1. SUMMARY

1.1. Background

Evidence from animal studies seems to indicate that the prenatal hormonal milieu, as a consequence of intrauterine positioning, is associated with the expression of sexual dimorphisms in morphology and behavior. This has been explained in terms of the diffusion of androgens, mainly testosterone, among womb mates. An equivalent of this phenomenon can be found in humans, in opposite-sex twins. The females of female-male twin pairs may be exposed to higher levels of testosterone *in utero* than female same-sex twins and thus provide a natural model to investigate the role of early testosterone in the development of sex differences. Well-documented animal studies provide a good basis for this assumption (e.g., Ryan and Vandenbergh, 2002; Vom Saal, 1989).

In the present dissertation, we attempted to study the influence of testosterone on the development of sex-typed behavior by using this opposite-sex twin paradigm. We first examined whether the sex of the fetus is associated with maternal serum steroid levels (chapter 2). In chapters 3 and 4 we presented two studies on the effects of prenatal exposure to testosterone in children aged 10 years, and in chapter 5 the results of a study of the same children when they were 13 years old. In chapter 6 we evaluated another indirect method of investigating the effects of prenatal exposure to testosterone, namely, the finger ratio in our twin sample, and also tested whether the finger ratio is associated with specific characteristics. In chapter 7 we explained the results on educational achievement in terms of the twin paradigm. The results are summarized in the following sections.

1.2. Are maternal serum steroid levels related to sex of the fetuses in twin pregnancies?

Although prenatal exposure to testosterone probably occurs, there are only limited data demonstrating the transfer of testosterone in human multiple pregnancies. In pregnancy, hormones from one fetus may reach another fetus via transmembrane transport (throughout pregnancy) or via the maternal-fetal circulation (Meulenberg and Hofman, 1991). In this study, we were interested in whether maternal serum steroid levels are affected by the sex of the twins the mother is carrying. Meulenberg and Hofman (1991) and Klinga et al. (1978) were the only investigators to show a difference in maternal testosterone levels during gestation between mothers pregnant with a male child and those pregnant with a female child, i.e. maternal testosterone levels were higher in boy pregnancies than in girl pregnancies. However, the pregnancies were singleton pregnancies, and there have been no studies of twin pregnancies. We hypothesized that if maternal serum steroid levels depend partly on the sex of the fetus, mothers pregnant with two boys would have different levels of steroids in their serum than mothers pregnant with opposite-sex twins or female-female twins.

We did not find an effect of the sex of the fetus on the serum steroid levels of mothers pregnant with twins. Possible explanations are that hormone concentrations were not measured at an appropriate moment or that the sex of the fetus does not influence maternal serum steroid levels. The latter is in accordance with many studies in singletons (e.g. Nagamani et al., 1979; Rodeck et al., 1985). Although another possibility for the nonfinding could be because of a sample size effect – the sub-group of male-male twins was (unexpectedly) rather small. If these (non-significant) results are replicated in a larger group, it may be that an increased prenatal exposure to testosterone in OS female fetuses occurs mainly through the direct feto-fetal route of hormone transfer in the amniotic sac rather than via the indirect maternal-fetal route. It has been shown rather convincingly in several studies (e.g., Nagamani et al., 1979; Rodeck et al., 1985; Van de Beek et al., 2004) that the levels of testosterone in amniotic fluid are higher with a male fetus than with a female fetus, and animal research has clearly shown that testosterone is transported across fetal membranes (e.g., Even et al., 1992).

1.3. Prenatal testosterone and cerebral lateralization

A further goal was to investigate whether prepubertal, 10-year-old opposite-sex (OS) twin girls as compared to same-sex (SS) twin girls, show a pattern of hemispheric specialization different from the typical female pattern as a result of their possible prenatal exposure to higher levels of testosterone due to fetal hormone transfer (the twin paradigm). To this end, we compared the performance of these two groups (only right-handed subjects) on a dichotic listening task (DLT; Bouma, 1998), which provided us with an indirect measure of functional cerebral lateralization.

The results indicated that the 10-year-old OS twin girls indeed showed a less feminine and more masculine pattern of functional cerebral lateralization, i.e. hemispheric specialization, than the SS twin girls. Their functional cerebral asymmetry, as reflected by a right-ear superiority (i.e. left-hemispheric dominance) when processing verbal-auditory stimuli, was greater than in SS twin girls. Differences between OS and SS twin girls in the extent of prenatal exposure to androgens could at least partly be responsible for these findings, given that prenatal testosterone has shown to influence brain organization and postnatal sex-dimorphic behavior (McGlone, 1980; Geschwind and Galaburda, 1987; Wisniewski, 1998).

A different line of research indicates that differences in performance on cerebral lateralization tasks can be attributed to current levels of sex hormones, which exert an activating effect (Sanders and Wenmoth, 1998). However, the current findings do not seem to be caused by differences in circulating levels of testosterone between the groups because testosterone levels were not correlated with functional cerebral laterality scores in our study. Our results showed not only that girls had higher testosterone levels than boys of similar age, a finding that has been reported before (Granger et al., 1999), but also, and more importantly for the interpretation of the current results, that there were no differences in testosterone levels between OS and SS twin girls. In other words, the difference in lateralization performance between OS and SS twin girls, as shown by the results on the DLT, could not be attributed to differences in the circulating level of testosterone between the two groups of girls. It seems therefore more likely that if testosterone influences lateralization performance, it exerts its effect prenatally.

However, when this measure was re-evaluated when the twins were 13 years old, the difference between the OS and SS girls had disappeared. There are two possible explanations for this. First, the sample may have become too small to detect such differences because fewer girls participated in this evaluation than in the evaluation when they were 10 years old. Statistical power analysis indeed confirmed our idea that the sample was too small to enable us to detect significant differences. Secondly, the prenatal effect may have actually faded with time or have been overshadowed by postnatal experiential influences.

1.4. Is there an organizational or activational effect of testosterone on verbal skills and spatial ability?

Clearly, the sexes differ on cognitive abilities in adulthood, with males excelling on spatial aspects such as mental rotation (e.g., Voyer et al., 1995) and females performing better on verbal tasks such as verbal fluency (Halpern, 2000; Hyde and Linn, 1988). The question is whether these sex differences appear in a young age as a consequence of prenatal sex hormones or whether they are dependent on the surge of sex hormones during puberty? To investigate this, we studied the performance of 10-year-old children on two spatial tasks and two verbal tasks. The results showed that on one task in each domain (i.e. spatial and verbal) the boys differed from the girls in the expected direction. We then investigated whether these differences were a result of prenatal exposure to testosterone. Although the OS females and SS females had a similar performance on these two tasks (the spatial and verbal tasks that showed sex differences), on the RAVLT long-term verbal memory the performance of the OS girls was worse than that of the SS girls. Because in these girls circulating testosterone levels did not correlate with test performance, we concluded that the difference was not caused by an activational effect of circulating testosterone. We hypothesized that the poorer, i.e. more male-typical, long-term verbal memory of the OS girls was caused by the effect of prenatal exposure to testosterone on brain structures involved in verbal memory. This is in line with our main idea that prenatal testosterone causes fetuses to become more male typical, possibly by affecting specific structures by stimulating neuronal proliferation and growth during critical periods of brain development (Goy and McEwen, 1980).

1.5. Is there an effect of prenatal testosterone on aggression and other behavioral traits?

In this study, we investigated whether sex differences in aggression, sensation seeking, and temperament are influenced by testosterone. The influence of testosterone on these behavioral traits could be exerted prenatally, although activational effects of circulating testosterone have been shown to be of a major importance. The latter is especially relevant because the hormone levels in the 13-year-old twins have changed as a result of puberty.

Whereas 10-year-old girls had higher testosterone levels than 10-year-old boys, at 13 years boys had overall higher circulating testosterone levels than the girls, as expected at this age. In both boys and girls, testosterone levels were higher in the morning than in the afternoon.

One study using the twin paradigm investigated the behavioral trait of sensation seeking (Resnick et al., 1993). In that study, female OS twins showed more sensation-seeking behavior, having higher scores on the total SSS scale and Disinhibition and Experience Seeking subscales than female SS twins. The authors concluded that this may have been due to prenatal hormones. We were unable to replicate these results, possibly because our subjects were younger and our sample was smaller (although the other study had a smaller OS group). However, we did find that the two groups of girls differed on the subscale Experience Seeking and showed a tendency to differ on the subscale Boredom Susceptibility, with SS girls having higher scores. We cannot explain these differences because normally these scales do not show any sex differences.

Although the other investigated traits (aggression and temperament) have been shown to be associated with circulating testosterone levels, similar studies using the twin paradigm have not yet been performed. We did not find SS and OS girls to differ with respect to temperamental behavior. However, one of the questionnaires used showed the OS girls to have more male-like aggression scores than the SS girls. This concerned an overt type of aggression, i.e. aggression proneness, whereas a measure of covert aggression did not reveal differences between the OS and SS girls. Thus the choice of behavioral trait and measurement method investigated appears to be important for detecting possible behavioral effects of prenatal exposure to testosterone. Other factors, such as circulating free testosterone levels, menarchal status, and pubertal stage, did not play a role in the outcome of our study.

1.6. Is the finger pattern masculinized in female opposite-sex twins?

Besides the use of atypical groups (e.g., girls with congenital adrenal hyperplasia (CAH)) and OS twins, and typical (prospective) studies to investigate the possible role of testosterone in the development of sex differences, there is also a fairly new line of investigation that involves biomarkers such as the finger ratio, i.e. the relative length of the second digit to the fourth digit (2D:4D). Researchers claim that the 2D:4D ratio is influenced by the effect of prenatal sex hormones up till week 14 of gestation, which is about the same period that is critical for the development of the brain. As the finger ratio and the twin paradigm are both considered to reflect the prenatal influence of hormones, we hypothesized that the two measures may be associated. Therefore we investigated whether OS girls had a more masculine finger pattern, i.e. lower finger ratio, than SS girls, but found this not to be the case. Possibly because the differences in prenatal exposure to testosterone were too low to affect finger pattern, or indeed no masculinizing effect on finger pattern exists. Still, there seems to be an association between finger ratio and testosterone in girls with CAH, because the finger ratio was lower in girls with CAH than in girls without CAH (Brown et al., 2002b; Ökten et al., 2002), although one study failed to detect significant differences between the two groups of girls (Buck et al., 2003) but this could be attributed to external factors.

1.7. Educational achievement in a national sample of Dutch female twins and female singletons

This study was designed to look for effects of prenatal exposure to hormones on educational achievement, by comparing the educational performance of OS and SS girls, and their matched singleton female classmates with an older brother or sister.

There was no difference between OS females and SS females in terms of educational achievement. However, there were large differences between the twin group and the singleton group. Our singleton girls performed not only better than our twin girls but also than the total Dutch female population. This may be because of bias, because schoolteachers selected the singleton classmates of the twin girls and may have unconsciously (or consciously) selected the cleverer girls. Importantly, we were unable to show an effect of prenatal testosterone in OS female twins, and we also did not detect a difference between singleton girls with an older brother and singleton girls with an older sister. This suggests that the "maleness" or "femaleness" of the environment in which a girl grows up does not affect her academic performance. Overall, this may strengthen the idea that postnatal environment could be of less importance to the development of sex differences.

2. IN CONCLUSION

Much research concerns the understanding of the behavioral consequences of prenatal exposure to sex hormones in humans. There is increasing evidence, obtained with different methods, that prenatal exposure to androgens has a masculinizing effect on the female offspring, especially at high doses of androgens and especially for sex-typed interests, spatial ability, and aspects of personality. Although it seems likely that androgens are responsible for some of the differences between the sexes in these traits, it is not clear how much they contribute to variations within males and within females. There is a need to continue to develop and validate methods for assessing these effects in typical samples, e.g., the twin paradigm.

The results of most studies using the twin paradigm have not been replicated by other investigators, and in many cases the studies have focused on only a few behavioral traits. The studies described in this thesis extend a line of research that has used OS twins to examine the role of prenatal exposure to testosterone in the development of sexual dimorphic behavior. This type of research offers a natural experimental setting to investigate the prenatal influence of hormones on sex differences. Although our research findings are mostly negative, carefully conducted studies, even when the results are negative, contribute to our understanding of the processes and causes behind sexual differentiation.