significant effect of gender, driven by the TM regressor ($t = 3.5$; $p = 0.013$ FWE-corrected) was revealed when comparing prepubertal boys with GD to control girls, indicating that prepubertal boys with GD showed a pattern of hypothalamic activation that was similar to that of the prepubertal control boys (see Table 3 and Figure 5.2B, 5.2D, 5.2E).

< FIGURE 5.2
page 102-103

When adolescent boys with GD were compared to adolescent control boys we observed a significant effect of condition (ON > OFF) ($t = 1.8$; $p = 0.046$) (see Table 2 and Figure 5.3C, 5.3G, 5.3H). Thus, adolescent boys with GD showed a significantly stronger, thus sex-atypical response to androstadienone compared to control boys, irrespective of the factor time. The reverse contrast (control girls > adolescent boys with GD), i.e. testing whether adolescent boys with GD would show an activation according to their natal sex, revealed no significant activations. Thus, adolescent boys with GD showed female-typical hypothalamic responses upon smelling androstadienone without, however, any effects due to sensitization.

< FIGURE 5.3
page 102-103

Discussion

SEX DIFFERENCES IN HYPOTHALAMIC ACTIVATION – EFFECTS OF PUBERTY

THE PRESENT STUDY IS, to our knowledge, the first to demonstrate that sex differences in hypothalamic activation upon smelling androstadienone are already present before puberty, and thus may be considered as a sex difference established during early brain development. We found that prepubertal as well as

adolescent control girls showed a stronger hypothalamic activation compared to boys (at both developmental stages), and that this sex difference was crucially modulated by effects of sensitization to androstadienone.

Previous psychophysiological studies in children and adolescents have shown that olfactory sensitivity to androgenic odors differed between boys and girls during development, due to the increased production of endogenous androgens by boys during puberty and the assumed resulting adaptation to their own body odors (Koelega and Köster 1974; Dorries et al. 1989; Hummel et al. 2005; Chopra et al. 2008). These findings suggested that puberty plays an important role in the development of sex differences in the olfactory sensitivity to androstadienone (Lundström et al. 2003). Therefore, we hypothesized that the hypothalamic response in males, in contrast to that in females, would similarly be subject to neuronal adaptation after repeated exposure to the steroid odor.

However, our results suggested only slight neuronal adaptation effects in boys, their activation remained relatively stable throughout the scanning session; instead, we found that girls showed an increase in hypothalamic responsiveness during the course of the odor stimulation. In line with the idea that androstadienone may function as a male modulator chemo-signal, our findings thus suggest that androstadienone indeed affects brain functions in females. Moreover, we showed that the sex difference in hypothalamic activation was not related to any hormonal or sexual maturation-related changes during puberty. Therefore, sex differences in neural responsiveness are probably not associated with differences in olfactory sensitivity to androstadienone and thus, its subjectively perceived intensity. Accordingly, we did not find any significant sex differences in the reported intensity ratings of androstadienone in both the prepubertal and adolescent groups.

GENDER IDENTITY & HYPOTHALAMIC ACTIVATION

GD HAS BEEN HYPOTHESIZED to develop due to an altered sexual differentiation of the body and the brain during early development (van Goozen et al. 2002; Swaab and Garcia-Falgueras 2009). Here we investigated a unique data set of individuals with GD at two different developmental stages, in order to determine whether they would respond to androstadienone in accordance with their natal sex, rather than their experienced gender. We found that both, adolescent girls and boys with GD showed hypothalamic activations that reflected their experienced

gender. The sex difference that we observed in the control groups, was absent in both age groups of boys and girls diagnosed with GD. However, prepubertal girls with GD showed no differences in hypothalamic activation compared to both control boys and girls, whereas prepubertal boys with GD showed significant sex-typical hypothalamic activations and thus, in accordance with their natal sex. These findings thus suggest that individuals with GD possess certain functional brain characteristics, i.e. hypothalamic activation to androstadienone, of their experienced gender and thus that they may have undergone atypical neuronal sexual differentiation. However, this can only be observed reliably in adolescents with GD.

GIRLS DIAGNOSED WITH GD

PREPUBERTAL GIRLS WITH GD did not differ in hypothalamic activation from control girls indicating that they did not show a male-typical response. However, they also did not show a female-typical hypothalamic activation, when compared to control boys, indicating that they did not differ from either of the control groups. It is possible that prepubertal girls with GD constitute a rather heterogeneous group with respect to future persisting GD. It has been shown that only about 15.8% of the childhood GD cases will eventually lead to adult GD (Steensma et al. 2011). However, women present more often early-onset cases (Nieder et al. 2011) and girls have overall higher persistency rates of GD into adolescence compared to boys (Steensma, McGuire, et al. 2013). Thus, our finding that prepubertal girls with GD did not show a clear-cut female- or male-typical response to androstadienone awaits further confirmation in the future, when it will be known who of our participants showed persisting GD into adolescence and adulthood.

The comparison of adolescent female controls versus adolescent girls with GD revealed very similar results as the control group comparisons, i.e. stronger hypothalamic activations in those subjects experiencing a female gender identity, and this effect was mainly driven by effects over time, reflecting sensitization. Thus, adolescent girls with GD responded remarkably like their experienced gender (control boys). While speculative, these findings fit with the idea that this group of girls with GD (who are more homogenous in terms of future persisting gender dysphoria compared to the prepubertal groups) may have had a more male-typical perinatal hormonal environment, resulting in the

development of certain typically male functional sex characteristics of the brain. The hypogonadal state of adolescent girls with GD using GnRHa at the moment of data acquisition is not likely to account for these findings, since our results in the prepubertal control groups suggest that hypothalamic responsiveness to androstadienone is probably independent of puberty, and thus not affected by circulating sex hormones. Furthermore, animal studies showed that sexually dimorphic responses to volatile urinary odors were not dependent on circulating sex steroids, but rather developed under the influence of organizational effects of sex hormones (Baum and Keverne 2002; Pierman et al. 2006).

It should be noted, though, that sexual orientation of the participants with GD might present a confounding factor. Berglund et al. (2006) showed that hypothalamic responsiveness to androstadienone in lesbian women was comparable to that of heterosexual men. In the literature, the majority of natal females with GD are reported to be gynephilic (Lawrence 2010; Nieder et al. 2011), which was true as well for our group of adolescent gender dysphoric girls. Therefore, it cannot be ruled out that the resemblance in hypothalamic activation with the control boys might be due to their shared sexual orientation rather than their shared gender identity.

BOYS DIAGNOSED WITH GD

THE HYPOTHALAMIC RESPONSE to androstadienone in the prepubertal boys with GD was not significantly stronger than that shown by the prepubertal control boys. Moreover, the reverse contrast (control girls > boys with GD) revealed that prepubertal control girls showed significantly stronger hypothalamic activation, implying that boys with GD responded according to their natal sex. Again, it is likely that the younger, prepubertal sample of boys with GD constitutes a rather heterogeneous group in terms of future persistence of their gender dysphoric feelings (Steensma et al. 2011; Steensma, McGuire, et al. 2013). Therefore, they may show more variability in hypothalamic responsiveness to androstadienone.

Adolescent boys with GD showed significantly stronger, thus sex-atypical hypothalamic activations, compared to control boys, although this effect was not modulated by any effects of time, thus sensitisation to androstadienone, as we did observe in the control girls. Accordingly, the reverse comparison (control girls > boys with GD) revealed no significant effects. The female-typical

activation in adolescent boys with GD is in line with a previous study by Berglund et al. (2008), who reported that adult men with GD differed significantly from a group of male controls in terms of hypothalamic activation during exposure to androstadienone. Their adult participants with GD were matched to control men with regard to hormonal status and gynephilic sexual orientation, whereas in our study adolescent boys with GD received GnRHa at the moment of data acquisition and the majority of our participants reported to have an androphilic sexual orientation. Thus, despite differences with regard to the participants' age, hormonal status, and sexual orientation, the findings of both studies suggest that males with GD possess certain female-typical brain functions, and may therefore have undergone sex-atypical early brain development.

Some limitations of the present study should be mentioned and may be addressed in future research. In most comparisons, the gender differences in hypothalamic response to androstadienone showed relatively small effect sizes. These effect sizes, in combination with relatively small group sizes (especially that of the prepubertal girls with GD with N = 17), suggest that a lack of statistical power may be related to our failure in finding any significant effects for particularly this group. Therefore, additional analyses in other functional and structural (e.g. gray and white matter volumes or diffusion tensor imaging data) MRI measures, which are in preparation, should corroborate the present preliminary findings. Sexual orientation was difficult to assess in our young groups of participants. We therefore estimated their (future) sexual orientation by asking whether he/she had ever been in love. However, any relationship between sexual orientation and the response to androstadienone could not reliably be investigated in our groups.

In summary, the present study is the first to demonstrate that sex differences in hypothalamic activation upon smelling the chemo-signal androstadienone are not acquired during sexual maturation, under the influence of gonadal hormones during puberty, but may be considered hard-wired responses, which already can be observed in prepubertal children. Moreover, the current study is the first to explore sex-atypical hypothalamic responses to androstadienone in male and female individuals with GD at two different developmental stages. Our results indeed suggest that individuals with GD possess certain functional brain characteristics of their experienced gender and may have undergone atypical neuronal sexual differentiation.

Acknowledgments

WE THANK PROF. DR. THOMAS HUMMEL and his Smell & Taste lab at the University Medical School in Dresden (Germany) for his hospitality and the kind introduction into the field of olfactory fMRI. We thank Paul Groot from the Academic Medical Centre Amsterdam for his help with the data analyses, and we thank Prof. dr. Michael Baum from Boston University for his helpful comments on earlier versions of this manuscript. Last but not least, we are especially thankful to Dipl.-Ing. Johannes Burke, who developed and built the olfactometer equipment and programmed the application software. This study was funded by a VICI grant (453-08-003) from the Dutch Science Foundation (Nederlandse Organisatie voor Wetenschappelijk Onderzoek) to Dr J. Bakker. Dr J. Bakker is a senior research associate of the Belgian Fonds National de la Recherche Scientifique.