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GENERAL INTRODUCTION AND RATIONALE
Sexual Differentiation of Brain & Behavior

FROM EARLY POSTNATAL LIFE onwards, males and females differ from each other in terms of behavior. As early as one day after birth, girls spent significantly more time looking at facial stimuli compared with boys, who prefer looking at mechanical objects (Connellan et al. 2000). During childhood, boys and girls differ with regard to their preferences for toys (Alexander 2003), playmates (Alexander and Hines 1994), and sex-typed activities (van de Beek et al. 2009; Lamminmäki et al. 2012). Later in life, other sex differences are observed, such as in cognitive strategy use (Capitani et al. 1999; Guillem and Mograss 2005; Butler et al. 2006; Clements et al. 2006; Keller and Menon 2009), motor skills (Jardine and Martin 1983; Watson and Kimura 1991; Hall and Kimura 1995), and in emotion processing (Schirmer et al. 2004; Schienle et al. 2005; Hofer et al. 2006; Koch et al. 2007). Moreover, men and women show differences in olfactory perception (Yousem et al. 1999; Doty and Cameron 2009), brain morphology (Cosgrove et al. 2007), and vulnerability for psychiatric disorders (Kessler et al. 2005; Paus et al. 2008; Bao and Swaab 2010).

THE PERINATAL SENSITIVE PERIOD

SEXUAL DIFFERENTIATION STARTS with the determination of the genetic sex. The nuclei of every human cell contain 22 autosomes and 2 sex chromosomes. In females, the sex chromosomes are two X chromosomes, males have one X and one Y chromosome. The chromosomes contain the genes that control the development of the reproductive organs. Whether a fetus develops in a male or female direction is determined by the presence of the sex chromosome Y and its gene SRY (Koopman et al. 1990; Koopman 1999). SRY induces the undifferentiated gonads to develop into testes and to produce testosterone beginning between 12 and 16 weeks of gestation. Testosterone, in turn, induces the differentiation of the internal and external male genitals. In absence of the Y chromosome (but two X chromosomes present) and without testosterone, the gonads differentiate into ovaries, and the fetus develops as female.

The production of gonadal hormones, which facilitate the differentiation of the sexual organs and also play an important role in the sexual differentiation of the brain, is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. The HPG-axis is active during fetal development and continues to function in
infancy; during childhood the HPG-axis is virtually quiescent, until it is reactivated at the onset of puberty (Grumbach 2002; Nathan and Palmert 2005) (Figure 1.1).

During fetal development, by the end of the first trimester of pregnancy (Grumbach 2002), gonadotropin-releasing hormone (GnRH) neurons migrate from the olfactory placode to the hypothalamus and start secreting GnRH. GnRH in turn stimulates neurosecretory neurons in the anterior pituitary to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) in a pulsatile manner. FSH and LH bind to ligand-specific receptors in the gonads resulting in the production of gonadal sex steroid precursors and sex steroids.

The sex difference in exposure to fetal testosterone presumably leads to a different fetal and neonatal pattern of LH/FSH secretion in boys and girls (Grumbach and Kaplan 1990). In males, a first prenatal surge of LH and testosterone takes place during the second half of pregnancy. In the brain, testosterone is synthesized to estradiol by the enzyme aromatase. Relatively higher local levels of estradiol in the male actively masculinize and defeminize brain structures and thus promote a male-typical sexual differentiation of the brain (Jost 1983; Bakker et al. 2006). During the first few weeks after birth, LH secretion, and to a lesser extent FSH secretion, again increases significantly resulting in a second, postnatal testosterone surge between 1–3 months of life, followed by a gradual decrease to pre-pubertal levels by 4–6 months (Waldhauser et al. 1981; Finegan 1989; Hrabovszky and Hutson 2002). In girls, the ovaries are relatively quiescent prenatally, but female infants show a similar, though somewhat later postnatal activation of the HPG-axis as boys, with, however, FSH instead of LH as the predominant hormone. During the first 4 months of postnatal life, high levels of FSH stimulate the maturation of ovarian follicles and, under modest influence of LH, estradiol is secreted by the ovaries during 6–12 months after birth. FSH and estradiol secretion starts to decline by 12 months of age, but continues until the age of 24 months (Waldhauser et al. 1981; Grumbach 2002; Quigley 2002). Thus, the pulsatile secretory patterns of gonadotropins during infancy are remarkably different between the sexes, both with regard to agent (LH in males versus FSH in females) and timing.

Animal studies suggest similar sex differences in the timing of sex hormone action. Active defeminization and masculinization by testosterone in males takes places during the prenatal developmental period (Quadros et al. 2002), whereas in females active feminization has been shown to proceed
Figure 1.1 Schematic representation of sex steroid concentrations during the sexual differentiation of the brain in female and male humans and rodents. Sexual differentiation takes place prenatally and early neonatally but probably also during adolescence. Figures adapted from Naninck et al. (2011).
under late postnatal and prepubertal influences of estradiol (Brock et al. 2010, 2011). However, whether estrogens are actively involved in female-typical sexual differentiation of the brain in humans is currently not known.

**ADOLESCENCE – A SECOND SENSITIVE PERIOD**

The transition from quiescence of HPG activity during childhood to the pubertal pattern is a gradual process that is marked by the reactivation of the HPG-axis and the production of gonadal sex steroids, also called gonadarche. Adrenarche, i.e. the production of adrenal androgens (androstenedione, dehydroepiandrosteron), together with gonadarche induce the physical changes associated with puberty, such as axillary and pubic hair growth, body odor, acne, and maturation of the secondary sex characteristics.
Adrenarche in both girls and boys usually starts (slightly) earlier than gonadarche, around 8 years of age. Gonadarche starts about 1.5 years earlier in girls (around 9 years of age) than in boys (around 11 years of age).

In males, LH stimulates the production of testosterone by the testes, which together with FSH is required for spermatogenesis. In females, the menstrual cycle is initiated (about 2 years after the start of gonadarche), during which LH induces ovulation and estradiol secretion, while FSH stimulates follicle maturation. The physical changes during puberty, such as pubic hair growth, breast development in girls, penile growth and testicular enlargement in boys have been categorized into 5 developmental stages by James Mourilyan Tanner (Marshall and Tanner 1969, 1970); the Tanner stages (Figure 1.2) are now widely applied by pediatric endocrinologists as a standard clinical instrument for the assessment and monitoring of pubertal maturation throughout adolescence.

The perinatal and the pubertal sensitive periods of sex hormone action coincide with critical periods of brain development. The effects of gonadal hormones on the sexual differentiation of the brain have traditionally been dichotomized into organizational and activational effects (Figure 1.3). Early, perinatal exposure to sex hormones has organizational, life-long irreversible effects on structure and function of the brain and consequently on behavior, whereas activational sex steroid action is typically associated with temporary and reversible effects on behavior (Phoenix et al. 1959). This basic categorization continues to serve as a valuable theoretical template, although more recent research stresses the presence of considerable variation in the magnitude of induced behavioral sex differences, the timing and number of critical time windows for steroid action (Bakker et al. 2002; McCarthy and Ball 2008; Brock et al. 2011), and the interaction with environmental and genetic factors (McCarthy et al. 2009).

The classic organizational-activational hypothesis has recently been challenged, since several studies conducted in rodents suggested that puberty might constitute an organizational period in itself. Sisk and colleagues (Sisk et al. 2003; Sisk and Foster 2004; Sisk and Zehr 2005; Ahmed et al. 2008; Schulz, Molenda-Figueira, et al. 2009) proposed that puberty may be regarded as a second sensitive period of sexual differentiation, during which the presence of sex hormones significantly influences changes in neuronal circuitry and brain morphology, resulting in the acquisition and/or accentuation of sex-specific behaviors. Sex hormones such as estrogen and testosterone have been shown to
impact brain developmental processes, such as cell migration (Tobet et al. 2009), neurite outgrowth (Toran-Allerand 1976), synaptic pruning (Markham et al. 2007), and dendritic sprouting (Zehr et al. 2008). Thus, hormonal changes during several (prenatal, neonatal, pubertal) critical periods of life essentially affect brain and psychosexual development.

SEX DIFFERENCES IN BRAIN DEVELOPMENT

LONGITUDINAL NEUROIMAGING STUDIES revealed that trajectories of neurodevelopment differ significantly between girls and boys (Lenroot et al. 2007; Goddings et al. 2013; Ingalhalikar et al. 2013). Both total brain volumes and gray matter volumes follow an inverted U-shape trajectory (size by age), with girls reaching peak sizes earlier than boys (3–4 years earlier for total brain size; 1–2 years for gray matter volume) (see Figure 1.4). Thus, the sex-specific neurodevelopmental changes during adolescence parallel the earlier occurring hormonal and physical changes during puberty in females. White matter volumes continue to increase throughout adolescence in both boys and girls (De Bellis et al. 2001). However, in males this increase occurs more rapidly, resulting in relatively larger adult white matter volumes compared with females (Goldstein et al. 2001). The sex difference in white matter brain volumes thus gradually increases throughout adolescence (Giedd et al. 2012).

Figure 1.3  Schematic representation of the developmental periods of organizational and activational influences of gonadal sex steroids on the male and female sexual differentiation. Figure adapted from Eva Naninck (unpublished).
Figure 1.4 Mean volume by age in years for males (N = 475 scans) and females (N = 354 scans). The (dotted) lines represent mean values, upper and lower 95% confidence intervals are represented by the grey areas. All curves differed significantly in height and shape with the exception of lateral ventricles, in which only height was different, and mid-sagittal area of the corpus callosum, in which neither height nor shape were different. 

- **a** Total brain volume (cc)
- **b** Gray matter (cc)
- **c** White matter (cc)
- **d** Lateral ventricles (cc)
- **e** Corpus Callosum Mid-Sagittal Area (cc²)
- **f** Caudate volume (cc)

Figure adapted from Lenroot et al. (2007).
The most consistently observed sexual dimorphism of the brain, in all pre-pubertal, adolescent, and adult samples is a significantly larger (9–12%) total brain size in males compared with females (Giedd et al. 1997; Nopoulos et al. 2000; Goldstein et al. 2001; Allen et al. 2003). Regional sex differences in brain morphology, adjusted for total brain volume, have repeatedly been found in areas of the frontal lobe, limbic structures, such as the amygdala, the hippocampus, basal ganglia, and the hypothalamus (Filipek et al. 1994; Giedd et al. 1997; Goldstein et al. 2001). Some of these morphological sex differences become apparent only in adulthood. For example, sex differences in volume and neuron number of the bed nucleus of the stria terminalis start to develop during or after adolescence (Chung et al. 2002). Moreover, boys and girls show different puberty-related maturational trajectories of these brain areas (Peper et al. 2008, 2011; Neufang et al. 2009; Bramen et al. 2011; Goddings et al. 2013; Ingalhalikar et al. 2013), which may contribute to sex differences in cognition and (social/emotional) behavior that are consolidated during adolescence.

**SEX DIFFERENCES IN BRAIN FUNCTION – THE ROLE OF OLFACTION**

Most of our knowledge on the sexual differentiation of the human brain derives from animal research. With regard to social communication, important for mate selection or determination of social status, most animals significantly rely on chemo-signaling, i.e. communication via the exchange of body odors, also called pheromones. The term *pheromone* was first introduced by Karlson and Lüscher (1959) for a substance secreted by an animal that causes a specific behavioral response in another animal. In mice, pheromones have been shown to induce important neuroendocrine changes affecting several aspects of reproduction (Lombardi and Vandenbergh 1977; Whitten 1999). These neuroendocrine effects of chemo-signals are thought to be mediated by GnRH neurons of the hypothalamus (Silverman et al. 1994). However, the debate surrounding the possible existence and definition of human pheromones has been very controversial (Pause 2004; Wysocki and Preti 2004). The odorous steroid 4,16-androstan-3-one (androstadienone) has been studied intensively as a putative human male modulator chemo-signal. Androstadienone 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, plasma, and semen (Brooksbank et al. 1969; Kwan et al. 1992). Higher concentrations of androstadien-
none in sweat have been found in men compared with women (Brooksbank et al., 1972; Gower and Ruparelia, 1993). Androstadienone has been suggested to be processed differently from ordinary odors (Jacob, Kinnunen, et al. 2001; Lundström, Olsson, et al. 2006), and several studies found that this steroid odor elicited sex- and sexual orientation-specific changes in the central nervous system, specifically in the hypothalamus (Savic et al. 2001, 2005; Berglund et al. 2006). Androstadienone has repeatedly been shown to impact women’s mood (Jacob and McClintock 2000; Jacob et al. 2002; Lundström et al. 2003; Lundström and Olsson 2005) and to affect cognitive functions and emotion processing (Hummer and McClintock 2009; Parma et al. 2012). Moreover, body odors (axillary extracts) have been reported to trigger endocrinological responses in both opposite sex (Preti et al. 2003; Miller and Maner 2011) and same-sex peers (Stern and McClintock 1998). Thus, chemo-signals play a significant role in human social interaction and communication. Research on the sex hormone-dependent central processing of chemo-signals such as androstadienone may contribute to our understanding of sex differences in (social-emotional) behavior, sexual attraction and sexual orientation in humans.

Gender Identity in Childhood & Adolescence

SELF-AWARENESS, CONSCIOUSNESS about oneself, the development of a personal identity is uniquely human, so is the sense of oneself of being male or female, i.e. gender identity. Gender identity is the subjective experience of one’s gender, the feeling of being a woman, man, or an alternative gender.

Between their first and second year of life, toddlers start to use gender labels in their speech, thereby starting to understand the concept of gender. By age 2–3, children show increasingly gender typical play behavior, prefer gender stereotyped toys (e.g. dolls versus trains), and refer to themselves as boy or girl (Kohlberg 1966). Similarly, social development is very much gender stereotyped during the preschool and primary school years, such that children choose to be friends mostly with same-sex peers (Slaby and Frey 1975). Certainly, the extent to which children conform to gender stereotypes is influenced by cultural values, their personality and temperament, by parenting style, and
their broader social environment children are raised. Along with the neurobiological and physical changes, adolescence is a period of cognitive development (with regard to e.g. moral reasoning, opinion formation), and consolidation of the social self, during which adolescents start to explore their sexual identity (Coleman and Hendry 1990) and gender role (masculine, feminine, androgynous) (Söchting et al. 1994; Bartle-Haring and Strimple 1996). Gender identity formation may not be fully established yet and still be influenced by gender role explorations during adolescence. However, in the general population, gender identity is generally assumed to be fairly fixed from early childhood on and most children develop a core sense of being male or female that is in congruence with their natal sex (Steensma, Kreukels, et al. 2013).

GENDER DYSPHORIA

INDIVIDUALS DIAGNOSED with Gender Dysphoria (GD), also referred to as transsexualism (ICD-10; World Health Organization 1992), according to the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; American Psychiatric Association, 2013) are characterized by a profound feeling of incongruence between their natal sex and their expressed/experienced gender. In order to meet the criteria for diagnosis, the gender dysphoric feelings should cause clinically significant distress or impairment in functioning (e.g. social, occupational). At least 6 out of 8 criteria defining the separate childhood diagnosis should be met, of which the first (criterion 1A: “strong desire to be of the other gender or an insistence that he or she is the other gender”) is required.

All participants with GD described in the studies of this thesis were diagnosed according to the criteria of the former edition of the DSM (DSM-IV-text revision; American Psychiatric Association 2000). In DSM-IV-TR, the diagnosis was referred to as Gender Identity Disorder, defined as 1) a strong and persistent cross-gender identification, 2) persistent discomfort with one’s biological sex or gender role behavior associated with one’s sex, 3) clinically significant distress or impairment in social, occupational or other important areas of functioning, and 4) the disturbance may not be concurrent with a physical intersex condition.

Most of the manuscripts described in this thesis were written by the time the new edition of the DSM was released. We therefore decided to use the new term Gender Dysphoria throughout this thesis, except for Chapter 2, which was
EPIDEMIOLOGY

ALTHOUGH FORMAL EPIDEMIOLOGICAL studies on the prevalence rates of GD in children, adolescents, or adults do not exist, severe and persisting GD seems to be relatively rare. Based on studies conducted in clinical samples (Zucker & Lawrence, 2009), the prevalence of GD in adults was estimated to range from 1:7,400 to 1:100,000 natal males and from 1:30,400 to 1:400,000 natal females. With regard to child and adolescent populations, estimates on the prevalence of GD have been based on a widely-used parental report questionnaire, the Child Behavior Checklist (CBCL) (Achenbach and Edelbrock 1983), assessing gender-variant behavior in children (between 4 – 11 years of age) in the general population. Overall, these population-based studies (Verhulst et al. 1996; Zucker et al. 1997; van Beijsterveldt et al. 2006) suggest that gender-variance in young children (until around 7 years of age) is relatively frequent, but is less often seen in older children (around 10 years of age) (van Beijsterveldt et al. 2006), and that it is more common in girls (5.0 – 5.3% endorsed item 5 “behaves like opposite sex”; 1.7 – 2.0% endorsed item 10 “wishes to be of opposite sex”) as compared to boys (item 5: 2.4 – 3.4%; item 10: 1.0 – 1.4%). However, these studies likely overestimate the number of children developing clinically relevant GD. Moreover, only a minority (approximately 15.8%) of the children who had been diagnosed with GD before puberty appear to have persisting GD in adolescence and adulthood (Steensma et al. 2011). Referral rates to gender identity services of children have consistently reported to be higher compared with those of adolescents (Zucker et al. 2008), although in Canada a sharp increase in referrals of adolescents has been observed since 2004 (Wood et al. 2013). Similarly, increasing numbers of referred adolescents, compared with referrals of children to the Center of Expertise on Gender Dysphoria in Amsterdam have been observed in recent years.

Similar to the prevalence estimates of GD, the sex ratios for the diagnosis vary considerably across childhood, adolescent, and adult cohorts. Next to the greater percentages of gender-variance that have been found in girls, girls also have an overall higher persistency rate of GD in adolescence compared with boys (Steensma, McGuire, et al. 2013), and adult natal females more often present early-onset cases of GD (Nieder et al. 2011). In contrast, within adult cohorts
natal males have been reported to be about 3 times more often diagnosed with GD compared with natal females (van Kesteren et al. 1996; De Cuypere et al. 2007), but sex ratios in referral rates may also be more balanced depending on socio-demographic and/or cultural factors (Kreukels et al. 2012).

**INTERVENTIONS**

**PROF. DR. PEGGY COHEN-KETTENIS** and Prof. Dr. Henriëtte Delemarre-van de Waal during the early 1990s developed a protocol for the treatment and clinical management of gender dysphoric adolescents (Gooren and Delemarre-van de Waal 1996; Cohen-Kettenis and van Goozen 1998). According to this *Dutch protocol*, adolescents who have experienced severe GD since early childhood are allowed, after a careful psychological diagnostic procedure, to start using GnRH agonists (GnRHa) in order to suppress pubertal maturation (Kreukels and Cohen-Kettenis 2011; de Vries and Cohen-Kettenis 2012). A minimum age of 12 years and at least Tanner stage 2/3 should be reached before any medical treatment is started. Advantages of this treatment are that further development of the secondary sex characteristics is inhibited, which improves psychological well-being (stress relief) and leaves more time to the adolescent to weigh the possibilities of an actual sex reassignment procedure. Moreover, arresting the development of the secondary sex characteristics positively influences later physical appearance in the preferred gender role.

From the age of 16 years on, as a first step in the actual sex reassignment, adolescents with persisting GD may receive cross-sex hormones (testosterone for natal girls and estradiol for natal boys), in addition to their treatment with GnRHa, in order to develop secondary sex characteristics of their experienced gender (Delemarre-van de Waal and Cohen-Kettenis 2006; Hembree et al. 2009).

While appealing, empirical evidence supporting the favorable effects of the treatment protocol is still needed in order to address ethical concerns regarding medical interventions in minors. It has been argued that experiencing all stages of (physical) puberty and having age-appropriate (socio-)sexual experiences is crucial for psychological (cognitive and emotional) maturation (Meyenburg 1999; Spriggs 2004; Korte et al. 2008). Others have pointed out that inhibition of endogenous sex hormone functioning may have detrimental effects on bone maturation, growth, and brain development (Viner et al. 2005; Houk and Lee 2006).
Only future studies can show whether medical interventions in young individuals with GD are advantageous or harmful, but the first reports on improved psychological well-being (fewer depressive symptoms and behavioral and emotional problems) and quality of life of adolescents treated with GnRHα and cross-sex hormones are encouraging (Cohen-Kettenis, Schagen, et al. 2011; de Vries, Steensma, et al. 2011).

ETIOLOGY

The etiology of GD is still largely unknown and a range of psychosocial (vulnerability) factors (Coates and Person 1985; Coates 1990; Zucker and Bradley 1995), stressful events during pregnancy (Ward et al. 2002; Seckl and Holmes 2007), and hormonal alterations during critical periods of development, which may be different for males and females (Cohen-Kettenis et al. 1998; Schagen et al. 2012) have been hypothesized to be involved in the development of GD.

The hypothalamus, a key brain area linking the endocrine and the nervous system (Baroncini et al. 2010), shows significant sex differences in both function (Savic et al. 2001; Taziaux et al. 2012) and morphology (Fernández-Guasti et al. 2000; Makris et al. 2013). Previous post-mortem histological research in adult individuals diagnosed with GD suggested sex-atypical hypothalamic neuron numbers in sexually dimorphic nuclei, such as the bed nucleus of the stria terminalis, (Zhou et al. 1995; Kruijver et al. 2000) the infundibular nucleus (Taziaux et al. 2012), and the interstitial nucleus of the anterior hypothalamus (Garcia-Falgueras and Swaab 2008). These studies suggest a neurobiological basis of GD and led to the hypothesis that the sexual differentiation of the brain in individuals with GD might not have followed the line of sexual differentiation of the rest of the body, and that somehow during development sex-atypical programming occurred (van Goozen et al. 2002; Swaab 2007). Since the differentiation of the genitals and the sexual differentiation of the brain take place during different time windows of pregnancy, these two processes may, theoretically, be independently affected by exposure to sex hormones (Garcia-Falgueras and Swaab 2010).

Evidence for genetic factors that have been hypothesized to play a role in the development of GD is inconsistent. Polymorphisms of the androgen receptor, the estrogen receptor, and aromatase genes have been linked to transsexualism (Henningsson et al. 2005; Bentz et al. 2008; Hare et al. 2009; Fernández...
et al. 2013). However, these association studies still await replication (Bentz et al. 2007; Ujike et al. 2009). Based on case reports of twins with GD (Heylens et al. 2012), it is argued that GD may indeed have a genetic component (Coolidge et al. 2002). However, cases of monozygotic twins who are discordant for GD (Segal 2006; Andreazza et al. 2013) support the notion that other factors, such as pre- and postnatal environmental effects, may also play a role in GD development.

Currently, we do not know the exact neuroendocrinological factors leading to a supposedly altered sexual differentiation in individuals with GD. Moreover, it is currently unclear to what extent puberty may be regarded as a critical period during which significant sex-specific changes in brain development take place, and to what degree brain sex differences, under the influence of sex hormones, may be consolidated during adolescence. For this thesis, the working hypothesis of the etiology of GD is that alterations in exposure to sex steroids, potentially due to (epi-)genetic factors, during different critical periods of sexual differentiation of the brain may underlie the strong sense of incongruence between one’s gender identity and natal sex.

**Thesis objectives**

The first objective of this work was to investigate whether individuals with GD might have undergone sex-atypical sexual differentiation, and thus whether their neurobiological characteristics reflected and better matched their experienced gender, rather than their natal sex.

The second aim of this thesis was to investigate whether pre-/perinatal and pubertal sex hormones exert any organizational and/or activational effects on the sex differences in brain structure and function. The goal therefore was to determine whether sex-related brain functions and sex differences in brain structure were already established during early development and would thus be present in prepubertal children, or whether sex-specific characteristics of the brain evolved during puberty, which is a second sex hormone-sensitive period.

This study is the first to investigate the sex-specific neurobiological characteristics of a large sample of young individuals with GD who were either treatment-naïve at the moment they participated, or received puberty suppressing or cross-sex hormone treatment. A third aim, therefore, was to explore the
effects of the hormonal treatments on their sex-specific (neuro-)biological characteristics and on brain development.

**Methods applied**

Two main methods were applied in the studies of this thesis: 1) recording otoacoustic emissions and 2) functional and structural MRI.

**Click-evoked Otoacoustic Emissions**

Click-evoked otoacoustic emissions (CeOAeS) are echo-like sounds that are produced by the inner ear in response to click-stimuli. CeOAeS generally show higher response amplitudes in women compared with men (Strickland et al. 1985; McFadden 1998), and this sex difference is already observed in neonates (Collet et al. 1993; Morlet et al. 1995). Weaker responses in males are therefore proposed to originate from elevated levels of testosterone during prenatal male sexual differentiation. Thus, CeOAeS can serve as a retrospective indicator of someone’s prenatal androgen exposure and may provide valuable, though indirect, information in relation to the possible etiology of GD. Moreover, whether androgens exert activational effects on CeOAeS during adolescence and adulthood is difficult to experimentally investigate in humans due to ethical constrains. Therefore, measuring CeOAeS in adolescents with GD who receive puberty suppressing medication and/or cross-sex hormones as part of their treatment offers unique research opportunities. Overall, 153 boys and girls with GD and 160 controls (participants without GD), therefore 626 ears, were examined.

**Magnetic Resonance Imaging**

Improvement and greater accessibility of non-invasive neuroimaging techniques such as MRI during the past 20 years have contributed to an enormous accumulation of knowledge about the development of function and structure of the human brain. An MRI scanner is in fact a powerful magnet that produces an intense and stable magnetic field.
Functional MRI (fMRI) measures brain activation based on local changes in blood flow over time. When a person lying in the scanner is asked to accomplish a certain task, neural activation and the associated blood flow to a particular brain area involved in that task is increased. The Blood Oxygenation Level Dependent (BOLD) signal results from magnetization changes associated with inflow of oxygen-rich relative to oxygen-poor blood and thus reflects the energy used by brain cells. This increase in hemodynamic response, which may be different between men and women during the performance of certain tasks, is picked up by the MRI scanner and can be extracted using statistical methods, resulting in brain activation maps.

Diffusion tensor imaging (DTI) is a relatively new magnetic resonance imaging (MRI) technique that has been used to characterize white matter fiber architecture on a microstructural level. Studies found sex differences in white matter diffusion characteristics suggesting that men generally possess a greater degree of myelination and axonal organization, whereas women show more white matter fiber crossing (Schmithorst et al. 2008; Inano et al. 2011; Menzler et al. 2011).

Although the technique is non-invasive and safe, undergoing an MRI scan is not necessarily very comfortable. The person lying in the scanner is subjected to loud noises, may experience claustrophobia, and is asked to lie perfectly still during the scanning session, because only a very slight movement of the head can cause image distortions. One can imagine that hardly moving at all for about one hour is quite a challenging task for young children. In addition, the scanner, an impressively big machine that makes loud noises, can be quite frightening. Therefore, in order to carefully prepare all our prepubertal participants for their MRI scan, we practiced the scanning session by means of a mock scanner (de Bie et al. 2010). The various parts of the scanner were demonstrated, each part of the MRI session was explained, and the children were familiarized with the MR sounds by playing the different MR frequencies via a built-in audio system. During the actual MRI investigation, in order to ensure minimal head motion, cartoons were shown to the children during the structural MRI sessions.

For the studies of this thesis, a total of 210 subjects participated in the MRI experiments, 74 of them even twice. Thirty-seven young adults participated in the olfactory fMRI experiments (Chapter 4) conducted in April 2010 at the Smell & Taste Clinic, Department of Otorhinolaryngology of the University
Medical School Dresden, in Germany. Eighty-three children and adolescents diagnosed with GD and 89 age-matched controls were scanned at the VU University Medical Center between October 2010 and June 2013. To date, this is the largest sample of children and adolescents diagnosed with GD participating in neuroimaging research. Moreover, a prospective functional MRI study on the effects of the hormonal interventions in GD adolescents (CHAPTER 7) has never been conducted before.

OLFACTORY FMRI

In chapters 4 and 5, a particular experimental fMRI setup was employed. During a 4 minute scanning session, subjects were asked just to lie passively in the scanner, and to breathe normally, while being exposed to the odorous steroid androstadienone. Androstadienone was delivered through a tubing system to the subjects’ nostrils by means of a custom-built air-dilution olfactometer (see Figure 1.5).
Previous research by Savic et al. (2001), using positron emission tomography (PET), suggested that olfactory stimulation with the chemo-signal androstadienone elicited sex-specific activation in the hypothalamus of heterosexual men and women. We aimed to employ this method in young children, and therefore had to replicate the previous PET findings first for using fMRI (Chapter 4), which is less invasive, compared with PET and thus better suitable for pediatric neuroimaging research.

Pre-pubertal children have not yet been exposed to chemo-signals in a sexual context, so finding any sex differences in the neural response to smelling androstadienone might represent the best evidence for organizational, hard-wired effects of gonadal hormones on this functional sex difference of the brain. Furthermore, potential differences in response to this steroid odor between individuals with GD and male and female controls would provide important arguments for a biological basis of GD.

Outline of this thesis

PART 1
OTOACOUSTIC EMISSIONS – A RETROSPECTIVE WINDOW TO EARLY ANDROGEN EXPOSURE

In Chapter 2, we retrospectively estimated the prenatal hormone environment by measuring CeOAes in a group of treatment-naïve children with GD, in order to test the hypothesis that individuals with GD may have undergone an atypical early sexual differentiation.

In Chapter 3 we investigated the potential effects of postnatal sex hormone exposure on CeOAes by testing CeOAE response amplitudes in groups of subjects receiving GnRHa and cross-sex hormones compared with treatment-naïve boys and girls with GD.

PART 2
THE CHEMO-SIGNAL ANDROSTADENONE SNIFFING THE SEX OF THE BRAIN

In Chapter 4 we determined whether and at which concentration a sexually dimorphic hypothalamic response to the chemo-signal androstadienone could be observed using fMRI.
Chapter 5 addressed the question whether the neurobiological response to this putative male chemo-signal is a hard-wired phenomenon that can already be observed in childhood, or evolves during puberty as part of sexual maturation. In addition, we tested whether children and adolescents with GD showed a sex-atypical response to androstadienone.

Part 3

Sex Differences and Effects of Sex Hormones on Adolescent Brain Structure & Function

In Chapter 6, adolescent boys and girls with GD, while receiving puberty suppressing medication (GnRHa) were compared with age-matched controls with regard to their white matter fiber microstructure.

Chapter 7 is a prospective study on the effects of the testosterone treatment on visuo-spatial cognitive functions in adolescent girls with GD.

Summary & Discussion

In Chapter 8 the main findings of the six studies described in this dissertation are summarized. In addition, limitations, remaining questions, and implications for future research are discussed.