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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.

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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 GnRHα. 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.

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 treatment-naïve children & adolescents with GD, by means of CEOAES recordings.

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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.

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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*

