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## Origins of gender differences in the human brain

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# Chapter 1

General introduction





Generally speaking, psychological characteristics in men and women are more alike than different. Nevertheless, notable gender differences exist in certain aspects of human behavior, cognition, brain structure, and function. Behavioral and cognitive gender differences have been extensively studied. So far the largest effect sizes have been found in toy and activity interests and spatial abilities (reviewed in Hines 2010). After the introduction of neuroimaging techniques such as magnetic resonance imaging (MRI) in scientific research, studies have also focused on the neural substrates of these gender differences. Functional MRI (fMRI) studies, measuring the activity of the brain while performing a task, have for instance shown that neural activity while viewing sexual images, emotional stimuli or during performance of spatial tasks differs between men and women (reviewed in Sacher et al. 2013). MRI studies of brain structure have shown gender differences in the volume of and structural connectivity between brain regions (Gong et al. 2011; Ruigrok et al. 2014 [meta-analysis]). The exact factors driving the development of these gender differences and their relative influence have yet to be elucidated.

In general, gender differences present a topic of great scientific and societal interest. Over the past decades, awareness of the importance of gender as a biological variable in research has grown. Historically, biomedical research has focused predominantly on males, while generalizing findings to both genders. This has led to a blind spot in many research fields, including neuroscience, with respect to knowledge of health and disease in females and differences between the genders (Lagro-Janssen 2012). To overcome this bias, funding agencies have changed their policies. An example is the recent policy change for NIH-funded research to ensure the inclusion of sex as a biological variable in vertebrate animal and human studies (notice number NOT-OD-15-102: <https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-102.html>).

In addition to the importance of detecting gender differences, it is essential to understand the factors and mechanisms underlying the development of these differences. With respect to the sexual differentiation of human brain structure and function, research can be motivated from a perspective of understanding healthy brain development, but also central nervous system-related disorders associated with gender differences in prevalence and/or symptom presentation. Psychiatric and neurological disorders with a higher incidence in boys and men include, for example, autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), dyslexia, and amyotrophic lateral sclerosis (ALS), while depression, anxiety disorders, eating disorders, and Alzheimer's disease occur more frequently in girls and women (reviewed in Zahn-Waxler et al. 2006; Bao and Swaab 2011; Zagni et al. 2016). Investigation of the factors that play a role in the development of gender differences in the healthy brain is likely to provide information relevant for these disorders. Before looking into the sexual differentiation of the brain, it is important to understand the development of the male and female physical phenotypes.

## 1.1 GONADAL AND GENITAL DEVELOPMENT

Mammalian sex-typed development of the reproductive system starts with fetal sex determination, followed by sex differentiation during the prenatal phase and puberty. In typical human fetal development, the gonads of both 46,XX and 46,XY fetuses are undifferentiated and bipotential until approximately 7 weeks of gestation. In addition to these bipotential gonads, precursors for both the male and female internal genital structures are present at this stage (e.g., reviewed in Hughes 2001). The Müllerian ducts are the precursors of the female internal genitalia, namely the fallopian tubes, uterus, and the upper part of the vagina. The Wolffian ducts are the precursors of several male internal genital structures, including the epididymis, vas deferens, and seminal vesicles.

Sex determination refers to the development of the bipotential gonads into male or female gonads, a process directly dependent upon the composition of the sex chromosomes. One of the major genes involved in male sex determination is the sex determining region Y (SRY gene), which is located on the Y-chromosome (Koopman et al. 1990). SRY gene expression is required to initiate a cascade of events resulting in testicular formation in a typically developing 46,XY fetus. In the absence of the SRY gene, i.e., in a 46,XX fetus, the gonads develop into ovaries. Although several genes are involved in ovarian formation (reviewed in Arboleda et al. 2014), no specific gene with a role equivalent to the SRY gene has currently been identified.

Upon sex determination, the testes start producing hormones responsible for male sex differentiation. Testosterone, secreted by the Leydig cells, and its more potent metabolite dihydrotestosterone (DHT), induce the development of the internal and external male genitalia, including the Wolffian duct derivatives, penis, and scrotum. Both androgens exert their effects by binding to, and activation of, androgen receptors (AR) in target tissues (Brinkmann et al. 1989). Anti-Müllerian hormone (AMH), secreted by the Sertoli cells, causes the degeneration of the Müllerian duct (Jost et al. 1973). In the 46,XX fetus, the absence of high androgen levels and AMH results in Müllerian duct differentiation and the development of female external genitalia. During puberty, sex hormones produced by the testes and ovaries are responsible for the development of secondary sex characteristics such as facial hair and breast development.

## 1.2 SEXUAL DIFFERENTIATION OF THE BRAIN

The exact factors and mechanisms underlying the sexual differentiation of the human brain is an active research area. Most of our current knowledge is based on experimental findings in animal models. In the second half of the 20<sup>th</sup> century, the main focus in this field was on the effects of androgens, similar to their role in the development of other somatic sex

differences. The foundation for the “classic” view of brain sexual differentiation was the work of Phoenix et al. (1959). They discovered that female offspring of guinea pigs that had received androgens during pregnancy, displayed male-typical sexual behavior in adulthood. By inference, the authors hypothesized that prenatal testosterone itself, or its metabolites, influence the development of neural tissue related to sexual behavior. More specifically, these results were suggestive of permanent effects of prenatal androgens, resulting in a male-typical organization of the brain. Circulating levels of sex hormones during adolescence and adulthood were proposed to have activational effects, which are transient and activate the prenatally organized tissues.

In the following decades, animal studies have convincingly supported the idea of an important role for androgens in the sexual differentiation of the brain and behavior (Wallen 2009a), and have provided further insight on the topic. Studies in rodents have shown that many male-typical organizational effects are not induced by testosterone directly, but by estradiol converted from testosterone locally in the brain by the aromatase enzyme (Baum 1979). In contrast, studies in nonhuman primates have suggested a prominent role for androgens, directly activating the AR (Wallen 2005). Although female brain organization was thought to occur by default in the absence of high androgen levels, an active role for estrogens in brain feminization, in a period later than the critical period for androgen effects, has been suggested in mice (Brock et al. 2011). With respect to the critical period in which the brain is susceptible to the organizational effects of hormones, it was found that the duration and timing of this period differs between species and can be limited to the prenatal phase, e.g., in rhesus macaques, but may also extend to early postnatal life, e.g., in rats and mice (reviewed in Wallen and Baum 2002; McCarthy 2013). Moreover, puberty has been proposed as a second sensitive period of neural development, in which sex hormones can still have organizational effects on the brain (Sisk and Zehr 2005; Schulz et al. 2009).

The initial focus on the role of sex hormones in sexual differentiation during the twentieth century changed when findings from animal studies suggested that not all sex differences could be explained by differences in sex hormone exposure alone (Arnold 1996). For instance, some sex differences were found to be present prior to gonadal hormone production. Therefore, hormone-independent effects of genes on the sex chromosomes were hypothesized to also play a role. With the development of sophisticated animal models, investigation of the potential contribution of sex chromosome genes was enabled. An important example is the four core genotypes (FCG) model, in which the Y chromosome no longer determines testes development, because the Sry gene has been deleted from the Y chromosome and is located on an autosome instead (Arnold and Chen 2009). Four types of offspring are produced with this model; XX with male or female gonads and XY with male or female gonads, allowing the assessment of direct genetic effects from genes on the sex chromosomes independent of sex hormone effects. Results obtained in this particular mouse model have not only confirmed important sex hormone effects, but have

also demonstrated a direct role for genes on the sex chromosomes in a number of neural and behavioral sex differences (Arnold and Chen 2009; Cox et al. 2014).

Following these recent advances in the field, a general theory of sexual differentiation has been proposed which does not only focus on the brain, but identifies biological factors that cause sex differences in any tissue (Arnold 2017). This theory states that the mechanism of sexual differentiation is multifactorial and includes both sex hormone and sex chromosome effects, acting in parallel or combined. Although this theory focuses exclusively on biological factors, environmental factors are acknowledged to also play a role. The latter is of particular importance for gender differences in the human brain and behavior, because of the highly gendered human social environment. The author points out that several aspects of the theory, specifically with regards to direct effects of genes on the sex chromosomes, have not yet been adequately studied in animals or humans.

### 1.3 STUDYING SEXUAL DIFFERENTIATION OF THE BRAIN IN HUMANS

Genetic, gonadal or hormonal manipulations, as used in animal models, cannot be applied in human studies. Therefore, identifying the factors underlying brain sexual differentiation and their relative influence is challenging in humans. Specifically, prenatal hormone effects and direct effects of genes on the sex chromosomes are difficult to study. Several methods have, however, been proposed to meet these challenges, involving studies in typical and clinical populations (Cohen-Bendahan et al. 2005; Berenbaum and Beltz 2011). Some of these methods will be discussed below to provide an overview of the variety of study designs that can be used in human studies, as well as their limitations.

#### 1.3.1 Studies in typical populations

In humans, the sensitive period for prenatal androgen effects on the brain is thought to be between 8 to 24 weeks of gestation, when testosterone levels are high in male fetuses (e.g., Reyes et al. 1974; Nagamani et al. 1979). The first months after birth are also marked by a testosterone surge in boys, peaking between 1 to 3 months postnatally, and increased estradiol levels in girls, which again decrease during the 2<sup>nd</sup> year of life (Winter et al. 1976; Kuiri-Hänninen et al. 2014). This period, often referred to as “mini-puberty”, might be another critical period for organizational sex hormone effects on the brain and behavior, although it has received far less attention in human studies than the prenatal phase. To assess the role of early sex hormone exposure, direct or indirect measures of early hormone levels can be used to correlate with sexually differentiated characteristics later in life.



### ***1.3.1.1 Prenatal hormone levels measured in amniotic fluid***

To study organizational effects of prenatal androgens or other sex hormones, direct measures of fetal hormone levels in amniotic fluid can be obtained in a prospective study design (Finegan et al. 1989). Samples can only be obtained in a clinical setting, because amniocentesis incurs fetal risk. Nevertheless, these samples are useful in studies on prenatal hormone effects, since the timing of amniocentesis for genetic analysis coincides with the timing of expected prenatal androgen effects. Disadvantages of this approach include the long-term prospective design, selection bias and the fact that only a single sample is obtained at varying gestational ages.

### ***1.3.1.2 Indirect measures of prenatal hormone levels***

Indirect indicators of prenatal hormone levels are an alternative method to study early hormone effects. First, in twin studies, prenatal hormone levels are inferred based on assumed intrauterine hormone transfer between the twins (Miller 1994). These studies are based on the (yet to be confirmed) hypothesis that females with a male co-twin are exposed to higher androgen levels than females with a female co-twin during gestation. Second, several measures that show gender differences have been hypothesized to reflect differences in prenatal or early androgen levels, because these gender differences are already present in neonates and, as far as this aspect has been measured, remain reasonably constant throughout life. These measures, including digit ratios, otoacoustic emissions (OAEs), and genital anatomy, have therefore been proposed as potential markers of early androgen exposure. The advantage of such markers is that they are non-invasive and relatively easy to obtain.

The most extensively studied is the 2D:4D ratio; the relative length of the 2<sup>nd</sup> to the 4<sup>th</sup> digit, which is larger in women than in men (Hönekopp and Watson 2010). Regardless of criticism with respect to the validity of this measure as a proxy of prenatal androgen exposure (Berenbaum et al. 2009; Wallen 2009b; Voracek 2014), for example based on studies in clinical populations that have indicated that the potential association between prenatal androgen exposure and the digit ratio is too small to reflect differences at the individual level (Berenbaum et al. 2009), the digit ratio is still widely used for this purpose.

OAEs are sounds produced by the cochlea, which can be measured in the inner ear canal (Kemp 1978, 2008; Davis 1983). Spontaneous OAEs (SOAEs) are more frequent and stronger in women, and OAEs evoked by click stimuli (click-evoked OAEs; CEOAEs) have larger amplitudes in women than in men (McFadden and Pasanen 1998; Snihur and Hampson 2011; for review see McFadden 2009). Further research on the hypothesized link between early androgen exposure and OAEs is needed to assess the validity of this marker.

Finally, certain aspects of genital anatomy have been proposed as markers of early androgen exposure. The anogenital distance (AGD), i.e., the distance between the anus and the scrotum in men and between the anus and vagina in women, is larger in boys than girls (Thankamony et al. 2009). Findings from animal and human studies suggest that AGD at

birth reflects prenatal androgen exposure (Dean and Sharpe 2013), although it is unclear if it could serve as a lifelong biomarker since postnatal hormones might modify AGD (Mitchell et al. 2015). Penile length measured during the first few months of life has been suggested as a marker of the postnatal testosterone surge in boys (Pasterski 2015).

### **1.3.2 Studies in clinical populations**

Valuable insights into the sexual differentiation of the human brain and behavior can also be obtained by studying disorders/differences of sex development (DSDs). DSDs are “congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical” (as cited in Hughes et al. 2006). Although many DSDs are not well studied in this context because they are extremely rare, several DSDs provide a unique opportunity to assess the factors involved in sexual differentiation. Examples include, but are not limited to, congenital adrenal hyperplasia (CAH), sex chromosome aneuploidies, and complete androgen insensitivity syndrome (CAIS).

#### ***1.3.2.1 Congenital adrenal hyperplasia***

CAH is an androgen excess DSD, most frequently caused by mutations in a gene coding for the 21-hydroxylase enzyme. Deficiency of this enzyme results in decreased cortisol biosynthesis and increased adrenal androgen production, beginning early in gestation (White and Speiser 2000). Although CAH affects both XX and XY fetuses, phenotypical consequences are most prominent in females due to varying degrees of genital virilization at birth depending on the severity of the enzyme deficiency. Excess prenatal androgen exposure in CAH provides the opportunity to assess the organizational effects of prenatal androgens on brain sexual differentiation. Limitations of the method include possible confounding factors related to the disease or its treatment, such as prenatal glucocorticoid deficiency, postnatal illnesses and medication, and atypical genital appearance (Speiser 2013).

#### ***1.3.2.2 Sex chromosome aneuploidies***

Individuals with sex chromosome aneuploidies (SCAs) have an atypical number of sex chromosomes and generally show relative deficits in certain cognitive domains (Printzlau et al. 2017), of which some are sexually differentiated in the general population. SCAs have therefore been suggested as a method to study possible sex chromosome gene effects in these cognitive domains and related brain regions. Two examples of well-studied SCAs, albeit not exclusively in this context, are Klinefelter syndrome (KS) and Turner syndrome (TS). KS is characterized by two or more X-chromosomes and a Y-chromosome, and TS by one X-chromosome and a lack of (all or part of) the second sex chromosome. Most SCAs also result in atypical sex hormone levels, such as hypogonadism in men with KS prior to testosterone supplementation (Davis et al. 2015) and decreased estrogen production in TS due to premature ovarian failure (Modi et al. 2003). Consequently, it is challenging to establish

if findings in these populations represent direct genetic effects related to sex chromosome gene dosage, sex hormone effects, or both.

### 1.3.2.3 Complete androgen insensitivity syndrome

Androgen insensitivity syndrome (AIS) is a 46,XY DSD with an estimated incidence between 1:40,800 and 1:99,000 (Boehmer et al. 2001). AIS is characterized by a mild (MAIS), partial (PAIS) or complete (CAIS) defect in androgen action caused by (a) mutation(s) in the X-linked androgen receptor (AR) gene (Hughes et al. 2012), resulting in reduced or completely abolished AR function. The degree of androgen resistance determines the phenotypical presentation, ranging from a male phenotype with fertility problems in MAIS, to mild or severe undermasculinization and ambiguous genitalia in PAIS and a female phenotype in CAIS. The combination of XY chromosomes with complete androgen resistance is of specific interest in research on sexual differentiation of the brain, because it provides a unique opportunity to assess the role of sex hormones versus sex chromosome genes.

In a fetus with CAIS, the gonads develop into testes under the influence of the SRY gene and start producing androgens and AMH. Due to the inability of androgens to activate the AR, the external genitalia develop in the female direction, while AMH causes regression of the Müllerian duct, resulting in a blind-ending vagina and absent uterus. CAIS is detected in infancy in case of an inguinal hernia, or in adolescence in case of primary amenorrhea (Hughes et al. 2012). The assigned gender and gender-of-rearing is typically female. With regards to secondary sex characteristics, pubic and axillary hair is either sparse or absent (Tadokoro-Cuccaro and Hughes 2014) and spontaneous breast development occurs when gonads are *in situ*. Because of an increased risk of gonadal tumor development in DSDs, including AIS, the general medical advice is to surgically remove the gonads (Cools et al. 2006; Lee et al. 2016). Gonadectomy in women with CAIS can be performed in infancy, but since the malignancy risk is estimated to be low in CAIS before puberty, this can also be postponed until adolescence (van der Zwan et al. 2015). After gonadectomy, estrogen replacement therapy is initiated to induce puberty, in case of prepubertal gonadectomy, and to optimize bone health (Bertelloni et al. 2011).

### 1.3.3 Findings from studies in typical and clinical populations

Studies on factors involved in the sexual differentiation of behavior, cognition, and the brain have been performed using many of the above-described methods. It is important to combine findings across sufficiently valid methods to reach conclusions, since each approach has its limitations, validity has not been confirmed for all methods, and most studies have been performed in small samples.

In general, most research in humans has focused on early sex hormone effects on behavioral gender differences and less on sex chromosome effects on behavior or the brain. There is converging evidence from both typical and clinical populations for a link between

early androgen levels and certain behavioral and cognitive gender differences, such as gender-typical activity interests and spatial abilities (Berenbaum and Beltz 2016). Findings supportive of this association for example include increased male toy and activity interests and better performance on spatial tasks in women with CAH relative to control women, as well as a positive association between fetal testosterone levels measured in amniotic fluid and male-typical childhood play behavior and certain aspects of spatial cognition (Auyeung et al. 2009, 2012; reviewed in Hines 2010; Berenbaum et al. 2012). Results from neuroimaging studies are less consistent and do not provide a clear pattern of results across methods. For example, fetal testosterone levels have been linked to the volume of several sexually differentiated brain regions later in life (Lombardo et al. 2012). In contrast, certain aspects of brain function in women with CAH were found not to differ from control women (Ciumas et al. 2009), and brain structure in both men and women with CAH differed from control groups in a similar way, which might reflect the influence of other disease-related mechanisms (Merke et al. 2003).

Direct effects of sex chromosomes, independent of sex hormones, have currently not been adequately addressed in human studies. Numerous studies on cognitive abilities and neuroimaging studies have been performed in SCAs, providing valuable information about cognitive and neural development in these populations (e.g., reviewed in Printzlau et al. 2017). Even though some of the findings coincide with cognitive abilities or brain regions that show gender differences in the general population, the exact implications of these findings are unclear as sex hormone levels are also affected in most SCAs.

At the time that the project described in this thesis was initiated, no neuroimaging studies had been performed in women with CAIS. Studies on the psychosexual development of women with CAIS have generally shown an androphilic sexual orientation (sexual attraction to men), a female gender identity, and female-typical gender-role behavior (Masica et al. 1971; Wisniewski et al. 2000; Hines et al. 2003), which is in line with the hypothesized role of androgens in the sexual differentiation of these characteristics. Recent work, however, has shown data that do not correspond with these observations (T'Sjoen et al. 2011; Brunner et al. 2016).

## 1.4 THESIS OBJECTIVES

Because the exact factors underlying the sexual differentiation of the human brain and their relative importance are currently unknown, studies in women with CAIS provide a unique opportunity to increase our understanding on this topic. Therefore, the **first aim** of this thesis was to investigate the role of sex hormones and genes on the sex chromosomes in the sexual differentiation of human brain structure and function. To address this question, brain structure and function in women with CAIS, assessed with fMRI and MRI scan techniques,

was compared with that of control men and women for selected neuroimaging modalities that have previously shown gender differences in typical populations.

The **second aim** of this thesis was to assess the validity of two proposed markers of prenatal androgen exposure; CEOAEs and 2D:4D ratios. These measures were also obtained in women with CAIS and compared with those of female and male control subjects.

## 1.5 THESIS OUTLINE

In **Chapter 2** we performed an fMRI study to investigate the role of sex hormones and sex chromosome genes on the sexual differentiation of brain functioning related to the performance of a visuospatial task; the mental rotation task. In **Chapter 3** we assessed the mechanisms underlying sexual differentiation of white matter microstructure. In **Chapter 4** we used data from larger samples of women with CAIS and control subjects to study the factors involved in the sexual differentiation of localized and spatially distributed patterns of brain structure, specifically gray matter volume. In **Chapter 5** we applied a multivariate pattern recognition method to structural and functional neuroimaging data obtained in women with CAIS and control subjects to further investigate the relative influence and importance of sex chromosome genes and sex hormones in the development of gender differences in various aspects of the brain. **Chapter 6** addresses the question whether CEOAEs and digit ratios measured in adulthood are valid retrospective markers of early androgen levels, by investigating the potential link between these measures and effective androgen exposure. In **Chapter 7** the main findings of these studies are summarized, and in **Chapter 8**, these findings are discussed, limitations are addressed and future directions are suggested for research on the mechanisms involved in the sexual differentiation of the human brain.

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