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SUMMARY & GENERAL DISCUSSION

THIS FINAL CHAPTER AIMS to summarize the main findings of the different studies of this thesis about the neuroendocrinological factors affecting gender identity and the sexual differentiation of the brain and behavior. Methodological considerations and implications are discussed in light of the existing literature. The discussion will conclude with recommendations for the clinical practice and future research.

The specific aims of this dissertation were threefold. First, we examined whether and to what extent the neurobiological characteristics of a group of young individuals, diagnosed with *Gender Dysphoria* (GD), reflected their

expressed/experienced gender, rather than their natal sex. As a second objective, we investigated whether pre-/perinatal and pubertal sex hormones exert any organizational and/or activational effects on sex differences of brain structure and function. Our third aim was to explore the contribution of (cross-)sex hormones to the development of sex differences in neuroanatomy and brain function.

The studies described in this thesis had as main objective to test the hypothesis of an altered sexual differentiation in – young – individuals with GD, and thus whether variations in sex hormones during critical periods of sexual differentiation contribute to the development of GD in childhood and adolescence.

Summary of the main findings

PART 1

A RETROSPECTIVE WINDOW TO EARLY ANDROGEN EXPOSURE - OTOACOUSTIC EMISSIONS

IN CHAPTER 2 WE measured *click-evoked otoacoustic emissions* (CEOAEs), i.e. echo-like sounds produced by the inner ear, showing generally higher response amplitudes in females, in a group of treatment-naïve children and adolescents with GD (24 natal boys, 23 natal girls) and control subjects (65 boys and 62 girls). Weaker responses in males were proposed to originate from elevated levels of testosterone during prenatal male sexual differentiation. Therefore, we employed CEOAE recordings in order to retrospectively estimate the potentially aberrant prenatal hormone environment of children with GD. We replicated the normative sex difference in CEOAE response amplitude, with significantly stronger emissions in the control girls compared to control boys. This sex difference, however, was absent in the gender dysphoric boys and girls. Boys with GD showed stronger, more female-typical CEOAEs, whereas girls with GD did not differ in emission strength compared with control girls. Based on the assumption that CEOAE amplitudes can be seen as an index of the relative prenatal androgen exposure, our findings provide some evidence for the idea that

boys with GD may have been exposed to relatively lower amounts of androgen during early development.

In CHAPTER 3, based on the assumption that sex hormones may also exert activational, postnatal effects on CEOAEs, we examined whether hormonal interventions (*gonadotropin-releasing hormone analogs* (GNRHa) for pubertal suppression or *cross-sex hormone* (CSH) treatment) in 43 natal boys and 62 natal girls, all diagnosed with GD, affected their CEOAEs. We hypothesized that suppression of endogenous testosterone production (by means of GNRHa) and administration of estradiol in natal males would result in stronger emissions, and thus in female-typical CEOAE response amplitudes in boys with GD. Conversely, suppressing endogenously high levels of estradiol (by means of GNRHa) and the administration of testosterone in natal females was assumed to result in diminished CEOAEs in girls with GD. Sex hormone suppression by means of GNRHa resulted in weaker CEOAEs, especially when suppressing endogenous estradiol levels in natal females. In line with the assumed diminishing effects of androgens on CEOAEs, natal girls who received testosterone treatment showed significantly weaker right ear CEOAEs compared with treatment-naïve natal girls. Contrary to our expectations, left ear CEOAEs in natal boys receiving estradiol administrations were also weaker than those of their treatment-naïve peers. Our findings suggest that both testosterone and estradiol seemed to be actively implicated in facilitating or inhibiting the cochlear amplification mechanism. We propose that postnatal variations in CEOAE amplitude are mediated by estradiol-regulated mechanisms, and that androgens are first aromatized into estradiol, in order to actively *masculinize* CEOAEs.

PART 2

THE CHEMO-SIGNAL ANDROSTADIENONE SNIFFING THE SEX OF THE BRAIN

THE ODOROUS STEROID COMPOUND androstadienone, a putative male chemo-signal that is found in axillary sweat, was previously reported to evoke hypothalamic activations in heterosexual women, but not in heterosexual men. Since androstadienone is centrally processed without any conscious awareness, investigating brain responses during exposure to the chemo-signal offers a relatively simple and objective experimental procedure for investigating functional sex differences in the human brain. In CHAPTER 4 we applied this method using

functional magnetic resonance imaging (fMRI) with the intention to replicate previous studies using positron emission tomography. Twenty-one women and 16 men, all heterosexual, were exposed to three different concentrations of androstadienone, in order to test whether the sex difference in response to the steroid odor was dose-dependent. Surprisingly, we found that both men and women showed hypothalamic activation when smelling androstadienone. In line with previous findings, women showed a stronger response compared with men when they were exposed to the *high* androstadienone concentration. However, a stronger hypothalamic response in heterosexual men compared with women, when exposed to the *medium* androstadienone concentration was unexpected, and points to the need for a more thorough investigation of possible behavioral and/ or physiological actions of this steroid compound in heterosexual men.

Based on the results of the study described in CHAPTER 4, we decided to use the *high* androstadienone concentration for the study described in CHAPTER 5, in which we investigated whether puberty and gender identity modulated the sex difference in hypothalamic response to androstadienone. We measured brain activation during exposure to the chemo-signal in 39 prepubertal and 41 adolescent boys and girls, and then investigated whether 36 prepubertal children and 38 adolescents diagnosed with GD exhibited sex-atypical, rather than sex-typical hypothalamic activations during olfactory stimulation with androstadienone. We showed that the sex difference in hypothalamic responsiveness to androstadienone was already present in prepubertal children, and thus likely developed during early development instead of during sexual maturation. Hypothalamic responses in both adolescent girls and boys with GD were remarkably similar to those of their experienced gender control groups, thus sex-atypical. In contrast, we found no evidence for sex-atypical neuronal processing of the chemo-signal in prepubertal boys with GD, while the young girls with GD showed neither a typically male nor typically female response pattern to androstadienone. The future persistence of GD into adulthood in the younger age groups will supposedly be relatively lower compared to the adolescent groups who already started using GnRHa. Therefore, we speculate that the prepubertal groups are more heterogeneous with regard to their future GD diagnosis, which in turn may hamper clear-cut results regarding their sex-typical or sex-atypical response to androstadienone. Our findings in the prepubertal GD samples, in light of the distinct sex differences in the prepu-

bertal controls and the robust sex-reversed pattern of activation in the adolescents with GD, suggest that factors (i.e. hormonal and/or psychological, environmental) other than early pre-/perinatal mechanisms of sexual differentiation may impact a non-normative gender identity development into adolescence.

PART 3

EFFECTS OF SEX HORMONES ON BRAIN STRUCTURE & FUNCTION IN ADOLESCENCE

DIFFUSION TENSOR IMAGING HAS been applied as a powerful magnetic resonance technique to map the three-dimensional diffusion of water in brain tissue. Diffusion measures are highly sensitive to changes of white matter cellular architecture, and have therefore been used to characterize changes in neurodevelopmental microstructural white matter. White matter diffusion characteristics were found to vary as a function of gender, suggesting differences in axonal organization and myelination between the sexes. In addition, pubertal development and circulating sex hormone levels were differentially associated with white matter diffusion characteristics in males and females. In CHAPTER 6, we investigated whether 21 adolescent girls and 17 adolescent boys, diagnosed with GD exhibited sex-atypical, rather than sex-typical white matter microstructural characteristics. We first identified several brain regions showing sex differences in white matter diffusion parameters in controls (21 girls and 20 boys). Then, we compared the mean diffusion values for each of these regions between groups. Boys with GD, who were receiving GNRHa, had intermediate values relative to control males and females in the majority of these brain areas, indicating that they showed neither full feminization nor full masculinization. This suggests that males with *early onset* GD may have had insufficient masculinization of their white matter fiber tissue during brain development, supporting the hypothesis of an atypical early sexual differentiation of the brain in individuals with GD. In contrast, girls with GD, also using GNRHa, had predominantly sex-typical white matter diffusion characteristics showing only slight masculinization in fiber organization. Our findings are at odds with a previous study showing that adult treatment-naïve women with GD had significantly masculinized white matter fiber organization. We therefore assume that variables such as sex hormones, natal sex, and GD diagnosis may

interact differently during adolescent brain maturation as compared with the adult situation.

In CHAPTER 7 a prospective fMRI study examining the effects of testosterone treatment on visuo-spatial cognitive functions in 21 adolescent girls with GD is described. A classical cognitive task eliciting robust behavioral sex differences, the mental rotation task, was performed twice by the natal girls with GD: after having received GnRHa for some time to suppress their endogenous sex hormones, just before the onset of cross-sex hormone treatment with testosterone, and then 10 months later while receiving testosterone. Two control groups of 20 boys and 21 girls participated twice as well. Thereby, within-subject effects other than the testosterone treatment, such as learning effects between sessions, or cognitive development, were accounted for. Between-group comparisons before the onset of the testosterone treatment suggested a more male-typical brain activation pattern in the girls with GD when performing the mental rotation task. Ten months after the start of testosterone administration, in a similar fashion as the control boys, girls with GD showed an increase in mental rotation task-associated brain activation compared with the pre-testosterone scan. Our findings thus suggest a priori masculinized visuo-spatial cognitive functions in girls with GD. In addition, we provide new evidence for activational effects of testosterone on visuo-spatial cognitive functioning.

Summary of the Main Findings

CHAPTER 2

AIM Estimate, retrospectively, prenatal androgen exposure in treatmentnaïve children & adolescents with GD, by means of CEOAEs recordings.

MAIN FINDINGS A significant sex difference in CEOAE response amplitude, present in the control groups, was not observed in boys and girls with GD. Boys with GD showed *demasculinized* (stronger), though not fully *feminized* (lower than control girls) CEOAE response amplitudes in comparison to controls. Girls with GD had sex-typical CEOAE strengths, similar to control girls.

CHAPTER 3

AIM Assuming sex hormones exert activational effects on CEOAEs, we examined whether the hormonal interventions (GnRHa and CSH treatment) in individuals with GD affected their CEOAEs

MAIN FINDINGS Sex hormone suppression had dampening effects on CEOAEs in the natal girls. Testosterone administration in natal girls and estradiol treatment in natal boys both had diminishing effects on CEOAEs.

CHAPTER 4

AIM Determine whether the chemo-signal androstadienone elicits sex-specific and dose-dependent effects on hypothalamic activation.

MAIN FINDINGS Women showed a stronger response to androstadienone than men, when exposed to the highest concentration of the steroid odor, whereas men showed stronger responses to the lower concentrations of androstadienone compared with women.

CHAPTER 5

AIM Determine whether the sex difference in hypothalamic response to androstadienone could be observed in prepubertal children, and whether children and adolescents with GD (receiving GnRHa) showed sex-atypical rather than sex-typical responses to the steroid odor.

MAIN FINDINGS Prepubertal girls, similar to adolescent and adult females, responded significantly stronger to androstadienone by means of hypothalamic activation than males, suggesting a *hardwired* functional sex difference of the brain. Adolescents with GD, both natal boys and natal girls, showed hypothalamic activations in accordance with their experienced gender, whereas the response of prepubertal boys with GD reflected their natal sex, and prepubertal girls with GD showed neither a typically male nor typically female response.

CHAPTER 6

AIM Investigate white matter diffusion characteristics of adolescent boys and girls with GD (receiving GnRHa) in predefined white matter brain areas showing sex differences in age-matched control groups

MAIN FINDINGS Adolescent boys with GD had diffusion values that were intermediate to those of the control girls and control boys. Adolescent girls with GD showed a predominantly female-typical white matter microstructure.

CHAPTER 7

AIM Compare a group of adolescent girls with GD (receiving GnRHa) to male and female controls with regard to visuo-spatial cognitive functioning and associated brain activation; determine the effects of testosterone treatment on visuo-spatial cognition

MAIN FINDINGS Girls with GD showed *a priori* masculinized visuo-spatial functioning, prior to the start of the testosterone treatment and while receiving GnRHa. Similar to control boys, girls with GD show testosterone-related increases in task-related brain activations after 10 months of testosterone administration.

CEOAE = click-evoked otoacoustic emissions; GD = Gender Dysphoria; GnRHa = gonadotropin-releasing hormone analog; CSH = cross-sex hormone

General Discussion

MORE THAN HALF A CENTURY of animal research on the sexual differentiation of the brain and behavior unequivocally demonstrated that the presence of sex hormones during early development critically affects the organization of sex differences in neural tissue and by consequence, in behavior (Phoenix et al. 1959; Wallen 2009). Males and females both have the potential to express behaviors considered male-typical or female-typical, because the sex-specific neural circuitry exists in both genotypes. However, factors that regulate the expression and the extent to which these systems are activated or suppressed by sex hormones, differ between males and females.

For obvious reasons, animal studies cannot elucidate the question on how hormonal mechanisms during critical periods of sexual differentiation determine and influence a very human sex-specific characteristic, the fundamental experience of oneself being a man or a woman, hence, gender identity. The work of John Money and his colleagues who, during the 1950s, started studying human gender-related behavior and GD in individuals with *Disorders of Sex Development* (DSD) (Money 1955; Money et al. 1955) importantly contributed to the now widely accepted view that also in human (psycho-) sexual differentiation a dose-response relationship exists between the degree of pre-/perinatal exposure to sex hormones and a resulting sex-typical postnatal gender identity and gendered behavior (Meyer-Bahlburg 2013). For example, genetically male individuals with *Complete Androgen Insensitivity Syndrome*, a condition characterized by nonfunctional androgen receptors and consequently a lack of male-typical testosterone action, develop a female phenotype, show female-typical gendered behavior, and, almost exclusively (Mazur 2005; T'Sjoen et al. 2011), a female gender identity, despite their male XY genotype. Similarly, for women with *Congenital Adrenal Hyperplasia* (CAH), which is characterized by excessive production of androgens prenatally, an association between the degree of their prenatal androgen exposure and later masculinized behavior has been demonstrated (Meyer-Bahlburg et al. 2006, 2008). However, the case of CAH illustrates that while these females may present significant behavioral masculinization, most girls and women still identify as female (Meyer-Bahlburg et al. 2004; Dessens et al. 2005). Thus, in addition to sex hormone-sensitive neurodevelopmental and genetic influences, other aspects, such as other

biological mechanisms, psychological factors, and the social environment are likely involved in the development of a sex-typical or sex-atypical gender identity.

Besides biological theories, psychodynamic theories stress the importance of social-environmental influences with regard to a (non-) normative gender identity development. Zucker and Bradley (1995) argue that a predisposition to develop GD may originate from more general child-related and environmental vulnerability factors, such as an insecure or anxious temperament of the child (Coates and Person 1985; Zucker et al. 1996; Wallien et al. 2007), in interaction with unfavorable environmental aspects, such as psychopathology of the parents (Marantz and Coates 1991). On top of these general vulnerability factors, specific (child- or environment related) factors are required that interact during a critical period of the child's gender identity formation and -consolidation, in order to trigger the development of GD (Zucker and Bradley 1995).

Others note that during childhood and adolescence, additional social environmental aspects, such as the quality of relationships with peers, a negative cultural climate towards, and social stigma due to the expression of gender-variant behavior, may further contribute to an atypical gender identity development (Steensma et al. 2013).

The etiology of GD is therefore assumed by some to be best described by means of a multifactorial model, encompassing genetic, endocrinological, neurodevelopmental, as well as social and systemic influences.

GENDER IDENTITY DEVELOPMENT: ORGANIZATIONAL EFFECTS OF PRE-/PERINATAL EXPOSURE TO SEX HORMONES

THE SEXUAL DIFFERENTIATION HYPOTHESIS of GD states that atypical levels of prenatal sex hormones during a sensitive period of sexual differentiation of the brain cause the non-normative development of gender identity and sex-atypical behavior (Swaab and Garcia-Falgueras 2009). However, GD becomes manifest only years after the early organizational sex hormone effects have taken place and because of the rarity of this condition prospective studies would require very large numbers of participating pregnant women, being monitored with regard to the prenatal exposure to testosterone of their unborn child. Intrauterine levels of testosterone have been associated with later masculinized behavior (Auyeung et al. 2009; van de Beek et al. 2009). However, they may not

necessarily reveal information on the actual testosterone metabolism in the fetal brain. In addition, measurements of testosterone in e.g. amniotic fluid may only be allowed during particular phases of pregnancy. Thus, at present, due to logistic and ethical reasons, there is no direct measure available that could test the relationship between an atypical prenatal sex hormone environment and the development of GD.

In CHAPTER 2 we took a second best approach and made use of an indirect indicator of the prenatal sex hormone environment, by measuring CEOAEs. We found that the normative sex difference in the control samples, i.e. higher response amplitudes in girls, was absent in the GD groups. Boys with GD had stronger emissions compared with control boys, suggesting relatively less prenatal androgen action, which may have contributed to their atypical gender identity development. Girls with GD showed female-typical CEOAE amplitudes, which may suggest that sex hormone alterations potentially affecting neuronal sexual differentiation in females may occur during a different, possibly postnatal sensitive period.

Sex differences in behavior and physiology that are present before the onset of puberty, and thus before the reactivation of gonadal hormone production, are considered to originate from sex hormone influences during the fetal and neonatal period. This would imply that sex differences in brain functions or neuroanatomy found in prepubertal children reflect hard-wired sex differences. In CHAPTER 5 we tested whether pubertal maturation was a prerequisite for the sexually dimorphic hypothalamic response when exposed to the androgenic odor androstadienone. We could demonstrate that prepubertal girls showed a stronger hypothalamic response to the chemo-signal than prepubertal boys, thus presenting similar sex differences as those observed in adolescent (CHAPTER 5) and adult samples (Savic et al. 2001; Burke et al. 2012 (CHAPTER 4)). Our findings thus suggest that this brain response reflects a functional sex difference that evolved under the influence of early perinatal sex hormones.

We found, however, no evidence for sex-atypical neuronal processing of the chemo-signal in the prepubertal boys with GD, while the young girls with GD showed neither a clear male nor female response pattern to androstadienone. One important factor that is hampering more definite conclusions about the relationship between GD and the prenatal hormone environment is the uncertainty about GD persistence in young children. At present, there is no unambiguous clinical or biological indicator available that predicts future persistence of

GD. Thus, despite our careful inclusion of children who clearly fulfilled all criteria for GD diagnosis, in some of them the gender dysphoric feelings may disappear (Steensma et al. 2013). Therefore, our groups of young boys and girls may be relatively heterogeneous with regard to future GD persistence. Our findings await confirmation in the future, when it will be known who of our young participants showed persisting gender dysphoric feelings into adolescence and adulthood.

ORGANIZATIONAL EFFECTS OF SEX HORMONES DURING PUBERTY

ORGANIZATIONAL EFFECTS OF SEX hormones have generally been considered to occur during early pre-/perinatal development, when the brain is undergoing rapid changes. Recent animal research (Sisk et al. 2003; Sisk and Zehr 2005; Schulz, Molenda-Figueira, et al. 2009) and longitudinal neuroimaging studies in humans (Giedd et al. 1999; Raznahan, Lee, Stidd, Long, Greenstein, Clasen, Addington, et al. 2010), however, have strengthened the notion that the brain continues to be organized well beyond the prenatal and early postnatal periods of sexual differentiation and that sex-specific brain development proceeds during adolescence under the influence of pubertal sex hormones.

For example, it was shown that exposure to testosterone, during a particular time window of early puberty, was essential for the development of male-typical adult social behaviors in the Syrian hamster animal model (Schulz and Sisk 2006). Intriguingly, hormone replacement treatments after this critical pubertal period (in animals that were castrated after the perinatal organizational period) failed to induce normal male-typical behavior in these animals. Thus, circulating sex hormones from adolescence onwards still affect brain development and behavior, although, the potential of sex hormones to influence sex-specific brain organization has been suggested to gradually decrease from prenatal development to early adulthood (Schulz, Molenda-Figueira, et al. 2009; Schulz, Zehr, et al. 2009). Moreover, female sexual differentiation in mice has been shown to be estrogen-dependent during a pre-pubertal sensitive developmental period (Brock et al. 2010, 2011). This suggests that the perinatal and pubertal phases may not be considered two separate periods of sexual differentiation (at least with regard to rodents), but rather form a continuous developmental trajectory of sex-specific and sex hormone-sensitive organization of the nervous system (Juraska et al. 2013).

In humans, adolescence is a period of significant improvement in cognitive control, advancement in executive functioning, and emotion regulation, which is paralleled by the maturation of the prefrontal cortex (Casey et al. 2000; Tamm et al. 2002; Giedd et al. 2006). At the same time, subcortical brain areas of the limbic system become increasingly responsive to social-emotional input (Nelson et al. 2005), resulting in higher emotional reactivity (Spear 2009), changes in social interactions, more risk-taking and impulsive behavior (Steinberg 2004), and an increasing sensitivity to evaluations by peers (Steinberg 2005). It has been suggested that particularly the reorganization of the limbic structures, showing high densities of sex hormone receptors (Goldstein et al. 2001; Cooke and Woolley 2005; Hajszan et al. 2007; Sarkey et al. 2008), may be sensitive to the modulating effects of gonadal hormones during puberty (Steinberg et al. 2008). Part of this cortical-subcortical reorganization is also a remodeling of the dopaminergic system of the prefrontal cortex and the striatum (Sisk and Zehr 2005). The redistribution of neurotransmitter systems, which is suggested to be influenced by gonadal hormones (Becker et al. 2005), may further result in sex-specific changes in the regulation of affective and motivational behaviors during adolescence. Adolescence might thus be a second important period during which sex hormones modulate the reorganization of neuronal systems regulating (sex differences in) cognition, affect and social development, which in turn may also impact gender identity development.

Apart from the different critical time windows for sexual differentiation, we know today that the mechanisms by which sex steroids and their metabolites act on the various target tissues is much more complex than was previously thought. The classic view holds that the presence of testosterone leads to masculinization of behavior, whereas the female nervous system develops as default, in absence of any influencing sex steroids. However, in rodents it has been shown that in neural tissue, a great deal of testosterone is locally converted to estradiol by aromatase, and thus that most of the organizing action of testosterone is estrogen-derived (Jost 1983; Baum and Tobet 1986; Bakker et al. 2006; McCarthy et al. 2012). Accordingly, our findings on the diminishing effects of the estradiol treatment on CEOAEs in natal boys with GD (CHAPTER 3) suggest that postnatal variations in CEOAE amplitude are mediated by estradiol-regulated mechanisms. Moreover, ovarian hormones have been proposed to be actively involved in the feminization of the (rodent) brain, in particular during (or just before) puberty (Bakker et al. 2002; Brock et al. 2011; Juraska

et al. 2013). Thus, estradiol can independently induce feminization, defeminization, and masculinization of discrete brain regions by activating a multitude of different cellular mechanisms (Kudwa et al. 2005; Bakker et al. 2006; Mccarthy 2008). The potentially resulting phenotypes allow for the presentation of a great diversity of sex differences in physiology and gendered behavior on a dimensional, rather than a dichotomous (male versus female) scale. However, it remains to be determined whether such estradiol-driven mechanisms are similarly involved in human sexual differentiation.

PUBERTY SUPPRESSION

ABOUT A DECADE AGO, delaying puberty by means of GnRHa was introduced in order to relieve gender dysphoric adolescents from the distress associated with the progressing growth of their unwanted physical secondary sex characteristics, and to provide them with extra time to explore their wish for actual sex reassignment (Cohen-Kettenis, Steensma, et al. 2011). Recent follow-up studies on the effects of this intervention suggest a significant improvement with regard to emotional and behavioral problems, and depressive symptoms after the start of GnRHa treatment (Cohen-Kettenis, Schagen, et al. 2011; de Vries, Doreleijers, et al. 2011).

Berenbaum & Beltz (2011) proposed that systematic studies on the effects of GnRHa treatment in adolescents with GD, by means of a randomized clinical trial, would offer the opportunity to investigate whether, and to which extent pubertal sex hormones exert organizational effects on neurodevelopment. They hypothesized that adolescents with GD, whose puberty was suppressed, in contrast to those who experienced sex-typical puberty, would exhibit less distinctive sex-typical cognitive functions, neuroanatomical and personality characteristics, including gender identity.

However, this proposed experimental model does not account for the cross-gender behavior adolescents with GD exhibit, and therefore, less distinct sex differences in brain function or structure in GD populations are very likely to be observed, regardless of any GnRHa intervention. In addition, the proposed randomized trials will not be feasible to conduct due to lack of the participants' commitment, because most gender dysphoric adolescents would refuse postponing the developmental arrest of their (unwanted) secondary sex characteristics. Thus, withholding GnRHa treatment might even be considered harmful (Kreukels and Cohen-Kettenis 2011).

In the studies described in this thesis, we tested the effects of pubertal suppression by means of cross-sectional comparisons. In CHAPTER 3, we showed that sex hormone suppressed individuals with GD had weaker CEOAEs compared with a group of treatment-naïve boys and girls with GD, especially (when suppressing endogenous estradiol levels) in natal females. These findings are in line with previous studies in pediatric non-GD populations (2–6 years of age) (Lamprecht-Dinnesen et al. 1998; Kapoor and Panda 2006), reporting weaker CEOAEs in girls and, therefore, less distinct sex differences in emission strengths during the childhood quiescence of GNRH secretion. However, whether the effects of GNRH action during puberty may be organizational with regard to variations in CEOAE response amplitudes, rather than activational, may not be determined based on cross-sectional comparisons.

In CHAPTER 5 we tested whether gender dysphoric adolescents who were receiving puberty suppression, would show a sex-atypical hypothalamic response to the chemo-signal androstadienone. Animal studies suggested that chemo-signals induce important neuroendocrine changes in GNRH neurons in the hypothalamus (Silverman et al. 1994), affecting several aspects of reproduction (Whitten 1956, 1999; Lombardi and Vandenbergh 1977). Therefore, it may be hypothesized that suppressing GNRH action would prevent a functional response to the chemo-signal androstadienone in our puberty suppressed groups. However, animal studies (Baum and Keverne 2002; Pierman et al. 2006, 2008), pointed to early organizational effects, particularly of estradiol, in the sex-specific processing of pheromonal odors. In line with these results, our findings of increased hypothalamic activation upon exposure to androstadienone in natal males receiving GNRHa and in the prepubertal girls suggest that this female-typical functional brain response is probably not affected by GNRH down-regulation. In prospective CSH treatment effect studies in boys and girls with GD, we plan to investigate whether estradiol or testosterone administrations may build on these pre-/perinatally programmed effects, affecting the hypothalamic responsiveness to androstadienone.

In line with the assumed organizational effects of pubertal sex hormones on gendered behavior and cognition, in CHAPTER 7 we found evidence for less female-typical brain activation during visuo-spatial functioning in adolescent girls with GD receiving GNRHa. The *masculinized/defeminized* brain activation pattern in natal girls may thus indeed be the result of the suppression of sex-typical estrogen action. Alternatively, sex-atypical cognitive functioning

may result from effects of early pre-/perinatal sex hormones. Also other environmental factors, such as personal experiences, and preferences for certain hobbies and activities, such as video-games or sports may have influenced the masculinization of brain functions in girls with GD.

In our diffusion tensor imaging study (CHAPTER 6), we found, contrary to the hypothesis formulated by Berenbaum & Beltz (2001), that girls with GD, receiving GnRHa, showed predominantly sex-typical white matter microstructural characteristics, whereas a previous study in adult women with GD (exposed to endogenous sex hormones), found sex-atypical diffusion characteristics of white matter microstructure (Rametti, Carrillo, Gomez-Gil, Junque, Segovia, et al. 2011). Of note, GnRHa treatment studies in sheep (Wojniusz et al. 2011) demonstrated that behavioral sex differences were exaggerated in those animals whose pubertal gonadal hormone production was suppressed. Intriguingly, very similarly, we observed more sex-typical brain activation patterns while performing the *Tower of London task*, recruiting executive functions, and thus more distinct sex differences in those adolescents with GD receiving GnRHa, compared with treatment-naïve boys and girls with GD (Staphorsius et al. unpublished).

A better alternative to the proposed randomized clinical trial by Berenbaum & Belz (2011) may be a longitudinal study design, investigating children and adolescents with GD in comparison to male and female control groups, during at least four developmental stages/ endocrinological conditions (prepuberty, early endogenous puberty, pubertal suppression, cross-sex hormone treatment). We believe that such a comprehensive approach will shed light on the differential contribution of early pre-/perinatal and pubertal sex hormone effects on gender-specific development of the brain and behavior.

CROSS-SEX HORMONE ADMINISTRATION

SEVERAL PREVIOUS STUDIES in adult individuals with GD suggested that cross-sex hormone administration may affect cognitive functioning (Van Goozen et al. 1994, 1995; Slabbekoorn et al. 1999) and brain structure (Rametti et al. 2012), molding gender-specific brain functions and behavioral performance in the direction of the experienced gender. For example, natal females receiving testosterone treatment showed significantly improved performance on a spatial ability task (generally favoring males), whereas their performance deterio-

rated with regard to verbal fluency (generally female-favoring) (Van Goozen et al. 1994). In CHAPTER 3 and 7 we provide additional evidence for the hypothesis that sex hormones exert significant postnatal, activational effects on auditory processing and visuo-spatial cognitive functions.

In CHAPTER 7, we found that girls with GD after on average 10 months of testosterone treatment showed a similar pattern of increased task-related brain activation as the male controls, whereas the control girls showed no changes in brain activation between scan sessions. All subjects obviously grew older between both test sessions and became physically more mature, which in boys is accompanied by an increase in endogenous testosterone secretion (Ankarberg-Lindgren and Norjavaara 2004). Thus, the testosterone administration in the adolescent girls with GD mirrored the effects of increasing endogenous androgen levels in the also still maturing male controls, affecting the development of gender-typical (visuo-spatial) cognitive functions during adolescence. Our findings are in line with previous studies suggesting activational effects of testosterone on male-typical cognitive functioning (Aleman et al. 2004; Cherrier et al. 2010). To the best of our knowledge, this is the first prospective neuroimaging study on the effects of testosterone administration in natal adolescent females. A study regarding the effects of estradiol treatment on visuo-spatial functioning in natal males is in preparation, and may reveal whether estrogens are similarly actively affecting brain functions and behavior.

In CHAPTER 3, in line with two previous studies in monkeys (McFadden, Pasanen, Raper, et al. 2006) and adult men (Snihur and Hampson 2012), we found, by means of cross-sectional comparisons, additional evidence for masculinizing effects of postnatal testosterone on the auditory system. Girls with GD, undergoing testosterone treatment, had significantly weaker, thus masculinized CEOAE response amplitudes compared with treatment-naïve girls with GD. Unexpectedly at first glance, we found that estradiol administration had significant dampening effects on CEOAEs in boys with GD. This finding, however, is in line with a study that observed lower CEOAE response amplitudes in women receiving oral contraceptives compared with normally cycling women (Snihur and Hampson 2012), suggesting that a continuous administration of estradiol results in more male-typical, thus weaker CEOAEs. Thus, an endogenously cyclic or pulsatile pattern of estradiol secretion seems to be a prerequisite for the *feminizing*, thus enhancing influences on CEOAEs to take effect.

ORGANIZATIONAL VERSUS ACTIVATIONAL EFFECTS OF SEX HORMONES

TAKEN TOGETHER, OUR findings suggest that both testosterone and estradiol exert significant postnatal effects on brain functioning and behavior. However, in view of our findings on spatial abilities (CHAPTER 7) and CEOAEs (CHAPTER 3), the postnatal effects of (cross-)sex hormones, and testosterone in particular, seem to be more of an activational rather than an organizational nature, emphasizing or accentuating gender-specific characteristics. Future neuroimaging studies testing the effects of estradiol administration in natal males with GD will provide additional information on the potential role of estradiol in active feminization of the brain.

Our findings furthermore suggest organizational effects of sex hormones to be associated with the development of an atypical gender identity. The more female-typical CEOAEs in treatment-naïve young boys with GD (CHAPTER 2), and the hypothalamic responsiveness to androstadienone in our prepubertal groups (CHAPTER 5) suggest pre-/perinatal organizational effects of sex hormones on the sexual differentiation of the brain and behavior. More speculatively, but in line with the lack of evidence for significant sex differences in white matter microstructure in pre-adolescent samples (Brouwer et al. 2012; Geng et al. 2012) our findings of sex-typical diffusion measures in adolescent natal females (with GD, receiving GnRHa) may suggest organizational effects of pubertal sex hormones on white matter development. However, since we observed slight feminization of white matter microstructure in the adolescent natal boys with GD (receiving GnRHa), such organizational influences of gonadal hormones may be sex-specific.

SEX DIFFERENCES IN SEX HORMONE EFFECTS

MANY OF OUR FINDINGS on the potential effects of sex hormones and differences between individuals with GD and control subjects were not generalizable to both sexes. In CHAPTER 2, we found no evidence for any prenatal hormone alterations in the girls with GD, because they had similarly high CEOAE response amplitudes as the female controls. Similarly, with regard to white matter microstructure (CHAPTER 7), while the natal males had intermediate diffusion parameters relative to the control groups, the natal girls with GD had sex-typical diffusion characteristics. Conversely, during the olfactory stimula-

tions with androstadienone (CHAPTER 5), the prepubertal girls with GD differed from neither of both control groups, whereas the prepubertal boys with GD showed clear sex-typical hypothalamic responses.

These sex differences in the supposed effects of sex hormones on an atypical gender identity development may be explained by different hypothetical scenarios. Sex differences in CEOAEs are assumed to reflect differences in prenatal androgen exposure. One possibility therefore is that some other sex hormone than testosterone, e.g. ovarian estradiol, which plays a major role during postnatal female development, may be the agent of masculinization of the nervous system leading to the development of GD in girls. Second, the target tissue for masculinization by androgens or estradiol action may be different between males and females; thus, e.g. *masculinization* of CEOAEs in natal males (with GD and thus, a female gender identity) receiving estradiol administration may not necessarily also reflect masculinization of neural tissue. As a third possibility, the time window for sex hormone action resulting in masculinization or feminization of the nervous system may be different between the sexes. For example, natal females with GD may have been exposed to aberrant neonatal (ovarian) hormone surges, whereas natal males with GD could have had relatively less prenatal androgen exposure. Moreover, animal studies suggested different developmental time windows for male and female sexual differentiation: the prepubertal phase of development may be particularly important for the female sexual differentiation, whereas for male sexual differentiation the pre- and early post-natal developmental period has been shown to be decisive (Brock et al. 2010, 2011). Taken together, the interaction of the different factors (hormone agent, target tissue, timing of hormone action) underlying the sexual differentiation of the brain and the associated (atypical) gender identity development may very well differ between males and females, and mechanisms found responsible in one sex do not necessarily have to mirror those being responsible in the other sex.

SEXUAL ORIENTATION & GENDER IDENTITY

AN IMPORTANT CONSIDERATION for the interpretation of our findings in gender dysphoric individuals is sexual orientation. The hypothesized etiology of GD and the assumed biological and environmental factors underlying the development of a homosexual, bisexual, or heterosexual orientation are very similar

(Zucker and Bradley 1995; Balthazart 2011). Regarding their presumed biological origin, both gender identity and sexual orientation are thought to be organized under the influence of prenatal sex hormone exposure during a sensitive period of sexual differentiation of the brain (Swaab and Garcia-Falgueras 2009; Hines 2011). Apart from the supposed biological factors involved, environmental factors, such as childhood family experiences (Ridge and Feeney 1998; Lung and Shu 2007), gender-nonconforming behavior during childhood (Zucker and Bradley 1995; Bearman and Bru 2002; Rieger et al. 2008), and cultural influences (Hendin 1978) have been associated with both the development of a non-heterosexual orientation and a non-normative gender identity development.

However, one important difference concerns the timing of their manifestation. Whereas the mental image of oneself being a boy or a girl is present from early childhood onwards, the feeling of romantic affection and sexual attraction towards males or females emerges during puberty and sexual maturation. Prospective studies have shown that most, but not all of the children and young adolescents diagnosed with GD will develop a sexual orientation directed to individuals of their natal sex later in life (Drummond et al. 2008; Wallien and Cohen-Kettenis 2008; Singh 2012). In adulthood, the majority of natal females with GD reports a *gynephilic* sexual orientation (sexually attracted to females), whereas natal males with GD are about evenly sexually attracted to males and females (Lawrence 2010; Nieder et al. 2011). In three of our studies, CHAPTER 2, 5 and 7 we noted that sexual orientation presented a confounding factor for the interpretation of our findings. The sex-atypical hypothalamic response to androstadienone (CHAPTER 5), the more male-typical pattern of brain activation during the mental rotation task in girls with GD (CHAPTER 7), and the feminized CEOAE response amplitudes in boys with GD (CHAPTER 2) may all reflect their cross-gender identity. However, the findings could just as well reflect their (disposition for a future) homosexual orientation, because all of these sex-specific characteristics have similarly been shown to vary as a function of sexual orientation (Loehlin and McFadden 2003; Savic et al. 2005; Berglund et al. 2006; Peters et al. 2007). Therefore, in order to disentangle the effects of gender identity and sexual orientation on sex-specific cognitive/(neuro-) biological functions, future studies should either include homosexual, non-gender dysphoric control groups, or compare groups of adult *gynephilic* males with GD to adult *androphilic* males with GD. The former comparison may be hard to manage in pediatric populations like in our studies, because sexual orientation is

difficult, though not entirely impossible to determine in childhood and early adolescence. However, the latter comparison would be an interesting option in order to elucidate the contribution of sexual orientation and gender identity to sex-specific brain functioning, at least in adults. Due to very small numbers of androphilic natal women with GD, comparative studies on the potential effects of sexual orientation on neuro-biological functioning in natal females with GD are difficult to conduct. To the best of our knowledge, no study to date directly compared individuals with GD who are sexually attracted to the same natal sex to individuals with GD, sexually attracted to the other natal sex. However, Savic and colleagues (Berglund et al. 2008; Savic and Arver 2011), seeking to avoid confounding homosexuality and gender identity, conducted a few neuroimaging studies including gynephilic, thus non-homosexual adult men with GD and compared them to heterosexual male and female controls. With regard to brain morphology and subcortical volumetric measures they found no evidence for any sex-atypical brain sexual differentiation (Savic and Arver 2011). This result is in contrast to studies including androphilic men with GD, which suggested that measures of white matter microstructure (Rametti, Carrillo, Gomez-Gil, Junque, Zubiaurre-Elorza, et al. 2011), cortical thickness (Zubiaurre-Elorza et al. 2012), and gray matter volumes (Simon et al. 2013) were (partially) feminized, thus, were comparable to controls sharing their gender identity. Interestingly, Savic et al. (Berglund, Lindström, Dhejne-Helmy, & Savic, 2008), in a functional neuroimaging study similar to our olfactory fMRI experiment (CHAPTER 5), tested the hypothalamic response to two potential chemo-signals androstadienone and *estra-1,3,5(10),16-tetraen-3-ol* in a group of treatment-naïve gynephilic men with GD. The authors observed that men with GD shared features of the response pattern to the steroid odors with both control men and women, but since their hypothalamic response to androstadienone differed significantly from that of the control men, the authors concluded that gynephilic men with GD showed more female-typical brain activation. Thus, although responsiveness to chemo-signals may, intuitively, be more likely associated with sexual orientation and sexual attraction, hypothalamic responsiveness to androstadienone seems to reflect gender identity as well. Taken together, the findings of the functional imaging studies (Berglund et al. 2008 and CHAPTER 5 of this thesis) suggest that natal males with GD, regardless of their sexual orientation, possess certain female-typical brain functions, whereas indications for an atypical sexual differentiation of brain structures are, to date, only confirmed for andro-

philic natal men with GD. These preliminary findings certainly warrant future replication in order to provide conclusive evidence for the question which sex-specific functional and structural brain characteristics vary as a function of gender identity, sexual orientation, or both.

METHODOLOGICAL CONSIDERATIONS

ADRENARCHE - ANOTHER SENSITIVE PERIOD?

Our experimental approach was to investigate neurobiological sex differences in two age groups, i.e. prepubertal children (7–11 years of age) versus pubertal adolescents (14–17 years of age). We assumed that any sex differences found in prepubertal children should originate from organizational effects of pre-/perinatal sex hormones.

However, puberty consists of two components, *adrenarche* and *gonadarche*. Whereas gonadarche starts around the age of 9 in girls and around age 11 in boys, adrenarche is a separate process that occurs during middle childhood, between 6 and 10 years of age. Adrenarche is the onset of adrenal androgen production, specifically *dehydroepiandrosterone* (DHEA) and its sulfate ester (DHEAS). The levels of DHEA(S) secreted during adrenarche are similar for boys and girls, but the onset of adrenal androgen production is considered to occur earlier in girls (around age 7) than in boys (around age 9). DHEA(S) is implicated in the development of pubic hair, sweat glands, and other somatic changes, such as a shift in glucose metabolism (Perrini et al. 2004; Goldstein and Kopin 2008), and increasing bone diameter (Remer et al. 2009). Moreover, adrenarche coincides with a period of important psychological development, characterized by significant changes in cognitive and affective functioning, and social behavior. Interestingly, the hormonal changes associated with adrenarche have been linked to the onset of sexual orientation development, forming the basis for the maturation of reproductive behavior and sexual attraction (Herdt and McClintock 2000; Del Giudice and Belsky 2010). DHEA(S) is assumed to play an important role in these brain maturational processes, and to mediate changes in social behavior and memory function, promoting identity consolidation (Campbell 2006). Of note, increasing levels of DHEA(S) promote the development of sebaceous and sweat glands, and therefore the development of body odors. The simultaneous appearance of axillary hair, enlarging the surface area for effective dispersion of odors by evaporation, suggests that body odor during

puberty becomes a relevant chemo-signal for social communication and may be linked to sexual development (Campbell 2011).

Given that these significant adrenal androgen-mediated processes partially overlap, but also partially precede gonadarche, an alternative interpretation of our findings of CHAPTER 5 may be considered. In that study, we demonstrated that a sex difference in hypothalamic responsiveness to the chemo-signal androstadienone, which is contained in axillary sweat, was already present in prepubertal children. We therefore concluded that this functional sex difference of the brain most likely evolved during early pre-/perinatal development. It is, however, conceivable that the hypothalamic response in these girls, who were between 7 and 10 years of age, reflected the effects of adrenarche, and thus the influence of DHEA(S) action on the brain, rather than a hard-wired gendered characteristic that developed pre-/perinatally. Markers for the onset of adrenarche, other than presence of pubic hair, such as changes in body odor and sweat production, or DHEA(S) levels in our participants were not (yet) available, although these would have been a relevant marker in the context of our chemo-signal experiment. However, inclusion criterion for participation was a prepubertal status, defined as Tanner stage 1. This means no glandular tissue, a small testicular volume and penis size, and absence of any pubic hair (Marshall and Tanner 1969, 1970). Therefore, levels of DHEA(S) in our study population were probably still low, limiting potential effects on the central processing of androstadienone.

We collected urine and saliva samples from all our study participants in order to assess sex hormone levels (testosterone, estradiol, LH, FSH, and DHEA(S)) that may be associated with any of our functional or structural brain characteristics. However, by the time of writing this thesis, the hormone assessments were not yet available. We plan to conduct follow-up correlation analysis between DHEA(S) levels in our prepubertal participants and their hypothalamic response to androstadienone.

Given the effects of DHEA(S) on brain development and the association of adrenarche with the formation of a sexual identity, it is not unlikely that these effects are sex-specific, and also impact gender identity development. The prepubertal girls with GD in our study showed no clear-cut female-typical or male-typical hypothalamic activation, indicating greater variability in their responsiveness to androstadienone. Although highly speculative, if adrenal androgen functioning would be implicated in the responsiveness to

androstadienone, this would suggest that adrenarche-linked maturational processes may be associated with the atypical gender identity development in the girls with GD. Adrenarche may thus form another sensitive period of brain sexual differentiation, preceding and interacting with the organizational effects of gonadal sex hormones, being also a new target period for the (potentially atypical) development of gender identity.

CLINICAL IMPLICATIONS & DIRECTIONS FOR FUTURE RESEARCH

THE STUDIES CONDUCTED and presented in this thesis support the existing literature on functional and structural sex differences of the brain, and provide new insights on potential neurobiological markers for the normative and atypical sexual differentiation of the brain and behavior. Yet, many clinically and scientifically relevant questions regarding gender identity development and GD remain to be answered. Three major goals for future research can be formulated as follows:

Identification and further characterization of the neurobiological and (social) environmental factors, implicated in the etiology of GD.

Evaluating whether suppressing puberty has any (harmful) effects on adolescent brain development.

Identification of determinants that predict the persistence of childhood GD into adolescence and adulthood.

As mentioned under the section *Organizational Effects of Sex Hormones during Puberty*, adolescence is a period of significant neuro-behavioral changes. We have shown that hypothalamic responsiveness and visuo-spatial cognitive functions of gender dysphoric adolescents, whose puberty is suppressed, are in accordance with their experienced gender, rather than their natal sex. The only structural MRI measure we investigated yet, white matter microstructure, suggested slight feminization in the natal boys and hardly any masculinization of fiber organization in the natal girls. However, drawing conclusions on the association between functional and structural sex-specific features of the brains of individuals with GD would be premature at this point. Future studies in adolescents with GD, which are in preparation, should elaborate our preliminary findings and further characterize the sex-specific neurodevelopmental changes, addressing other structural brain characteristics, such as gray and white matter densities, cortical thickness, or functional and structural brain connectivity measures.

Given the significant reorganization of neuronal systems regulating executive control, affect, and social cognition during adolescence, an intriguing but still open question is whether the suppression of sex hormone action in adolescents with GD, during years of significant and steroid-dependent neurodevelopmental changes, may have any delaying, or adverse effects on cognitive and social-emotional maturation. We have also acquired data of an emotional face processing fMRI paradigm, and will investigate the effects of endogenous sex hormones, GnRHa, and CSH treatment on the social-emotional functioning in young individuals with GD.

Regarding the maturation of social-emotional behavior in gender dysphoric children and adolescents, the recently noted co-occurrence of GD and disorders from the autistic spectrum is remarkable (de Vries et al. 2010; Bejerot et al. 2011; Lemaire et al. 2013). Both conditions are suggested to reflect aberrant endocrine pre-/perinatal developments (Whitehouse et al. 2010; Baron-Cohen et al. 2011; Cheslack-Postava and Jordan-Young 2012) and show atypical sex-specific developmental trajectories during childhood and adolescence (Auyeung et al. 2013). GD and autism spectrum disorders may even share certain neuronal correlates (Beacher et al. 2012; Lai et al. 2013) and may therefore share some common neurodevelopmental pathway. Future research on the sex-specific markers and the neurodevelopmental aspects concerning social-emotional and affective functioning in both GD and autism should ultimately enhance our knowledge on the multitude of factors involved in the sexual differentiation of the brain, behavior and gender identity.

An intriguing, though still unanswered question concerns the possibly harmful effects of GnRHa administrations on adolescent brain development during a critical developmental period. Future prospective (neuroimaging) studies should therefore follow adolescents with GD throughout the different phases of their hormonal treatment trajectory. Studies need to evaluate whether adolescents with GD, compared with their non-GD peers, show any delay with regard to brain maturation and associated cognitive functioning, and whether they catch-up later, as soon as the CSH treatment is started.

Many of the gender dysphoric adolescents and parents whose child, diagnosed with GD, participated in the MRI experiments, asked me whether their brain scan could prove that they indeed had an *opposite sex brain*. They were hoping for a clearly visible brain marker, a *gender-gyrus* reflecting their experi-

enced gender that would explain their extreme feelings of incongruence with their natal sex.

Unfortunately, we are far from using MRI scans as an aid in the diagnosis and prediction of the course of GD. We can only make inferences based on mean differences between groups, given a certain likelihood of that difference.

For the clinician giving advice and counseling, as well as for the parents rearing their gender dysphoric child, it is of great importance to know as to whether the gender dysphoric feelings of a particular child are likely to persist into adolescence. Qualitative (Steensma et al. 2011), as well as quantitative (Steensma et al. 2013) follow-up studies of childhood GD cases suggest that factors such as 1) relatively more cross-gender behavior, 2) a higher intensity of GD, and 3) the cognitive gender labeling of the child of being the *other* sex, rather than expressing the wish to have the body of the *other* sex, are the strongest predictors for persistence of GD into adolescence. These studies further revealed that the period between 10 and 13 years of age, during which physical changes affect psychological factors, such as self-confidence, body image, and sensitivity for peer group acceptance, was perceived as decisive for the persistence of the feelings of GD into adolescence. This suggests that early puberty is a crucial phase for the consolidation of gender identity. Our neurobiological findings confirm the significant differences between prepubertal and adolescent individuals with GD on functional and structural sexually dimorphic neurobiological markers.

Our findings provide preliminary evidence for sex-atypical neurobiological characteristics in gender dysphoric children and adolescents. One of the challenges of future research is to further understand the exact timing and independent contributions of the postnatal sensitive periods and environmental factors to the sexual differentiation of the brain during childhood and adolescence. Given the recent converging developments in the fields of machine learning and neuroimaging, new analysis techniques may have diagnostic and predictive value (Demirci et al. 2008), and may be applied to decode information such as structural brain sex differences (Wang et al. 2012; Feis et al. 2013). The ultimate future goal would be a multimodal integrative approach, in which different functional and structural neurobiological features together with clinical/diagnostic information are used for the identification of GD biomarkers and the prediction of its course on an individual basis.