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Neurobiological characteristics associated with gender identity: Findings from neuroimaging studies in the Amsterdam cohort of children and adolescents experiencing gender incongruence

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ABSTRACT

This review has been based on my invited lecture at the annual meeting of the Society for Behavioral Neuroendocrinology in 2023. Gender incongruence is defined as a marked and persistent incongruence between an individual's experienced gender and the sex assigned at birth. A prominent hypothesis on the etiology of gender incongruence proposes that it is related to an altered or less pronounced sexual differentiation of the brain. This hypothesis has primarily been based on postmortem studies of the hypothalamus in transgender individuals. To further address this hypothesis, a series of structural and functional neuroimaging studies were conducted in the Amsterdam cohort of children and adolescents experiencing gender incongruence. Additional research objectives were to determine whether any sex and gender differences are established before or after puberty, as well as whether gender affirming hormone treatment would affect brain development and function. We found some evidence in favor of the sexual differentiation hypothesis at the functional level, but this was less evident at the structural level. We also observed some specific transgender neural signatures, suggesting that they might present a unique brain phenotype rather than being shifted towards either end of the male-female spectrum. Our results further suggest that the years between childhood and mid-adolescence represent an important period in which puberty-related factors influence several neural characteristics, such as white matter development and functional connectivity patterns, in both a sex and gender identity specific way. These latter observations thus lead to the important question about the possible negative consequences of delaying puberty on neurodevelopment. To further address this question, larger-scale, longitudinal studies are required to increase our understanding of the possible neurodevelopmental impacts of delaying puberty in transgender youth.

Gender incongruence (ICD-11), also known as gender dysphoria (DSM-5), is defined as a marked and persistent incongruence between an individual's experienced gender and the sex assigned at birth. Feelings of gender incongruence can already be present during childhood, since it is generally thought that gender identity, i.e., someone's fundamental sense of self as being male or female, develops early in life, between 2 and 3 years of age (Kohlberg, 1966). Gender identity needs to be further consolidated during adolescence, which is a period of significant cognitive and social-emotional change as adolescents start to explore their sexual identity (Coleman and Hendry, 2003). For individuals experiencing gender incongruence as young children, early adolescence (between 10 and 13 years of age) appears to be a critical period for the (dis-) continuation of gender dysphoric feelings since less than half of them (+/− 40 %; Wiepjes et al., 2018) experience persisting gender dysphoria and start gender affirming hormone treatment (GAHT), including puberty suppression.

A recent study (Wiepjes et al., 2018) reviewed the prevalence of gender incongruence using the medical files of all people attending the center of expertise on gender incongruence at the Vrije Universiteit (VU) Medical Center, Amsterdam, The Netherlands, between 1972 and 2015. It was found that the estimated prevalence in the Netherlands in 2015 was 1: 3800 for people assigned male at birth (AMAB) and 1: 5200 in people assigned female at birth (AFAB). However, these estimates were based on adults turning to health services and thus seeking medical treatment. Since some individuals might be hesitant to seek medical care about their gender incongruence or simply do not wish to undergo any medical treatment, these studies probably underestimated the prevalence of gender incongruence. Indeed, a Dutch study (Kuyper and Wijsen, 2014) based on self-report reported that 4.6 % of natal men and 3.2 % of natal women have an ambivalent gender identity (i.e. equal

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identification with other sex as with sex assigned at birth) and 1.1 % of natal men and 0.8 % of natal women an incongruent gender identity (i.e. strong identification with another sex than with sex assigned at birth). However, lower percentages reported a dislike of their natal body and/or a wish for hormones/surgery to change their body. Combining these figures estimated the percentage of men reporting an ambivalent or incongruent gender identity combined with a dislike of their male body and a wish to obtain hormones/surgery at 0.6 %. For women, this was 0.2 %. Recently, the number of applicants to gender clinics has increased rapidly in many countries (Pang et al., 2020; Zucker, 2019) which mainly seems to reflect an increase in the number of relatively "older" AFAB individuals (> 10 years of age at their first visit; van der Loos et al., 2023). Furthermore, the overall ratio of AMAB to AFAB people has shifted: prior to 2007 the predominant proportion of referrals concerned AMAB people, but this began to shift around 2009 to favoring more AFAB people seeking care and by 2018 these individuals outnumber AMAB by almost 3 to 1 (Van der Loos et al., 2023).

The dramatic and continuing increase in requests for medical treatments such as GAHT, including puberty suppression of transgender youth, demands further examination of the development of gender identity and knowledge about the effects of hormonal interventions on brain development and function. An overview will be provided here of a series of structural and functional neuroimaging studies which were conducted in children and adolescents experiencing gender incongruence at the VU Medical School Amsterdam between 2010 and 2016.

1. Etiology of gender incongruence

A prominent hypothesis on the etiology of gender incongruence proposes that it is related to the sexual differentiation of the brain and particularly to the observation that different critical periods exist for the development of the reproductive organs versus the brain. The thinking is that these two processes might have been affected differentially in individuals with gender incongruence (Swaab, 2007).

Most of our knowledge on the sexual differentiation of the brain has been derived from studies in animal models. However, these studies do not shed any light on the mechanisms underlying the development of gender identity since gender identity is an exclusively human trait. Therefore, any knowledge of the neural origins of gender development might be derived from studies conducted in humans, such as clinical observations in individuals diagnosed with a difference in sex development (DSD). Complete androgen insensitivity syndrome (CAIS; incidence: 1:99000; Hughes et al., 2012) is characterized by a 46,XY karyotype with a mutation in the androgen receptor with the consequence that all tissues, including the brain, are insensitive to testosterone. Individuals with CAIS are born with external female genitalia since the development of the male external genitalia depends entirely on testosterone, but they have testes and internal components of the male reproductive tract since they develop independent of testosterone action during embryonic development due to the presence of the SRY gene on the Y chromosome and the secretion of anti-Müllerian hormone by the developing testes. Most people with CAIS report an androphilic sexual orientation (i.e., sexual attraction to men), a female gender identity and a female-typical gender role (Masica et al., 1971; Wisniewski et al., 2000; Hines et al., 2003), supporting the notion that, in the absence of androgens, typical female psychosexual characteristics develop. However, a small percentage of people with CAIS do not report a female gender role and/or identity or an exclusively androphilic sexual orientation (T'sjoen et al., 2011; Brunner et al., 2016). Gender development might thus not always be typically female in people with CAIS, suggesting a potential contribution of the sex chromosome genes as well. Finally, it is important to note that people with CAIS are generally being reared as girls so it is well possible that their female gender identity reflects female-typical socialization.

Congenital adrenal hyperplasia is one of the most common DSDs (1:10000; Merke and Kabbani, 2001) and is characterized by a mutation in the gene encoding the enzyme 21-hydroxylase, which is important for the conversion of progesterone to deoxycorticosterone. As a result, progesterone is converted into 17-hydroxyprogesterone and ultimately into androgens. Therefore, genetic women and girls (46, XX) with CAH are frequently studied to determine whether androgens affect the brain and behavior since they have been exposed prenatally to excessive androgens. Girls with CAH are often born with ambiguous genitalia such as an enlarged clitoris and partially fused labia, the degree to which depends on the amount of androgen exposure during fetal development. At birth, the hormonal imbalance is corrected, terminating the period of excess androgen exposure. In adulthood, the vast majority (95 %) of women diagnosed with CAH at birth but raised as girls, identify themselves as women (Dessens et al., 2005). Only a small percentage (5 %) report serious discrepancies with their gender identity, indicating that there may be no direct relationship between prenatal testosterone exposure and the development of a male gender identity. The female gender identity of women experiencing CAH could be the result of estrogen action on the developing brain, but there is no direct evidence of a feminizing role of estrogens in women at present (Bakker, 2019). Women with Turner Syndrome (TS), who often have only one copy of the X-chromosome, are exposed less to estrogens due to ovarian dysgenesis. They often need estrogen therapy to induce pubertal maturation and later fertility. Since women with TS do not have ambiguous genitalia at birth, systematic studies on their psychosexual development are scarce. A recent publication (Kreukels et al., 2018) from the DSDlife group on gender incongruence and gender changes in DSD, found no cases of gender incongruence in women with TS thereby questioning the importance of estrogens in the development of a female gender identity.

Post-mortem studies investigating the brains of individuals with gender incongruence have been the basis of the sexual differentiation hypothesis. For example, a reduced, female-typical volume and number of neurons in the central subdivision of the bed nucleus of the stria terminalis and the third interstitial nucleus of the anterior hypothalamus have been observed in transwomen (AMAB, female gender identity; Zhou et al., 1995; Garcia-Falgueras and Swaab, 2008; Kruijver et al., 2000). In addition, a female-typical expression of neurokinin B and kisspeptin, two neuropeptides important in the regulation of gonadotropin-releasing hormone (GnRH) neuronal activity, and thus reproductive functioning, has been detected in the infundibular nucleus of the hypothalamus of transwomen (AMAB, female gender identity; Taziaux et al., 2012, 2016). These postmortem studies have provided valuable information on potential neurobiological contributors to gender incongruence. However, caution is warranted when interpreting the results because of the relatively small sample size, as well as the potential influence of hormonal therapy that most transwomen receive at some point during their life, and social influences. Thus female-typical hypothalamic volumes and neuropeptide expression levels might be the result of, rather than the cause of, life-long gender incongruence and/or hormone use.

The introduction of neuroimaging techniques has made it possible to conduct larger-scale studies involving more substantial subject samples and a better-controlled study design that includes controlling for hormonal treatment as well as sexual orientation. Since 2010, the number of both structural and functional neuroimaging studies on gender incongruence has been steadily increasing, although it remains rather low in comparison with other topics covered in neuroscience. Furthermore, the techniques used, the design, and study samples remain very diverse across studies, particularly in regard to age, onset of age of feelings of gender incongruence, hormone treatment, and sexual orientation. A recent meta-analysis (Mueller et al., 2021) of structural magnetic resonance imaging (MRI) data (n = 803) found that transgender persons differed significantly from cisgender persons with respect to several (sub) cortical brain volumes and surface area, but not in cortical thickness. A variety of patterns was observed that not only depended on the direction of the gender identity (towards male or towards female) but also on the brain measure as well as the brain region examined.

Transgender persons appear to have a brain phenotype that differs on average from cisgender persons, but not one that is shifted towards either end of the male-female spectrum. A specific transgender phenotype has also been suggested by several recent resting state MRI studies based on functional connectivity data showing a disconnection of fronto-parietal networks implicated in own-body, self-referential processing (Lin et al., 2014; Manzouri et al., 2017; Feusner et al., 2016; Burke et al., 2018).

2. Neuroimaging studies in transgender children and adolescents

The advantage of the non-invasive nature of MRI is that one can study brain function and structure in young subjects. This is particularly interesting when studying gender incongruence, since it often manifests at early prepubertal stages. It also creates an additional investigational opportunity since only some (+/− 40 %) of the children will express persisting gender dysphoria and only some will start GAHT (Wallien and Cohen-Kettenis, 2008; Wiepjes et al., 2018).

Sex reassignment for individuals with gender incongruence has been met with a great deal of skepticism and this is even more pronounced when it involves the treatment of minors. In the Netherlands, Cohen-Kettenis and Delemarre-van de Waal have been pioneers in establishing a treatment protocol for minors: early adolescents experiencing severe gender incongruence, are allowed, upon a careful psychological diagnostic procedure, to start using GnRH agonists (GnRHa) to suppress pubertal maturation. The advantages of GnRHa treatment are that any further development of the secondary sex characteristics is paused which positively influences later physical appearance providing more time to reflect upon a sex reassignment procedure which improves general psychological well-being (Kreukels and Cohen-Kettenis, 2011). After this pause, individuals can receive cross-sex hormones (androgens for AFAB and estrogens for AMAB), for which the timing depends on when GnRHa treatment was started. However, this treatment protocol has always received strong criticisms, which has only been increasing in the recent years. First, arresting pubertal development has been seen as problematic for several reasons. It has been argued that experiencing all stages of puberty and having age-appropriate socio-sexual experiences is crucial for psychological maturation, both at the cognitive and emotional level (Meyenburg, 1999; Spriggs, 2004; Korte et al., 2008). It is well-established that the prefrontal cortex, a brain area critical for cognitive control, emotion regulation and social cognition, shows protracted development during adolescence (Casey et al., 2002; Huttenlocher, 1979). It can thus be argued that inhibiting pubertal development might interfere with the marked adolescent-specific developmental changes in cognition, and consequently, their ability to reflect upon something as important as sex reassignment. Nevertheless, the protocol has become common practice in gender identity clinics throughout the Western world and has been incorporated into the Endocrine Society's guideline for the medical treatment of GD from the earliest edition (Hembree et al., 2017) and into the standards of care by the World Professional Association for Transgender Health (https://www.wpath.org/publications/soc). Available clinical data (van der Loos et al., 2023) in the Netherlands so far suggest that almost everyone (93 %) who was treated with puberty blockers decided to proceed to cross-sex hormones. The question is thus whether GnRHa treatment would actually "buy time" or whether starting GnRH treatment already predetermines the outcome. Second, the effects of cross-sex hormones can be irreversible, in particularly regarding physical appearances, such as breast development induced by estrogens and voice breaking/lowering induced by testosterone. As a result, the current treatment protocol has become more and more controversial with the main argument being that there is insufficient experimental evidence to properly weigh the risks and benefits of hormonal interventions, especially considering the recent exponential increase in teenage girls coming to gender clinics. As a result there is increased concern about young people regretting the transition and consequently, asking for a detransition.

The current literature on puberty and brain development in humans is limited and has not specifically addressed any differential effects of pubertal timing and whether delaying puberty, as is the case with GnRHa treatment, might affect brain development. There is evidence that developmental trajectories of certain brain areas align better with pubertal changes, and in particular, more so with hormone levels than with age, although age-related effects have not been specifically investigated. At present, there appears to be only one study (Beltz and Berenbaum, 2013) that has explicitly tested the relationship between pubertal timing and sex-typical cognitive development, reporting that late pubertal timing was correlated with poorer performance in a mental rotation task in men. These results suggest that pubertal hormones may be needed for the development of full cognitive functioning as well as that there might be a decreasing window of sensitivity to gonadal hormones throughout adolescence. The latter is, of course, important considering the current treatment protocol of delaying puberty in transgender youth.

Taken together, our first research objective was to determine whether sex differences in brain function and structure are established during pre- and early postnatal development indicating that they should already be present in prepubertal children, or whether these sex differences emerge only (or possibly increase) during puberty, supporting the hypothesis that puberty might represent an additional organizational period in the sexual differentiation of the brain (Bakker, 2019; Schulz et al., 2009; Sisk et al., 2003). Secondly, we wanted to determine whether individuals experiencing gender incongruence have undergone an altered sexual differentiation of the brain as suggested by postmortem studies, and if so, whether they would show neurobiological characteristics typical of their experienced gender before puberty or whether they only emerge during puberty. Finally, we wanted to determine whether hormonal interventions, such as puberty suppression and cross-sex hormones, affect brain function and structure. To address these research objectives, we used two different cohorts of participants, i.e., one cohort consisted of prepubertal children and adolescents experiencing gender incongruence, which was still called gender dysphoria in 2010, using the criteria of the DSM-5 (Table 1). They were recruited through the Center of Expertise on Gender Dysphoria at the VU University Medical Center (now part of the Amsterdam University Medical Center), in Amsterdam, The Netherlands. The second cohort (Table 2) consisted of adolescents experiencing gender incongruence (gender dysphoria, DSM-5), who had either started GnRHa treatment or were still waiting to receive it. They were also recruited through the Center of Expertise on Gender Dysphoria. Cisgender participants were recruited via several primary and secondary schools in The Netherlands, and by inviting friends and relatives of the participants with gender incongruence. For Cohort 1, all prepubertal children took part in one MRI session whereas all adolescents were scanned twice, i.e., for the first time while being on GnRHa treatment and for the second time, after receiving cross-sex hormone treatment for at least 10 months. Cisgender adolescents were also scanned twice. For Cohort 2, all participants were only scanned once. For Cohort 1, brain images were acquired on a 3 T Signa HDxt scanner (General Electric, Milwaukee, WI, USA) present at the VU University Medical Center Amsterdam, whereas for Cohort 2, brain images were acquired on a 3 T Philips Intera (Best,The Netherlands) MRI scanner, present at the Academic Medical Center Amsterdam. The next sections will give an overview of the results obtained in these two different cohorts.

2.1. Hypothalamic activation to the putative male chemosignal androstadienone in trans boys and girls

In humans, the odorous steroid 4,16-androstadien-3-one has been proposed to be a putative male chemosignal. Androstadienone which is 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

Table 1
Subject characteristics of Cohort 1.

			Pre-puberal Children				Adolescents			
			Cis girls	Cis boys	Trans boys	Trans girls	Cis girls	Cis boys	Trans boys	Trans girls
Group size	*N*		19	20	17	19	21	20	21	17
Age in years	Mean (SD)		9.7 (0.9)	9.5 (1.1)	9.6 (1.1)	10.4 (0.9)	16.3 (0.9)	15.9 (0.6)	16.1 (0.8)	15.3 (1.2)
Pubertal stage	Mean (SD)	P	1	1	1	1	4.2 (0.7)	4.7 (0.7)	4.7 (0.6)	3.1 (1.1)
		G/M	1	1	1	1	4.1 (0.8)	4.1 (0.8)	4.1 (1.1)	3.1 (0.8)
Sexual orientation	% *(N)*	Gynephilic	–	66.0 (13)	17.6 (5)	21.1 (4)	–	100 (20)	100 (21)	–
		Androphilic	89.5 (17)	–	29.4 (3)	42.1 (8)	100 (21)	–	–	70.6 (12)
		Ambiphilic	–	–	5.9 (1)	–	–	–	–	5.9 (1)
		Don't know	10.5 (2)	34 (7)	47.1 (8)	36.8 (7)	–	–	–	17.6 (3)
		Missing								5.9 (1)
Hormones		LH (IU/mmol)	0.01 (0.03)	0.01 (0.01)	0.00 (0.02)	0.04 (0.11)				
		FSH (IU/mmol)	0.44 (0.34)	0.16 (0.08)	0.32 (0.23)	0.37 (0.25)				
		DHEA (umol/mmol)	0.00 (0.01)	0.00 (0.02)	0.01 (0.02)	0.01 (0.02)				
		Testosterone (pmol/l)					40.5 (60.4)	307.0 (106.5)	–	–
		Estradiol (pmol/mmol)					529.6 (643.3)	245.3 (183.1)	–	–
		Cumulative GnRHa dose (mg)					–	–	78.8 (60.8)	88.1 (46.2)

N = group size; SD = standard deviation; P = pubic hair growth; G = genital development (applies for AMAB); M = breast development (applies for AFAB). Pubertal stages were assessed by means of the five-point (1 = pre-pubertal; 5 = post-pubertal) Tanner maturation scale. Sexual orientation was difficult to assess, especially in the prepubertal sample. 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. Luteinizing hormone (LH), follicle-stimulating hormone (FSH), dehydroepiandrosterone (DHEA), and Estradiol are corrected for creatine. For more details (i.e., educational background, ethnicity) see Burke et al., 2014; Heeswijk et al., 2021).

Table 2
Subject characteristics of Cohort 2.

Group	Treatment stage	N	Age (M ± SD)	Tanner (M ± SD)	IQ (M ± SD)	Duration GnRH (days) (M ± SD)	Duration Cross-sex (days) (M ± SD)
Cis boys		44	16.4 ± 2.8	4.4 ± 1.1	110.5 ± 19.9	–	–
Cis girls		52	16.3 ± 3.0	4.6 ± 0.8	105.2 ± 16.3	–	–
Trans boys	Untreated	17	15.2 ± 2.8	4.1 ± 1.2	101.8 ± 14.7	–	–
	GnRH	16	15.9 ± 1.5	3.6 ± 1.4	93.3 ± 14.7	611 ± 435	–
	Cross-sex*	21	19.1 ± 1.3	2.9 ± 1.9	105.3 ± 17.3	1596 ± 786	1025 ± 436
Trans girls	Untreated	11	13.8 ± 2.4	3.5 ± 1.1	112.5 ± 22.7	–	–
	GnRH	14	15.3 ± 0.7	3.7 ± 1.0	93.9 ± 10.8	692 ± 248	–
	Cross-sex*	12	19.0 ± 2.2	4.2 ± 0.8	107.8 ± 19.0	1620 ± 398	912 ± 497

Note that this is the total sample used for measuring GM volumes (Hoekzema et al., 2015). In the study of Staphorsius et al. (2015), only a subsample was scanned performing the Tower of London Sample, see the paper for more details. For more information (i.e., educational background, ethnicity) on this cohort, please see Hoekzema et al. (2015) and Staphorsius et al. (2015).

* Cross-sex hormones for trans boys consisted of testosterone (Sustanon) and for trans girls of estradiol (Progynova/Meno-implant).

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 have been found in men compared to women (Brooksbank et al., 1972; Gower and Ruparelia, 1993). Studies by Savic et al. (2001) using positron emission tomography (PET) found that smelling androstadienone (in pure crystalline form placed under the nose) induced a response in the hypothalamus of heterosexual women but not in heterosexual men. The latter only showed an activation in brain areas associated with the olfactory system, such as the piriform cortex and the amygdala. Thus, olfactory stimulation with androstadienone offered a relatively simple and objective experimental procedure to investigate functional sex differences in the human brain. Therefore, we used this olfactory task in our studies in children and adolescents (Cohort 1; Burke et al., 2014) to investigate whether 1) sex differences are already present before puberty and 2) children and adolescents experiencing GI have hypothalamic responses according to their experienced gender identity. To do so, we first had to determine whether we could observe any sex differences in hypothalamic activation upon smelling androstadienone using functional MRI, since PET acquisitions are not allowed in children and adolescents without any medical reason. We were able to replicate the findings of Savic et al. (2001) but we also observed hypothalamic activation in men depending on the concentration of androstadienone (Burke et al., 2012). At the highest concentration we observed a strong sex difference with women showing hypothalamic activation but not men. Therefore, we used this concentration in our study with children and adolescents. Interestingly, sex differences in hypothalamic activation (girls > boys) were already present in the prepubertal sample, suggesting that this sex difference is most likely organized early in development, i.e., during the fetal and neonatal period (Burke et al., 2014). In addition, it suggests that circulating gonadal hormones are not necessary to activate the brain response to the chemosignal. By contrast, we found no evidence for hypothalamic responses in line with their experienced gender in prepubertal boys with GI, whereas prepubertal girls with GI did not differ from cisgender girls

and boys in their response pattern to androstadienone, suggesting an "intermediate" pattern of activation. It should be taken into consideration when interpreting the results that we do not know whether our prepubertal participants with GI had persistent GI. At present, there is no unambiguous clinical or biological indicator available that predicts future persistence of GI. Thus, despite a very careful inclusion of prepubertal children who fulfilled all the criteria for GI, feelings of gender incongruence might have dissipated in a significant proportion of them. Therefore, our groups of prepubertal girls and boys with GI may have been relatively heterogeneous regarding GI persistence.

The sex difference in hypothalamic activation by androstadienone was also present in the adolescents sampled and interestingly, both boys and girls with GI showed hypothalamic responses typical of their experienced gender (Burke et al., 2014). Thus, androstadienone induced hypothalamic activation in boys with GI, like cisgender girls, but not in girls with GI, like cisgender boys. The adolescents represented are more homogeneous than the prepubertal individuals since they have all started GATH. Both boys and girls with GI were taking GnRH agonists at the time of the scan, further confirming that circulating gonadal hormones are not needed to show a brain response to androstadienone.

Taken together, our results suggest that sex differences in hypothalamic activation upon smelling the chemosignal androstadienone are not acquired during sexual maturation and under the influence of gonadal hormones during puberty but may be considered developmentally programmed responses observed in prepubertal children. Furthermore, our results indicate that children and adolescents experiencing GI possess certain functional brain characteristics of their experienced gender, which is in line with the sexual differentiation hypothesis.

The sexual orientation of our subjects with GI could present a potential confounding factor in the interpretation of our results. Early onset GI is often accompanied by a homosexual orientation, i.e., boys with GI tend to be androphilic and girls with GI tend to be gynephilic. Savic and co-workers (Savic et al., 2005; Berglund et al., 2006) reported that homosexual men, like heterosexual women, showed a hypothalamic response to androstadienone, and that homosexual women, like heterosexual men, did not show this response. All adolescent trans boys reported to be gynephillic and about 70 % of the trans girls reported to be androphillic (Table 1). So perhaps our results reflect the sexual orientation of our subjects rather than their transgender identity.

2.2. Brain activation related to cognitive functioning in trans boys

Sex differences in visuo-spatial cognitive functioning have been reported repeatedly, with the mental rotation task showing the most robust sex differences with men generally outperforming women, both in reaction time and the number of correct answers (Linn and Petersen, 1985). Brain activation patterns while performing this task have also shown robust sex differences with greater activation in parietal regions in men than in women (e.g., Jordan et al., 2002; Weiss et al., 2003; Butler et al., 2006; Gizewski et al., 2006; Schöning et al., 2007; Hoppe et al., 2012). It has been proposed that these sex differences reflect organizational actions of androgens during early development (Janowsky et al., 1998; Zitzman, 2006). Women with CAIS exhibited female-typical neural activation patterns when performing this task (van Hemmen et al., 2016). By contrast, a meta-analysis showed that spatial abilities were not masculinized in women with CAH (Collaer and Hines, 2020). Sex differences in mental rotation performance are observed in children, although the magnitude of the sex difference is smaller in comparison to adolescents and adults, suggesting that circulating gonadal hormones may affect the performance on this task. Mental rotation performance and associated brain activation patterns have repeatedly been shown to vary as a function of circulating gonadal hormone levels in adults (e.g., Hausmann et al., 2000; Schöning et al., 2007; Toffoletto et al., 2014), although it is important to note that this relationship has not been consistently observed (e.g., Epting and Overman, 1998; Halari et al., 2005; Pletzer et al., 2024;). However, it should be noted that the mental rotation task is very difficult to perform and thus that the smaller sex difference observed in children might reflect their difficulties in performing in this task. Of note, our children sample (both cis- and transgenders) in the Amsterdam cohort performed very poorly on this task and by consequence no reliable results could be obtained (unpublished results).

The mental rotation task has been implemented in persons with GI to address the hypothesis of a possible altered sexual differentiation in these people, as well as to study whether cross sex hormones would affect their performance. These behavioral studies (Zucker and Bradley, 1995; Cohen-Kettenis et al., 1998; van Goozen et al., 2002) in adults with gender incongruence find that people mostly performed like their experienced gender, which was even more pronounced when receiving cross-sex hormones (Van Goozen et al., 1994, 1995; Slabbekoorn et al., 1999). However, no effects of cross-sex hormones on mental rotation in GI were observed in the studies by Miles et al. (1998, 2006). Few fMRI studies (Carrillo et al., 2010; Schöning et al., 2010) have investigated brain activation while performing the mental rotation task in adults with GI and found less activation in parietal brain regions in adult men with GI, independent of hormonal status, compared to cisgender men (Schöning et al., 2010), as well as increased activation in frontal brain regions following estradiol treatment compared to cisgender women (Carrillo et al., 2010). Remarkably, no effects were observed in women with GI. Here, we conducted a prospective fMRI study in which we investigated the effects of testosterone treatment on visuo-spatial cognitive abilities in adolescent girls with GI (Cohort 1; Burke et al., 2016). The mental rotation task was performed twice: 1) when having received GnRHa treatment for 24 months on average to suppress endogenous gonadal hormones, just before the onset of testosterone treatment, and 2) 10 months later during testosterone treatment. Two cisgender groups of boys and girls participated as well to control for any learning effects of the task. We confirmed sex differences in neural activation while performing the mental rotation task (Burke et al., 2016). Cisgender girls had significantly increased right inferior frontal (precentral gyrus and frontal inferior operculum) and left parietal (cuneus) activation compared with cisgender boys. Likewise, cisgender girls showed increased right frontal activation compared to girls with GI who had not yet started testosterone treatment. Group comparisons of brain activation patterns between cisgender boys and girls with GI did not reveal any significant differences. Following 10 months of testosterone treatment, girls with GI showed significantly increased bilateral parietal and left frontal activation while performing the mental rotation task. A very similar pattern of increased frontal and parietal activation was observed in the cisgender boys in the second session, whereas brain activation patterns remained unchanged between sessions in cisgender girls. These findings suggest that brain activation patterns of girls with GD were in line with their experienced gender identity. As in the study of androstadiene, sexual orientation may be a confounding factor since all trans boys reported to be gynephillic. Performance on the mental rotation task can vary as a function of sexual orientation (Peters et al., 2007; Maylor et al., 2007) but effects of sexual orientation have only been shown for behavioral responses, not at the level of brain activity patterns. Furthermore, these effects were primarily observed in men and were more moderate or even negligible in women. However, we cannot rule out that the activation patterns observed in girls with GI reflect their previous experiences with visuospatial tasks since we observed that girls with GI shared similar interests and preferences for certain hobbies and activities with cisgender boys, such as video games and sports.

2.3. Functional amygdala lateralization in trans boys: effects of testosterone

The two hemispheres of the brain differ in structure and function, with small but robust differences between the sexes in functional lateralization (meta-analysis Hirnstein et al., 2018). Based on this sex

difference, the development of brain lateralization has long been thought to be under the influence of testosterone (for a review see Pfannkuche et al., 2009). However, this hypothesis has been debated since it has been mostly based on correlational studies. Here, we used a quasi-experimental approach to address this question by measuring functional lateralization in trans boys (AFAB) before and after testosterone treatment and comparing them with cisgender boys and girls (Cohort 1; Beking et al., 2020). We focused on the amygdala, a brain region important in emotion and memory and for which strong asymmetries have been reported). Lateralized sex differences in structure have been reported with larger right amygdala volume compared to the left amygdala in boys, whereas there was no difference between the left and the right in girls (Uematsu et al., 2012). This sex difference in structural asymmetry seems to disappear in adulthood (based on a large meta-analysis; Guadalupe et al., 2016). Both amygdalae are involved in emotion processing but consistent sex differences in lateralized activity are observed with more activation in the right amygdala in men and in the left amygdala in women during emotion processing (Cahill et al., 2004). Therefore, in this study (Beking et al., 2020), trans boys and cisgender boys and girls performed an fMRI face-matching task that has been shown to robustly engage the amygdala. We chose to use fearful and angry faces as these emotions elicit strong amygdala activation, on which effects of testosterone have been demonstrated. We observed that testosterone treatment shifted the lateralization index towards the right amygdala in trans boys. We also found that the cumulative dose of testosterone treatment correlated significantly with amygdala lateralization after treatment. However, rather unexpectedly, we did not observe any sex differences in lateralization or any correlation with testosterone in cisgender boys and girls. These inconsistencies might reflect sex differences in sensitivity to testosterone or its metabolites.

2.4. Gray matter volumes in trans boys and girls

Structural sex differences in the human brain have been frequently described. A meta-analysis (Ruigrok et al., 2014) of 16 whole-brain-voxel-based MRI studies reported that the adult male brain is on average 11 % larger than the adult female brain, as well as that adult men have larger bilateral gray matter (GM) volumes in limbic regions, including the amygdala, hippocampus, parahippocampal and cingulate gyrus, the temporal pole, precuneus, putamen, and cerebellum, whereas adult women have larger volumes in the bilateral thalamus and precuneus, right planum temporale/parietal operculum, insula Heschl's and anterior cingulate gyrus, parts of the frontal cortex, and in the left parahippocampal gyrus and lateral occipital cortex. By contrast, a more recent study (Ritchie et al., 2018) found no brain subregions where females had larger volumes than males. The discrepancy might be explained by differences in study size and heterogeneity. Ruigrok et al. (2014) used data generated by many separate studies, on separate scanners, generally with small sample sizes, whereas Ritchie et al. (2018) was based on one very large, single-scanner study, including a total of 5216 participants (2750 women and 2466 men).

Some sex differences in regional GM volumes can be observed in children (8–11 years of age) and can be correlated to fetal testosterone levels measured in amniotic fluid (Lombardo et al., 2012). In our study (Cohort 2; Hoekzema et al., 2015), we compared regional GM volumes between transgender and cisgender adolescents to examine whether there would be an association between pubertal hormones and sex differences in GM volumes as well as to determine whether transgender adolescents exhibit GM volumes typical of their natal sex or experienced gender. We compared GM volumes in transgender boys and girls who were either treatment naïve (i.e. exposed to endogenous sex hormones) with those who were on GnRH agonists to suppress puberty and with those on cross sex hormones. We also had two groups of age-matched cisgender boys and girls. When comparing the latter, we observed clusters of enlarged GM volume in the hypothalamus and both superior posterior lobes of the cerebellar hemisphere in cisgender boys, whereas cisgender girls had more GM volume in the superior medial frontal cortex. These clusters are centered on previously reported regions of sex differences in the human brain (Ruigrok et al., 2014). In the transgender groups, when examining the complete sample, i.e., including all subjects at different stages of treatment, we observed regional GM volumes that were largely concordant with their natal sex. Thus, at the whole brain level, we found no signs of an altered sexual differentiation of the brain. By contrast, when specifically examining structures showing sex differences in the cisgender children using region of interest (ROI) analyses, we detected in both transgender boys and girls, subtle alterations in GM volumes in the direction of their experienced gender. More specifically, trans boys had significantly more volume in the right cerebellum and less volume in the left superior medial frontal cortex in comparison with cisgender girls, whereas trans girls had significantly less volume in the bilateral cerebellum and hypothalamus and a trend for more medial frontal volume than cisgender boys (Hoekzema et al., 2015). These alterations were observed in the treatment naïve subsample and were not affected by GAHT. Taken together, the neuroanatomical characteristics of transgender boys and girls were primarily concordant with cisgender boys and girls. Only very subtle changes were observed in brain regions that showed sex differences, thereby only partially confirming the sexual differentiation hypothesis. These results are in line with MRI studies conducted in adult transgender populations (Kreukels and Guillamon, 2016; Guillamon et al., 2016), suggesting that there are most likely additional neural mechanisms underlying gender incongruence.

2.5. White matter microstructure and executive functioning in trans boys and girls

Several studies (e.g. Rametti et al., 2011a, 2011b; Kranz et al., 2014; Burke et al., 2017) addressing the neural mechanisms underlying gender incongruence have focused on white matter microstructure by using diffusion tensor imaging. Robust sex differences have been reported in fractional anisotropy (FA) values. Relatively higher overall, as well as region-specific FA values have been found in cisgender men compared to cisgender women (e.g. Bava et al., 2011; Cox et al., 2016; van Hemmen et al., 2016), indicating relatively more longitudinal organization, fiber coherence, and myelination in men compared to women. Furthermore, pubertal development (e.g. Bava et al., 2011; Chahal et al., 2018), as well as sex hormones (Herting et al., 2012; Ho et al., 2020; Peper et al., 2015) have been differentially associated with white matter diffusion characteristics in male and female adolescents. A 2-year longitudinal study in adolescents reported FA increases in cisgender boys and decreases in cisgender girls that were predicted by adrenal- and gonadal hormone-based changes (Herting et al., 2017). This suggests that puberty, independently of chronological age, influences white matter development differently as a function of sex.

A prospective case study of an 11-year-old transgender girl who received puberty suppression treatment, found that the typical, testosterone-related white-matter maturation (i.e. an increase in FA with older age) was not observed (Schneider et al., 2017). In addition, performance intelligence quotient and memory deteriorated over a period of 28 months. Single case findings warrant further study of the effects of puberty suppression treatment in transgender adolescents on white matter development and cognitive functioning in larger samples. Towards that end, we cross-sectionally examined sex and gender differences in regional FA values and the relationship to puberty with alterations in the white-matter organization of treatment-naïve prepubertal children and adolescents with gender incongruence, receiving puberty suppression (Cohort 1; Van Heesewijk et al., 2022). The mean responses in transgender individuals were compared with age-matched cisgender peers. In accordance with the literature, we detected significant sex effects in FA values, with birth assigned males having higher FA values than birth assigned females in the left cortico-spinal tract (CST) and right superior longitudinal fasciculus (SLF). We also observed a

relationship with pubertal status, with higher FA values in adolescents than in children. Furthermore, we found that adolescent transgender natal boys (trans girls) had lower FA values in the bilateral inferior fronto-occipital fasciculus (IFOF) compared to adolescent cisgender boys. This finding is in line with a study conducted in transgender adults (Burke et al., 2017) suggesting a salient neural correlate of gender incongruence. Rather unexpectedly, in the prepubertal sample, we observed higher FA values in children with gender incongruence compared with their age-matched cisgender peers. This novel observation is difficult to interpret, due to the lack of comparison literature. It might be related to the fact that the prepubertal groups are more heterogeneous since only part of them will continue with GAHT.

To explore whether our findings of relatively lower FA in the adolescent transgender groups might be explained by GnRHa treatment, correlation analyses were conducted between FA values and the cumulative doses (in milligrams) of GnRHa received. These analyses revealed a negative association between FA values and the cumulative doses of GnRHa for the right IFOF, i.e. the more GnRHa received, the lower the FA values were, although this effect did not survive Bonferroni correction for multiple testing.

In a separate study on Cohort 2 (Staphorsius et al., 2015), we examined the effects of pubertal suppression on executive functioning by measuring brain activity using fMRI when performing the Tower of London task. Performance on this task improves with age until early adulthood and correlates nicely with the development of the prefrontal cortex (De Luca et al., 2003; Huizinga et al., 2006; Asato et al., 2006). Reduced performance has been observed on this task in patients with pathology of the prefrontal cortex (Carlin et al., 2000). Thus, we compared patterns of activation of adolescents with gender incongruence who received GnRHa with those of cisgender adolescents. In addition, to determine whether potential differences between the adolescents might be the result of GnRHa treatment, a group of age-matched adolescents with gender incongruence who were not yet receiving GnRHa but were already in puberty, was included. ROI analyses revealed sex differences with cisgender boys having significantly greater activation than cisgender girls in the bilateral precuneus and a trend for greater activation in the dorsolateral prefrontal cortex when performing the Tower of London task. Interestingly, brain activation patterns of untreated transgender boys and girls were in between those of the two cisgender groups in those brain areas that showed significant sex differences. By contrast, puberty suppressed trans girls (AMAB) had greater activation than the puberty suppressed trans boys (AFAB), not only in the same regions that were more active in cisgender boys than cisgender girls (i.e. the bilateral precuneus), but also in other brain regions that did not show sex differences, i.e. the prefrontal cortex (left dorsolateral and bilateral rostrolateral). This exaggerated activation, particularly in the dorsolateral prefrontal cortex in trans girls, might reflect an increased effort to complete the task since at the behavioral level, they made the most mistakes (Staphorsius et al., 2015). However, in trans boys in which puberty was suppressed, precuneus activation was lower than cisgender girls but did not differ in accuracy or reaction time from the cisgender girls. Clearly, larger-scale, longitudinal studies are required to understand possible neurodevelopmental impacts of delaying puberty using GnRHa treatment over time in transgender youth (Chen et al., 2020).

2.6. Brain functional connectivity patterns in trans boys and girls

Based on functional MRI connectivity data (e.g., Lin et al., 2014; Manzouri et al., 2017; Feusner et al., 2016; Burke et al., 2018), it has been proposed that gender incongruence could reflect a disconnection of fronto-parietal networks implicated in own-body self-referential processing. Thus, the discomfort with their own body in transgender youth may reflect alterations in this cerebral network, leading to the request for GAHT. However, why there would be no incorporation of the typical physical traits of the natal sex into the own body representation remains unknown at present. In general, very little is known about the factors/genes that are important in establishing this specific cerebral network. In addition, no sex differences have been observed in brain activation patterns during own body perception compared to a scrambled control image (Burke et al., 2019).

To shed more light on the neurobiological factors underlying the development of the body perception network in transgendered youth, we analyzed functional connectivity (FC) patterns in prepubertal children and adolescents experiencing GI, and in age-matched cisgender boys and girls (Cohort 1; Nota et al., 2017). We selected the visual network (VN), sensorimotor networks (SMNs), default mode network (DMN), and the salience network (SN) as our resting state networks (RSN) of interest. We used independent component analyses to obtain the different RSNs and to compare across groups. Sex differences in FC between the cisgender adolescents were observed in the right supplementary motor area within one of the two SMNs (SMN-II; girls > boys) and the right posterior cingulate gyrus within the posterior DMN (boys > girls). Within these networks, adolescent trans girls had FC patterns similar to their experienced gender, whereas adolescent trans boys showed a FC pattern similar to their experienced gender in the SMN-II only. These findings are thus partially in line with the sexual differentiation hypothesis. Interestingly, we observed a specific FC pattern in transgender youth within one of the three VNs, i.e. the VN-I, with adolescent trans girls showing stronger FC in the right cerebellum in comparison with all other adolescent groups. No such specific FC pattern was observed in trans boys. The cerebellum is important in motor control but has also been proposed to play a role in the processing of mostly negative emotional stimuli (Schraa-Tam et al., 2012). Thus, perhaps prolonged distress such as a negative perception of self may have led to alterations of FC between brain regions involved in emotional processing in adolescents experiencing gender incongruence.

We did not observe any sex differences or differences between trans- and cisgenders in prepubertal children. This suggests that they only emerge with aging, during brain maturation. Indeed, several functional neuroimaging studies suggest that the occurrence of sex differences is dependent on age (Scheinost et al., 2015) or brain maturation (Rubia, 2013). Whether pubertal hormones play a role in establishing these sex differences remain unknown. All adolescent transgender individuals were taking GnRHa, so perhaps some of the FC patterns observed were due to suppression of gonadal hormones. We performed supplementary regression analyses in which we used testosterone levels in cisgender boys, estradiol levels in cisgender girls, and weeks of GnRHa treatment in transgenders of both sexes to determine possible effects of circulating sex hormones on FC, but no significant relationships were found.

3. Strengths and limitations

A strength of these studies is the inclusion of a unique sample of prepuberal children and adolescents with gender incongruence as well as our hypothesis-driven analysis approach. To our knowledge, they present the only neuroimaging studies conducted in such relatively large groups of children and adolescents with gender incongruence at present. However, sample sizes remain rather modest, which limit the generalizability of our findings. Overall, neuroimaging studies have been subject to lack of statistical power and publication bias (David et al., 2013, 2018). Furthermore, when differences were observed between cis- and transgenders, it cannot be ruled out that these differences reflect other factors, such as socialization and learning, rather than the hormonal status and/or gender incongruence. Finally, although neuroimaging techniques have been very useful in studying the neural correlates associated with gender incongruence, they cannot be used to replicate previous postmortem findings due to their limited resolution.

4. Concluding remarks and future directions

The primary goal of our neuroimaging studies in children and

adolescents experiencing gender incongruence was to determine whether they may have undergone an altered sexual differentiation of the brain, as suggested by postmortem studies. In addition, we wanted to determine whether sex differences in the brain are established before puberty and might thus reflect early, organizational effects of hormones during development or whether they are established under the influence of pubertal hormones, either through activation or as part of an additional period of brain development.

We found some evidence in favor of the sexual differentiation hypothesis at the functional level, i.e., the hypothalamic responses to androstadienone and brain activation patterns when performing the mental rotation task. This was, however, less evident at the structural level, such as gray matter volumes. We also observed some neural signatures specific to transgender individuals, i.e., regarding white matter microstructure and functional connectivity patterns, suggesting that they might present a unique brain phenotype rather than being shifted towards either end of the male-female spectrum as is widely believed. We observed that some sex differences reflect early organizational actions of hormones on the brain such as the hypothalamic response to androstadienone since the sex difference was observed in prepubertal children. By contrast, we did not observe an effect of gender incongruence on these hypothalamic responses before puberty. A possible explanation remains that the prepubertal group individuals were quite heterogeneous since it was unknown at the time of the fMRI acquisitions and subsequent analyses whether their gender incongruence would persist and whether they would start GAHT. It is also possible that these specific hypothalamic responses require pubertal maturation in this group of transgender individuals. It should be noted that all transgender subjects had undergone some pubertal development since they were not allowed to start GnRHa treatment before being in Tanner stage 2/3.

Our cross-sectional design limits any conclusions regarding brain development, but our results suggest that the years between childhood and mid-adolescence represent an important period in which puberty-related factors, including hormonal, social, and age-related factors, influence several neural features, such as white matter development and functional connectivity patterns, in both a sex and gender identity specific way. These observations lead to the important question about possible negative consequences of delaying/suppressing puberty on neurodevelopment. There was a trend for a negative association between white matter development and cumulative dosages of GnRHa, but it did not survive Bonferroni correction for multiple analyses. Furthermore, our study of the effects of GnRHa treatment on executive functioning did not find any evidence of detrimental effects of long-term puberty suppression on this measure, although our results of exaggerated sex differences in transgenders taking GnRHa were somewhat surprising and might reflect increased cognitive efforts to be able to complete the task. This effect could also be a chance finding since the number of participants in this study was rather small. Larger-scale, longitudinal studies are required to better understand the possible neurodevelopmental impacts of pubertal suppression over time in transgender youth. This was also the main conclusion of a Delphi consensus procedure which engaged numerous international experts in neurodevelopment, gender development, puberty & adolescence, neuroendocrinology, and statistics & psychometrics (Chen et al., 2020). At present, there is an urgent need for these studies considering the dramatic but unexplained rise in number of referrals to specialized gender identity clinics worldwide and associated increasing demands for hormonal interventions such as puberty suppression and cross-sex hormones. In addition, the current treatment protocols are increasingly being criticized due to the lack of sufficient solid scientific evidence to weigh the risks and benefits of these hormonal interventions in young people. There is thus increasing concern of young people regretting the transition and consequently, asking for a detransition (reviewed in Jorgensen, 2023).

Finally, we did not always observe the same patterns in trans boys versus trans girls in those studies in which we were able to compare both groups. For example, some of the transgender specific neural correlates, i.e., increased FC in the VN-I and decreased FA values in the IFOF, were only observed in trans girls and not in trans boys. This cannot be explained by any differences in circulating gonadal hormones since both trans girls and boys were receiving GnRHa treatment. This may suggest the existence of different mechanisms underlying gender incongruence in natal boys versus natal girls. However, it should be considered that overall samples sizes were rather modest, so any inconsistencies can also arise from Type I errors due to the lack of statistical power. Some polymorphisms in steroid hormone receptors have been associated with gender incongruence with an inversed allele interaction between the estradiol beta receptor and the androgen receptor in trans women, and both estradiol receptors being involved in trans men (Fernandez et al., 2018). However, at present, how these steroid receptor polymorphisms lead to altered steroid hormone actions during brain development, and by consequence, to gender incongruence, remain unknown.

In sum, more research is clearly needed to better understand the development of gender identity and the effects of hormonal interventions on brain development and function. This is challenging because these studies require the participation of significant numbers of subjects experiencing gender incongruence at different ages to obtain a longitudinal design. Furthermore, it would require the willingness of at least half of the participants not to receive any hormonal interventions to be able to act as controls. Finally, it should be acknowledged that overall neuromaging studies suffer from the lack of replication due to the relatively small number of participants. This is even more problematic when studying gender incongruence which remains relatively rare.

CRediT authorship contribution statement

Julie Bakker: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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