The early pubertal brain: work in progress

A study on genetic and hormonal influences

Het brein in de vroege puberteit: werk in uitvoering

Een studie naar genetische en hormonale invloeden

(met een samenvatting in het Nederlands)

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TABLE OF CONTENTS

Chapter 1

General introduction

General introduction

Puberty (Latin for *pubes*, meaning hair) is an important period during development and hallmarks the biological transition from a non-reproductive state into a reproductive state (Nussey and Whitehead, 2001). Puberty partly overlaps with the adolescent period and both terms are often used interchangeably. However, adolescence also refers to the period of drastic cognitive and emotional development (Sisk and Zehr, 2005). Children entering puberty and adolescence rapidly advance in abstract reasoning, focusing of attention, response inhibition (or cognitive control) and goal-directed behavior (from reviews of Spear, 2000; Casey et al., 2005; Yurgelun-Todd, 2007). Furthermore, they show development of risk evaluation (Crone and Van der Molen, 2007) and develop complex social skills like understanding others' emotions and mental states (Blakemore, 2008). Research currently attempts to relate these functional changes to underlying neural substrates (for reviews see Paus, 2005; Durston and Casey, 2006).

The timing and speed of developmental processes during puberty might be of critical importance to optimal adult functioning. Indeed, diseases that affect the brain at a young age, such as schizophrenia, are likely to have their first symptoms in this period (Andreasen, 1995; Kessler et al., 2005), although there is a wide range in age of onset (Rapoport et al., 2005). It has been argued that in schizophrenia, there could be a "developmental arrest" of functions that should be developing during adolescence (e.g. executive functioning), resulting from pathological processes at various developmental stages (Pantelis et al., 2005). Yet, before hypotheses about 'abnormal' maturation of clinical populations can be tested, it is required to study normative development in healthy (pubertal) individuals (Luna and Sweeney, 2001).

In this chapter, an overview will be presented discussing a number of aspects associated with healthy pubertal development. First, the endocrinological changes during puberty are discussed. Second, literature on pediatric and adolescent brain development will be reviewed. Third and finally, possible genetic and hormonal

mechanisms associated with human brain development during puberty will be introduced which will be the main topic of this thesis.

1.1 Hormonal and secondary sexual aspects of puberty

Human puberty is typified by the (re)activation of the hypothalamic-pituitary-gonadal (HPG) axis. It is called *re*activation, since prior to the relative quiescence during childhood, the HPG-axis is also active around birth (Grumbach, 2002). At the onset of puberty the hypothalamus starts to produce gonado-releasing hormone (GnRH) in a pulsatile manner. GnRH then signals the pituitary gland to produce both luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are called gonadotropins and in males stimulate the testis to produce the androgen testosterone. Specifically, LH increases production of testosterone by Leydig cells in the testes. FSH in conjunction with testosterone is required for spermatogenesis in the course of puberty. In females, gonadotropins stimulate the ovaries to produce the estrogen estradiol. LH is predominantly responsible for ovulation, while FSH develops maturing follicles (Grumbach and Styne, 2003). The gonadal or sex steroids testosterone and estradiol act on GnRH via a negative feedback mechanism (**Figure 1.1**).

The production of sex steroids results in the maturation of sex organs and cause secondary sexual characteristics. Secondary sexual characteristics in girls and boys can be divided into different categories devised by James Mourilyan Tanner (Tanner, 1962). In girls, breast development is induced by estradiol and is called ‘thelarche’. Thelarche is usually followed by pubic hair development (Note that 15% of girls show pubic hair development before the onset of breast development (Tanner and Davies, 1985)). Growth of pubic hair in girls is called ‘pubarche’ and is initiated by testosterone that is secreted by the adrenal glands (maturation of adrenal gland, i.e. ’adrenarche’) (Grumbach and Styne, 2003). Menstruation usually occurs within 2 years of thelarche and is called ‘menarche’. In boys, the first secondary sexual characteristic is testicular enlargement (together with penis growth this is called ‘gonadarche’), induced by increased levels of testosterone. Gonadarche is followed by pubic hair growth.

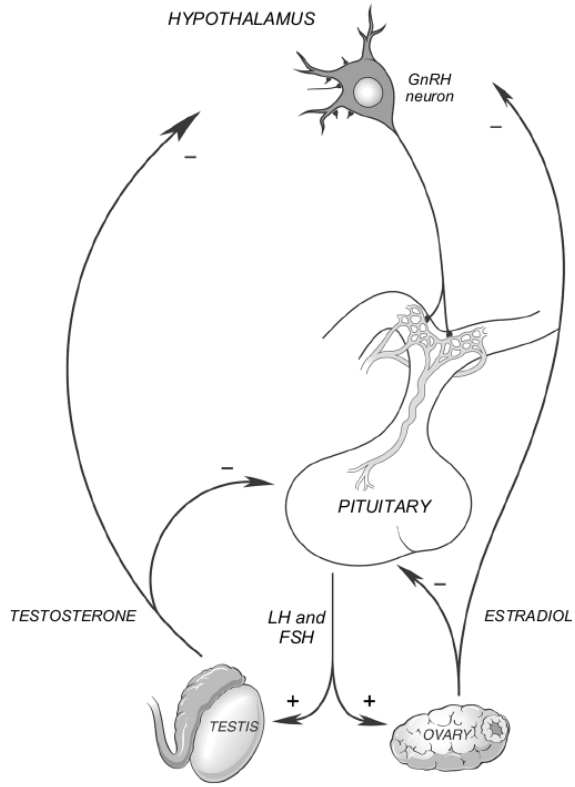

Figure 1.1 Hypothalamus Pituitary Gonadal (HPG)-axis
The reproductive axis: interactions between the hypothalamus, pituitary gland and gonads (testes and ovaries). Interrelationships between hormones are depicted as stimulatory (+) or inhibitory (-) (Adapted from Cameron (2004)).

Within both sexes, part of testosterone is converted via the enzyme aromatase into estradiol. Estrogens in boys and girls have been associated with the pubertal growth spurt and assumed to be mediated by growth hormone (GH) and insulin growth factor-1 (IGF-1) (Clark and Rogol, 1996).

Although exact estimates of puberty onset vary between countries, it is generally assumed that girls show pubertal signs at an earlier age than boys. In the Netherlands, the average onset of secondary sexual characteristics in girls is 10.7

years (SD 2.3 years), and 11.5 years (SD 1.8 years) in boys (Mul et al., 2001). Typically, the production of pubertal hormones precedes the development of secondary sexual characteristics by 1 to 2 years. GnRH levels are almost impossible to detect (it has a short half-life estimated at 4-8 minutes (Redding et al., 1973)), and it is only measurable directly from the portal venous system in the pituitary (Delemarre-Van de Waal, 2002). Therefore, its following nocturnal LH production is usually the first endocrinological marker for the onset of puberty in both boys and girls (Delemarre-Van de Waal et al., 1991; Demir et al., 1996). With the progression of puberty, LH secretion gradually increases during both day and night (Delemarre-Van de Waal, 1991).

There is ongoing debate on the mechanisms that trigger reactivation of the HPG-axis related to the onset of puberty (e.g. Grumbach, 2002; Ojeda et al., 2006a; Banerjee and Clayton, 2007). However, evidence is accumulating that the KiSS1-gene and its peptide-product kisspeptine play an important role in the initiation of puberty: together with the G-protein-coupled receptor 54 (GPR54) they have been involved in the molecular mechanisms underlying the reactivating of GnRH neurons at puberty (for review see Smith and Clarke, 2007). In addition, levels of the main adipose derived hormone leptin, which plays a key role in energy intake and appetite, have been found to rise preceding the onset of puberty (Mantzoros et al., 1997; review by Kaplowitz, 2008). Interestingly, evidence has been provided that leptin is able to modulate activity of KiSS-1 neurons (Smith et al., 2006; Morelli et al., 2008).

In addition to preprogrammed genetic mechanisms, external factors such as psychological stress, influence (the maturation process of) the HPG axis. Stress-related activity of the hypothalamus-pituitary-adrenal (HPA) axis activity interacts with HPG-axis activity (for review see Viau, 2002). For example, corticotrophin releasing hormone (CRH) which is secreted from the hypothalamus in response to stress can inhibit the firing of GnRH neurons, which in turn suppress reproductive hormone secretion (Olster and Ferin, 1987; Feng et al., 1991).

1.2 Brain morphology during childhood and adolescence

Most likely related to the remarkable physical and behavioral changes during the pubertal and adolescent period, are the dynamic changes in brain structure during this critical period of life.

At birth, the healthy human brain is not completed. A widely held misconception is the fact that the total number of neurons (i.e. gray matter, together with dendrites) does not increase anymore after birth. There is now accumulating evidence that neurogenesis in fact takes place well into adulthood within a number of brain regions, including the hippocampus, striatum and cerebral cortex (Gould, 2007; Zhao et al., 2008). A brain area of particular interest is the prefrontal cortex. For example, research has shown that although synaptic density within most cortical areas reaches a maximum at around 3.5 years after birth, pruning (i.e., reduction in the overshoot of unconnected or "unused" neurons) in especially the prefrontal cortex continues well into adolescence (Huttenlocher, 1979; Huttenlocher, 1990). Furthermore, studies suggest that myelination of axons (i.e. white matter) takes place into adolescence and adulthood in a posterior-to-anterior regional manner (Yakovlev and Lecours, 1967). These above mentioned studies are based on post-mortem data. However, with the development of structural brain imaging techniques such as Magnetic Resonance Imaging (MRI), it is now possible to study human brain structure in vivo. Unlike imaging techniques as computed tomography (CT) or positron emission tomography (PET), MRI is not based on invasive procedures involving ionizing radiation, but makes use of large magnetic fields and radio waves. This is a safe and painless way to non-invasively obtain neuroanatomical information. Particularly important in the current context is the fact that MRI does not pose any ethical problems with measuring healthy volunteers -of all ages, including children- repeatedly over time.

Using volumetric MRI, global brain volumes including total gray and white matter can be quantified. Furthermore, regional estimates of brain areas can be obtained with hand-segmentation of regions of interest (ROI), voxel-based morphometry (VBM) or by measuring cortical thickness. VBM is a segmentation technique which measures the relative gray or white matter concentration ('density') within a

voxel (i.e., a three-dimensional volume element). As opposed to the time consuming 'hand' segmentation approach VBM can be applied to investigate the whole brain in an unbiased way.

Although MRI-data suggest that at the age of 6, total cerebral volume has already reached 95% of its total adult volume (Giedd et al., 1999), the composition of gray and white matter within the cortex is still undergoing rapid changes. Specifically, cross-sectional studies over the course of childhood and adolescence have reported a consistent increase in global white matter and a decrease in global gray matter as a function of age (Jernigan et al., 1991; Caviness et al; 1996; Giedd et al., 1996; Paus et al., 1999; Sowell et al., 1999; Thompson et al., 2000). Moreover, between 7 and 16 years, the overall rate of gray matter decrease is slower than the increase of white matter (Sowell et al., 2002). Longitudinal studies demonstrated a region specific decrease of gray matter within the cortical lobes (Giedd et al., 1999; Gogtay et al, 2004, Hua et al., 2007; Lenroot et al., 2007a). For example, frontal and parietal gray matter was found to decrease faster than temporal and occipital gray matter (Giedd et al., 1999; Lenroot et al., 2007a). In addition, cortical thinning was reported in several regions of the cerebral cortex (Sowell et al., 2003). Interestingly, cortical growth was found to be distinct across the cortex in relation to their complexity of cortical layers: i.e. limbic areas with simpler laminar architecture showed simpler growth trajectories as opposed to cortical areas with a more complex laminar architecture (Shaw et al., 2008). Using VBM, regional patterns of gray matter density decreases (i.e. in frontal and parietal areas (Sowell et al., 2001) and white matter density increases were reported over the course of childhood and adolescence (Sowell et al., 2001; Wilke and Holland, 2003; Wilke et al., 2007). Diffusion tensor imaging (DTI) is a relatively new technique which gives information on orientation and/or coherence of white matter tracts. DTI measures fractional anisotropy (FA), of which higher values are suggested to be a measure of higher integrity of white matter bundles. Studies using DTI in children and adolescents reported increased FA values with age within the corpus callosum, frontal cortex (Barnea-Goraly et al., 2005; Giorgio et al., 2008), basal ganglia, thalamic pathways and internal capsule (Barnea-Goraly et al., 2005).

1.3 Possible mechanisms underlying brain structure in puberty

1.3.1 Genetic and environmental influences

As argued in the previous paragraphs the brain is still 'under construction' during puberty. It would be of particular interest and importance to explore the genetic and environmental contributions underlying individual differences in brain volumes and morphmetry during this period.

Roughly, one can distinguish two main sources of variance contributing to inter-individual differences of an observed phenotype (i.e. trait): genetic and environmental variance. If genes influence a certain phenotype, then variation in those genes can introduce variation in that specific phenotype. For example, genetic variance can be caused by genetic polymorphisms, such as naturally occurring variations in the sequence of genetic information on a segment of DNA among individuals. Such variants, if they occur within gene loci, are called alleles. Genetic effects can be additive (effects of different alleles 'add up'), dominant (interaction of alleles at a locus,) or epistatic (interaction between alleles at different loci) (Plomin et al., 2001). The other main source of variance in a phenotype is caused by environmental factors. These include for instance prenatal events, non-genetic biological events after birth, but also parenting, nutrition and infections.

To measure the contribution of genetic and environmental influences to variance of a certain trait, genetically related individuals need to be studied. If genetic factors influence a trait, then the phenotypic resemblance of relatives should increase with increasing degree of genetic resemblance. The statistical approach to analyzing genetic influences through family-, adoption -and twin designs is called genetic epidemiology, or behavior genetics, and is founded in the theory of quantitative genetics (e.g. Falconer, 1989). However, the observation that family members show a high resemblance for a certain trait does not necessarily mean that this trait is heritable: family members also share their family environment which can make them more alike.

The twin design

A commonly used design to disentangle genetic influences and (shared) environmental influences is the classical twin-design. In this design, the resemblance of monozygotic (MZ, identical) twin pairs is compared to the resemblance of dizygotic (DZ, fraternal) twin pairs (Boomsma et al., 2002). MZ twins develop from one zygote and share (almost) 100% of their genes, whereas DZ twins derive from two zygotes and share on average only half of their segregating genes (like singleton brothers and sisters) (Martin et al., 1997). Both types of twins are usually reared together and consequently share their family environment as well. Examples of shared environmental factors are parental care, social economical status, pattern of nutrition. Both MZ and DZ twins also experience unique environmental influences, such as diseases, random life events and non-shared experiences. Thus, the difference in resemblance between MZ and DZ pairs (based on twin pair correlations: rMZ for the correlation between twins of MZ pairs and rDZ for the correlation between twins of DZ pairs) gives information about the relative influence of genes or shared/unique environment on variance of a certain trait. In brief, a higher resemblance in MZ than in DZ twin pairs (rMZ>rDZ), is indicative of genetic influences.

A complex trait like brain structure is likely to be influenced by multiple genes. To search for specific genes, a trait needs to be heritable. Heritability means the relative proportion of variance in a trait which is caused by additive and non-additive (dominant) genetic variance ("A" and "D"). If gene action is additive, a first estimate of heritability is obtained as: 2 (rMZ-rDZ). In case rMZ > 2 (rDZ), than non-additive genetic effects are suggested, which can be calculated as 2 (rMZ)-4 (rDZ). The influence of common environmental factors ("C") is suggested when rDZ>½ rMZ, and can be calculated as 2(rDZ)-(rMZ). The part of the variance that MZ twins do not resemble each other can be attributed to unique environmental factors ("E"), assessed with 1-(rMZ) These first estimates are known as "the Falconer method" (Falconer, 1989). Based on this principle, using cross-trait cross-twin correlations, it can be investigated whether the *association* between two traits, like IQ and brain volume, has a genetic origin. When the cross-correlation between IQ in twin 1 and brain volume in twin 2 is higher in MZ twins

than in DZ twins, this indicates that a common genetic factor influences IQ and brain volume. The amount of overlap is reflected by the genetic correlation (r_g). r_g gives the correlation between genetic factors influencing both phenotypes.

Structural equation modeling

Structural equation modeling (SEM) is a statistical technique which is progressively more used in twin studies to disentangle the relative importance of A, C and E on influencing individual variation of a certain trait or association between traits. In contrast to the Falconer method that merely calculates A, C and E from twin correlations, SEM allows researchers to explicitly test whether genetic or environmental factors contribute significantly to explaining individual differences. **Figure 1.2** shows a path diagram of a structural model in which an observed phenotype is influenced by A, C and E.

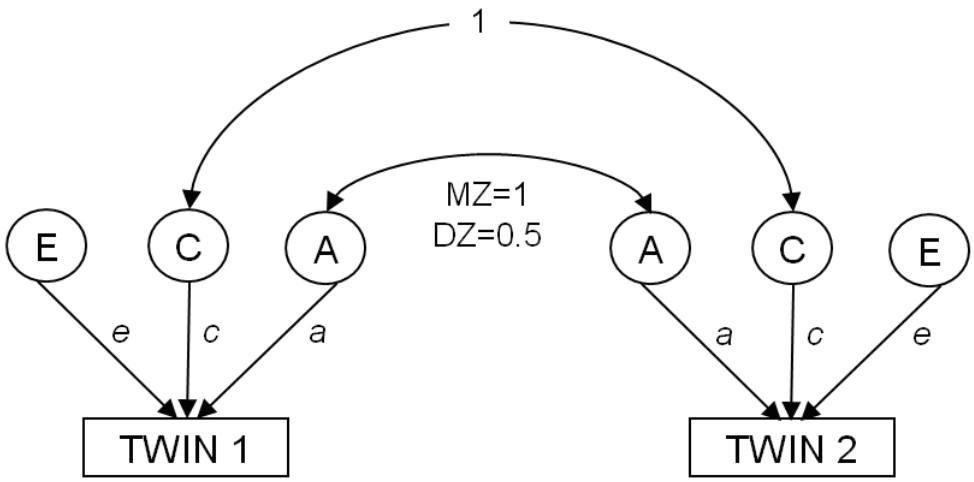

Figure 1.2 Univariate path diagram of the classical twin design
Phenotypes of both twins (twin1 and twin2) are influenced by additive genetic (A), common environmental (C) and unique environmental variance (E). The total variance within each twin is equal to A + C + E. The correlation between C_twin1 and C_twin2 is 1, the correlation between A_twin1 and A_twin2 is 1 for MZ, and 0.5 for DZ. Thus, the expected covariance between MZ twins is A + C, and the expected covariance between DZ twins is 0.5A + C.

When the observed variances and covariances differ only slightly from those predicted by the model, than the model will show a good fit according to χ^2-tests. The aim is to find the most parsimonious model with the least number of parameters that can most accurately describe the observed data. For example, the ACE model is compared to the AE-model that excludes common environmental factors, the CE-model that excludes additive genetic effects and the E- model that excludes all familial resemblance. The 'best' model is the model with the fewest parameters that does not fit significantly worse than the more complex model. From the maximum likelihood parameters of the various models, the heritability estimate is calculated as A/(A+C+E). The relative influences of C and E are estimated similarly. In genetically related individuals who are also raised together, influences of C and D (genetic dominance) can not be disentangled.

Using SEM, twin studies with adults (Baaré et al., 2001; Wright et al., 2002) as well as with the elderly (Pfefferbaum et al., 2000; 2004) have shown that individual variation in global brain volume is highly genetically determined. Heritability estimates that have been reported are above 80%. On the other hand, heritability estimates of regional brain areas can vary from a high heritability in the frontal cortex to moderate or low heritabilities within subcortical regions or areas surrounding the lateral ventricles (Hulshoff Pol et al., 2006a; Chapter 2 of this thesis). Over the course of childhood and adolescence, global brain volumes (Wallace et al., 2006; Schmitt et al., 2007) as well as cortical thickness in frontal, parietal and temporal areas (Lenroot et al. 2007b) were reported highly heritable.

However, at the specific period of puberty onset, the genetic contributions to individual differences human brain volumes are still unknown. Also, heritability of regional white matter has never been investigated. Importantly, as the expression of genes changes with age (Plomin et al., 1997, Lenroot et al., 2007b; Wallace et al., 2006; Hoekstra et al., 2007), research investigating the genetic contributions to brain development should be conducted in an age-restricted sample.

Apart from the genetic contributions, environmental factors shape brain structure also. It has, for instance, been shown that the exposure to certain activities, such as musical training (Bengtsson et al., 2005), motor training (Draganski et al., 2004) or

certain diets (Isaacs et al., 2008; Erickson et al., 2008) can influence brain structure.
Furthermore, being in an enriched environment (McNair et al., 2007) or early life stress (Bhansali et al., 2007) can alter gene-expression and influence brain morphology. These interactions demonstrate the complex interplay between genes and environment.
Puberty is a period during which a child's environment changes from parent-dependent to more self- or peer-dependent (Steinberg, 2005). As the developing brain exhibits increased plasticity during this period (Huttenlocher, 1979), it might be argued that the brain is particularly vulnerable to a shift in stimulating or activity-dependent environmental effects (Spear, 2000; Andersen, 2003).

1.3.2 Pubertal hormones and brain structure

The increased production of HPG-axis hormones LH and FSH or testosterone and estradiol at the onset of puberty could play a critical role in shaping the brain's morphology.
Beside their obvious implication in the development of sex organs, the brain is also a major target for the sex steroids testosterone and estradiol. Sex steroids can act on the brain via binding to receptors which are present throughout the whole brain, but receptor binding sites are especially dense in the hypothalamus, thalamus, amygdala and hippocampus (Simerly et al., 1990) and cerebral cortex (Finley & Kritzer, 1999; Simerly et al., 1990). Steroid receptors are associated with components within the cell-nucleus that can alter gene-expression (i.e. genomic action) (McEwen, 1984). Generally two types of hormonal action on the brain can be distinguished: a) organizational effects (i.e. steroids act on the central nervous system to organize neural pathways (irreversible effects)) and b) activational effects (hormonal stimulation act on neural pathways to activate certain behaviors (Goy and McEwen, 1980). Organizational effects are thought to take place during so-called 'sensitive periods' during development. This implies that only in a certain time window brain tissue is susceptible to changes by hormone exposure. These sensitive periods can vary across species, but in humans, such a sensitive period for

organizational effects of testosterone on brain structure is thought to be between week 8 and 24 of gestation (Collaer and Hines, 1995). Animal studies have shown that sex steroids are able to influence number of dendrites, soma size, neurogenesis and neurite outgrowth (reviewed by Romeo and McEwen, 2004). In addition, within the sensitive periods when testosterone is higher in developing males versus females, it has been suggested that testosterone exposure may be responsible for the development of sex-related brain differences. Indeed, animal research suggests that prenatal exposure to testosterone or testosterone converted to estradiol via the enzyme aromatase, leads to 'masculinization' of the brain (McEwen, 1984; Pilgrim and Hutchison, 1994; De Vries & Simerly, 2002). 'Feminization' is thought to occur in the absence of testicular hormones (MacLusky and Naftolin, 1981). The relation between LH or FSH and brain morphology is unclear, although it has been found that LH can cross the blood-brain barrier (Lukacs et al., 1995) and LH-receptors have been found in the brain (Lei et al., 1993).

Beside the prenatal period, fluctuations in hormonal levels at later stages of life might affect brain tissue as well (Pilgrim and Hutchison, 1994). Puberty, a period characterized by neural development, has been hypothesized to be another sensitive period for gonadal steroids to shape the brain (Romeo et al., 2003; Sisk and Zehr, 2005; Giedd et al., 2006; Blakemore, 2008). Animal studies have, for instance, shown that rats castrated before puberty have a greater number of androgen receptor cells in the amygdala than rats that have been castrated after puberty (Romeo et al., 2000). Also, during puberty pruning of dendrites and spines, in combination with axonal changes have been observed within the medial amygdala (Zehr et al., 2006; Cooke et al., 2007). In addition, androgen administration to pubertal rats induced an increase in neuronal spine density within the amygdala and hippocampus (Cunningham et al., 2007). In a first recent study in humans pubertal hormones were found to be associated with the sexually dimorphic amygdala and hippocampus (Neufang et al., 2008). However, due to the relatively small sample size of this study (15 boys and 15 girls, between 8 and 15 years) replication of their results in boys and girls separately is warranted.

Taken together, it can be concluded that the (relative) influence of genetic and hormonal factors on human brain volume and structure around the onset of puberty remains to be elucidated.

1.4 The present study: aim and sample

The general aim of this thesis is to explore possible mechanisms contributing to individual variation in brain structure at the brink of puberty. To address this issue two lines of research have been conducted:

(1) *The relative importance of genetic and environmental influences on global and regional brain volume (**Chapters 2 and 3**)*

(2) *The association between HPG-axis hormones and brain structure (**Chapter 4, 5 and 6**).*

To my knowledge, this thesis describes the largest sample to date of healthy twin pairs within a restricted age-range (all 9 years of age) that has been scanned with structural MRI. Furthermore, a direct link between pubertal hormones and human brain structure has up till now never been investigated in such a large sample.
Results discussed in most chapters of the current thesis are based on a sample of children coming from 107 twin-families recruited from the Netherlands Twin Registry (Boomsma et al., 2006; Van Leeuwen et al., 2008; www.tweelingenregister.nl) (**Figure 1.3**). The families consisted of healthy 9-year-old twin pairs (45 MZ and 62 DZ pairs) and one of their full siblings (N=85) aged between 10 and 15 years. Children were carefully screened for mental and physical illnesses. Data collection took place between September 2004 and January 2006.

Figure 1.3 Participating twin-families across the Netherlands**.**
Each small dot represents one twin-family who visited the UMC Utrecht for an MRI session; a larger dot represents two families. They were recruited from the Netherlands Twin Registry (Boomsma et al., 2006; www.tweelingenregister.nl).

First, children underwent extensive cognitive testing at the VU University in Amsterdam, including assessment of IQ, different aspects of memory (long/short term, spatial and working memory), processing speed, cognitive control, executive functioning and social cognition. These data are described elsewhere (van Leeuwen, 2008). Before visiting the VU University in Amsterdam, children collected saliva and urine samples directly after waking up on two consecutive days, in which testosterone levels (from saliva), FSH, LH and estradiol levels (from urine) were determined. On average, within 40 days of the cognitive assessments, MRI measurements and quantification of secondary sexual characteristics took place at the Utrecht University Medical Center.

Before entering the MRI scanner, children first practiced in an imitation scanner to get used to the real MRI scanner. The imitation scanner is an exact copy of the real device including the sounds but without the magnetic field (www.niche-lab.nl). This practice procedure yielded a very low number of children to drop out during the MRI session (2 out of 299) (see **Figure 1.4** for a flowchart of the sample).

1.5 Outline of this thesis

In **Chapter 2**, an overview is given of studies on genetic influences on individual variation (heritability) in brain structure. To that end, a total number of 18 brain morphological twin studies across the life-span is reviewed. In this chapter, some methodological aspects of the twin design are considered in more detail as well. The heritability of global and focal brain structures at the onset of puberty is discussed in **Chapter 3.** For that purpose, 9-year-old twin pairs (n=195 individuals) are investigated using volumetric MRI and voxel-based morphometry. Also, in this chapter it is explored whether the onset of secondary sexual characteristics is implicated in regional brain structure, using the Tanner scales of pubertal development. **Chapter 4** focuses on the early endocrinological marker of puberty, luteinizing hormone (LH), measured in first morning urine samples. The association between LH concentrations and brain structure is investigated within 104 9-year-old twins (57 boys, 47 girls). In addition, the etiology of such an association is explored. That is, whether or not there exists a common (genetic) origin of this association. In **Chapter 5**, associations between sex steroids and brain structure is investigated in a more advanced pubertal sample of older siblings of the earlier described 9-year-old twin pairs. Interrelations between testosterone, estradiol, gray and white matter is explored in 78 children between 10 and 15 years old (37 boys, 41 girls). Furthermore, it is investigated whether these hormones could be related to sex differences in brain structure. In **Chapter 6**, the influence of the intrauterine presence of a male co-twin on masculinization of brain volume is studied, possibly mediated by prenatal testosterone exposure. Four groups of 9-year-old dizygotic twins are investigated, differing on suggested prenatal testosterone exposure: (i) boys from same sex pairs, (ii) boys from opposite sex

pairs (iii) girls from opposite sex pairs and (iv) girls from same-sex pairs. In **Chapter 7** the studies will be briefly summarized and the main results will be discussed. Finally, implications and methodological considerations for future research will be discussed.

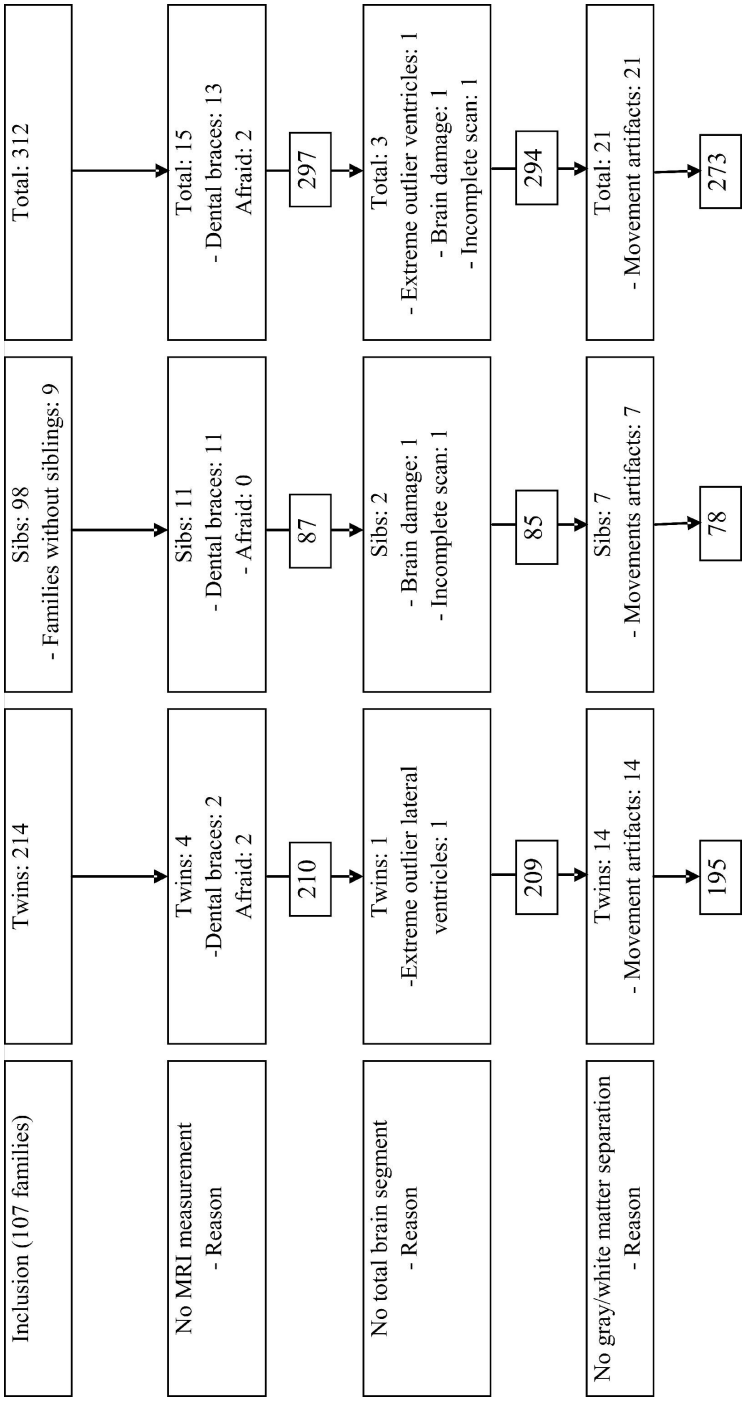

Figure 1.4 Flow chart of included and excluded subjects.
Inclusion and exclusion of subjects are shown in different stages of MRI-acquisition and/or processing-steps of the data. Within each row, number of excluded subjects is shown plus the reason for exclusion. In the small boxes between each arrow the number of included subjects is depicted.

Chapter 2

Genetic influences on human brain structure: a review of brain imaging studies in twins

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Abstract

Twin studies suggest that variation in human brain volume is genetically influenced. The genes involved in human brain volume variation are still largely unknown, but several candidate genes have been suggested. An overview of structural Magnetic Resonance (brain) Imaging studies in twins is presented, which focuses on the influence of genetic factors on variation in healthy human brain volume. Twin studies have shown that genetic effects varied regionally within the brain, with high heritabilities of frontal lobe volumes (90-95%), moderate estimates in the hippocampus (40-69%), and environmental factors influencing medial brain areas. High heritability estimates of brain structures were revealed for regional amounts of grey matter (density) in medial frontal cortex and Heschl's gyrus. In addition, moderate to high heritabilies for densities of Broca's area, anterior cingulate, hippocampus, amygdala, gray matter of the parahippocampal gyrus and white matter of the superior occipitofrontal fasciculus were reported. The high heritability for (global) brain volumes, including the intracranium, total brain, cerebral gray and white matter, seems to be present throughout life. Estimates of genetic and environmental influences on age-related changes in brain structure in children and adults await further longitudinal twin studies. For prefrontal cortex volume, white matter and hippocampus volumes a number of candidate genes have been identified, whereas for other brain areas only a few or even a single candidate gene has been found so far. New techniques such as genome-wide scans may become helpful in the search for genes that are involved in the regulation of human brain volume throughout life.

2.1 Introduction

While showing an impressive prenatal growth, the human brain is not completed at birth. There is considerable brain growth during childhood. Interestingly, our brain is not fully matured after adolescence either. Evidence is accumulating of dynamic changes taking place in the human brain throughout life. Such lifetime dynamic changes in the human brain might allow us to optimally adapt our lives to our environments and experiences. Evidence is also accumulating that brain structure is under considerable genetic influence. The mechanisms by which interaction between genes and environment occur and dynamics of brain structure and its association with brain functioning still remain unknown. However, twin and family studies and new evolving genetic approaches start to give us a glimpse as to which genes and (interacting) environmental influences are shaping our brains.

In a recent study head circumference was measured in healthy children at different ages. Compared to measures at birth (mean, SD was 34.9, 1.1 cm), head circumference was found to increase with more than 30 percent in the first year (46.6, 1.3 cm); between 1 and 4 years of age with another 9 percent (50.9, 1.4 cm) and between 4 and 8 years with an additional 4 percent (53.4, 1.4 cm) (Gale et al., 2006). Magnetic Resonance Imaging (MRI) studies have shown that at 6 years of age total cerebral volume has reached 95 percent of its adult volume (Giedd et al., 1999). Considering that a medium-large adult head size is estimated at 58 cm and expected to remain stable, the volumetric findings are quite compatible with a small increase in head circumference after childhood. Within the brain however, dynamic changes continue from childhood into adulthood. In the landmark studies of a cohort of children between 4-20 years of age using magnetic resonance imaging it was shown that gray matter and white matter both increase in volume until early adolescence. In early adolescence gray matter volume starts to decrease, except for gray matter of the temporal lobe, which was found to increase into late adolescence (Giedd et al., 1999). Overall white matter volume continues to increase (Giedd et al., 1999; Paus et al., 1999). Thus, while overall brain size has almost reached its adult size by 6 years of age, the gray/white matter ratio

decreases in adolescence and into adulthood (for reviews see Toga et al., 2006; Paus et al., 2005; Durston et al., 2001).

In adulthood, brain volume continues to change. Cross-sectional volumetric studies consistently showed a steady decrease in total brain volume, predominantly due to decreases in gray matter (e.g., Allen et al., 2005a; Raz et al., 2004; Hulshoff Pol et al., 2002a). Density mapping of gray matter revealed that the trajectories of age-effects varied over the cortex, with visual, auditory and limbic cortices showing a more linear pattern of aging than the frontal and parietal neocortices (Sowell et al., 2002). Longitudinal studies have confirmed that indeed several of the age-associated changes in brain volumes are probably non-linear. White matter volume was found to increase until age 45 before it starts to decrease (Bartzokis et al., 2001). In a 5-year MRI follow-up study we recently observed continued growth in total brain volume in 43 percent of healthy adult subjects until 40 years of age, suggesting that at least in a subpopulation of adults, brain growth continues into adulthood (Brans et al., 2007; Van Haren et al., 2008). Thus, in adulthood significant dynamic changes in brain structure continue to take place, with continued growth of white matter and nonlinear decreases in total brain volume and gray matter volumes and densities.

Brain structure as we measure it macroscopically using MRI, and the dynamic changes therein, have a functional relevance. In healthy subjects the level of intellectual functioning has been positively associated with whole brain, gray and white matter volumes (Thompson et al., 2001; Posthuma et al., 2002). More focally, several brain areas were found to be correlated with intelligence (Frangou et al., 2004; Haier et al., 2004; Reiss et al., 1996). Interestingly, in a recent study it was shown that the trajectory changes in cortical thickness throughout adolescence are associated with level of intelligence (Shaw et al., 2006). It is important to know which genes are involved in brain structure and if common genes are involved in both in brain structure and cognitive functioning so as to possibly explain their association.

Studies in quantitative genetics describe the decomposition of (observed) phenotypic variance into genetic and environmental sources. Genetically related individuals are studied to disentangle these sources of phenotypic variance.

Heritability is the proportion of genetic variance over the total variance. Environmental variance can be decomposed into environmental variance shared by members of a family (common environment) or non-shared variance which is unique to a certain individual (unique environment). To determine the relative contribution of genetic, common and unique environmental influences on variation in brain structures, the (extended) twin model is a particularly powerful approach (Posthuma and Boomsma, 2000). More specifically, heritability estimates of brain structure are usually based on data from monozygotic twin pairs (MZ, who are nearly always genetically identical) and dizygotic twin pairs (DZ, who share on average 50 percent of their segregating genes). If for a certain brain measure monozygotic twin pairs resemble each other more closely than dizygotic twin pairs, it can be inferred that variation of the brain measure is heritable. However, in addition to genetic influences, common (or shared) environmental influences may play a role in explaining resemblances. The presence of shared environmental factors is suggested when correlations in DZ twins are larger than half the MZ correlation (Boomsma et al., 2002). A first impression of the importance of unique environmental factors is obtained from the extent to which MZ twins do not resemble each other.

To estimate contributions of additive genetic (A) effects, common (or shared) environmental (C) and unique environmental effects (E) to variation in a phenotype, structural equation modeling (SEM) is increasingly used in twin studies. SEM is a more advanced method which, in contrast to the Falconer method, is capable of explicitly testing whether genetic or environmental factors contribute significantly to explaining individual differences. In other words, SEM allows for parameter estimations, while the Falconer (correlational) method merely allows parameter calculation. In extended twin studies the siblings, or sometimes other relatives of twins, are included in the study design. This increases the statistical power to detect the influences of common environmental influences shared by members from the same family.

We review the findings from (extended) twin studies on normal human brain structure and discuss the impact of these findings on future studies into specific

genes and environmental factors that are involved in the continuing development of our brains throughout life.

2.2 Methods

A PubMed indexed search was carried out with a limitation for human subjects and the following keywords: (brain volume) or (white/gray matter) and ((twin) or (heritability)). For inclusion, papers had to be written in English, and use structural magnetic resonance imaging (MRI) or computer tomography (CT). These included volumetric MRI (both global and focal measures), voxel-based morphometry (VBM) and diffusion tensor imaging (DTI) (for information on white matter integrity). Case studies or qualitative studies were not included. If available, information on the number of subjects, average age and age range of the sample, type of analysis (Structural Equation Modeling or Falconer method) and heritability estimates with their 95% confidence intervals were extracted from the papers.

2.3 Results

2.3.1 Heritability of brain volumes

A total of fourteen twin studies measuring brain volume have been carried out, of which eleven were analyzed using SEM and another three used the Falconer method for calculating heritability. Brain structure in healthy MZ and DZ twin pairs was first quantitatively studied using computed Tomography (CT) (Reveley et al., 1984) (**Table 2.1**). In this study it was found that lateral ventricle variation was mostly explained by genetic factors. Later studies using MRI, found high heritability estimates of global brain measures including intracranial volume (>81%) (Baaré et al., 2001; Pfefferbaum et al., 2000; Carmelli et al., 1998) and total brain volume (66%-97%) (Baaré et al., 2001; Bartley et al., 1997; Pennington et al., 2000; Wright et al., 2002). The first twin-sibling study to measure the genetic contributions to variation in global gray and white matter, found heritability

estimates of 82% for gray matter and 88% for white matter (Baaré et al., 2001). The volumes of each cerebral hemisphere showed 65% heritability (Geschwind et al., 2002). For variation in cerebellar volume a heritability of 88% was reported (Posthuma et al. 2000). Area measurements of the corpus callosum revealed heritability estimates between 79% and 94% (Pfefferbaum et al., 2000; Scamvougeras et al., 2003). In a study that did not include DZ twin pairs, MZ twin pair correlations were high (>.90 for cerebellum, total brain, gray and white matter and >.75 for caudate nucleus, putamen, thalamus and cortical depth) as compared with a healthy comparison group, indicating an upper limit of heritability (White et al., 2002).

In the only published twin study to date in children consistent with previous adult studies, additive genetic effects accounted for a substantial portion of variability in nearly all brain regions with the notable exception of the cerebellum (Wallace et al., 2006).

A number of global brain areas seem to be mainly under environmental control. The overall gyral patterning of the cortex was found to be under environmental control (Bartley et al., 1997; Eckert et al., 2002). Moreover, common and unique environmental factors explained individual variation in lateral ventricle volumes (Baare et al., 2001; Wright et al., 2002). However, individual differences in lateral ventricle size were mainly of genetic origin in a study consisting of elderly subjects (Pfefferbaum et al., 2000; Pfefferbaum et al., 2004). While the volume of the cerebellum was found to be mainly under influence of genes in adults (Posthuma et al., 2000; Wright et al., 2002), in children it was found to be largely under common (30%) and unique environmental (21%) control (Wallace et al., 2006). The only study to date measuring heritability of hippocampus volume, found a moderate heritability of 40% (Sullivan et al., 2001).

Table 2.1: Twin studies on human brain volumes

Authors	Subjects	Age in years (range)	Brain region	Heritability (A) in % (95% CI)
Reveley et al (1984) §	18 MZ, 18 DZ	*NA*	LV	82%-85% (*NA*)
Bartley et al (1997)	10 MZ (6 M), 9 DZ (3 M)	MZ:31 (19-54) DZ: 23 (18-29)	TB Gyral patterns	94 % (*NA*) 7-17% (*NA*)
Carmelli et al (1998)	74 MZ, 71 DZ (M)	68-79 years	IC	91 % (*NA*)
Pennington et al (2000) §	RD 25 MZ (12 M), 23 DZ (16 M) Non-RD: 9 MZ (4 M), 9 DZ (4 M)	RD MZ 17.1, DZ: 16.8 Non-RD MZ 19.4, DZ 18.7	TB Neocortex subcortex	97% (*NA*) 56% (*NA*) 70% (*NA*)
Pfefferbaum et al (2000)	45 MZ, 40 DZ (M)	MZ 72.2 DZ 71.4 68-78	IC CC LV	81% (72-90) 79% (69-89) 79% (55-100)
Baaré et al (2001)	54 MZ (33 M) 58 DZ (17 M, 21 OS) 34 sibs	MZM: 31.2, MZF: 34.1, DZM: 30.3, DZF: 30.6, OS: 30.3 sibs: 29 (19-69 years)	IC TB GM WM LV	88% (82-92) 90% (85-93) 82% (73-88) 87% (80-91) C: 59% (47-69), E: 41% (31-53)
Pfefferbaum et al (2001)	15 MZ, 18 DZ	75.7 ± 2.7 years	Size CC Microstructure CC (DTI)	5:1 (*NA*) 3:1 (*NA*) (relative proportion A:E)
Sullivan et al (2001)	See Pfefferbaum et al. (2000)	See Pfefferbaum et al. (2000)	HIP	40% (*NA*)

Thompson et al (2001) §	10 MZ (5 M), 10 DZ (5 M)	48.2 (± 3.4)	middle frontal sensorimotor and anterior temporal cortices asymmetry heritability for Broca's and Wernicke's (cortical thickness)	90-95% (*NA*)
Eckert et al (2002) §	27 MZ, 12 DZ (all M)	MZ: 6.9-16.4 DZ: 6.1-15.0	Planum temporale asymmetry	*NA (NA)*
Geschwind et al (2002)	72 MZ 67 DZ (M)	MZ: 72.3 DZ: 71.8 (68-78)	Cerebral hemispheres Cerebral asymmetry LH/RH handedness	65% (*NA*) LHS:More C influence LH: less genetic control
Posthuma et al (2000)	See Baaré et al (2001)	(see Baaré et al., 2001)	CB	88% (81-92)
Wright et al (2002) #	10 MZ (6 m), 10 DZ (4 m)	MZ: 31 (19-54) DZ: 23 (18-29)	TB LV CB Ventrolateral FR, cingulate, anterior & superior and transverse temp, retro-splenium	66% (17-100) C: 48%(0-97), E:50%(32-84) 63%, E: 22% (*NA*) 58-73% (*NA*)
White et al (2002)#	12 MZ (6 m), 12 control pairs (6 m) **No DZ**	MZ: 24.5 ± 7.2 Controls: 24.4 ± 7.2	TB, GM, WM, CB CAU, PUT, THAL cortical depth	r>.90 r>.75 low correlations (MZ corr)
Scamvougeras et (2003)	14 MZ, 12 DZ	MZ 16-41 DZ: 18-32	CC	94% (*NA*)
Pfefferbaum et al (2004)	34 MZ, 37 DZ.	T1:68-80 years T2: 72-84 years 4 year longitudinal follow-up	CC (T1) CC (T2) LV (T1) LV (T2)	89% (*NA*) 92% (*NA*) 92% (*NA*) 88% (*NA*)

Hulshoff Pol et al (2006) #	See Baaré et al (2001)	See Baaré et al. (2001)	WM (SOF, CC, CST) GM MFL, SFL, STL, CING, PARAHIP, AMYG, OCC	69-82% (*NA*) 55-85% (*NA*)
Wallace et al (2006)	90 MZ (52 M, 38 F), 37 DZ (22 M, 15 F)	MZ: 11.9 DZ : 10.9 (5-19)	TB GM WM FR, TEMP, PAR CB LV	89% (67-92) 82% (50-87) 85% (56-90) 77-88% (50-90) 49% (13-83) 31% (0-67), C:24% (0-58), E:45% (33-60).

#=Voxel-based Morphometry, §= Falconer Method of Heritability, A=additive genetic, AMYG=Amygdala, C=common environment, CAU=Caudate, CB=Cerebellum, CC=Corpus Callosum, CI=Confidence interval, CING=Cingulate Cortex, CST= Corticospinal tract, DTI=Diffusion Tensor Imaging, DZ=dizygotic, DZF=dizygotic female, DZM=dizygotic male, E=unique environment, F=female, FR=Frontal Lobe, GM=gray matter, HIP=Hippocampus, IC=Intracranial Volume, LH=Left Handed, LV=Lateral Ventricles, M=male, MFL=Medial Frontal Lobe, MZ=monozygotic, MZF=monozygotic female, MZM=monozygotic male, *NA*=not available, OCC=Occipital Lobe, Occ-front-temp=occipito-fronto temporal, OS=opposite sex, PAR=Parietal Lobe, PARAHIP=Parahippocampal gyrus, PUT=Putamen, RD=reading disability, RH=Right Handed, Sibs=siblings, SOF=Superior Orbitofrontal, TB=Total brain, TEMP=Temporal Lobe, THAL=Thalamus, SFL=Superior Frontal Lobe, STL=Superior Temporal Lobe, WM=white matter

2.3.2 Heritability of regional brain areas using voxel-based morphometry

Three studies have examined possible genetic effects on more specific brain areas using voxel-based morphometry and cortical thickness measures. Two of these studies were analyzed with SEM. One study additionally used a path analysis on the results (Wright et al, 2002), and one study used a stringent correction of multiple comparisons (Hulshoff Pol et al., 2006a) according to the Random Field Theory (Worsley et al., 1996). Cortical thickness of middle frontal, sensorimotor and anterior temporal cortices, as well as Wernicke's region was found to be particularly influenced by genetic factors (Thompson et al., 2001). Voxel-based morphometry revealed high heritabilities for paralimbic structures and temporal/parietal neocortical areas (Wright et al., 2002). Furthermore, brain density of the medial and superior frontal, superior temporal and occipital gray matter (see **Figure 2.1**) and connecting white matter of the superior occipito-frontal fasciculus and corpus callosum (see **Figure 2.2**) were particularly influenced by genetic factors (Hulshoff Pol et al., 2006a). Unique environmental factors influenced vast gray matter and white matter areas surrounding the lateral ventricles (up to 50%) (Hulshoff Pol et al., 2006a).

2.3.3 Heritability of changes in brain structure with age

In the only study to date that measured heritability estimates of changes in brain volumes over time, genetic contributions to variability in intracranial volume, corpus callosum and lateral ventricles were found to be high in healthy elderly (Pfefferbaum et al., 2000) and remained high at longitudinal follow-up of 4 years (Pfefferbaum et al., 2004).

2.3.4 Heritability of brain structure and the association with brain function

Total brain volume, gray matter and white matter are positively correlated with general intelligence, and these structures and verbal and performal intelligence share a common genetic origin (Posthuma et al., 2002).

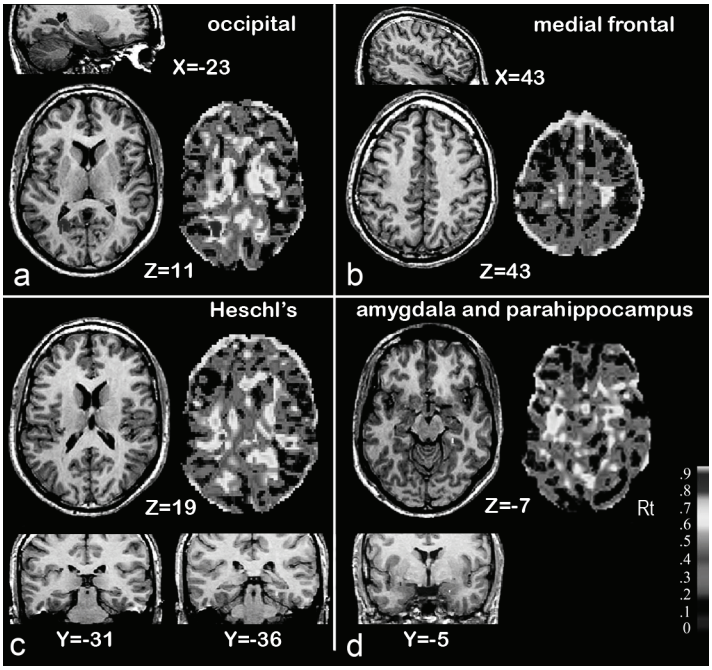

Figure 2.1 Genetically influenced focal gray matter density brain areas.
Significant heritability maps are superimposed on axial and sagittal sections through the magnetic resonance image of the standardized reference brain (left). The complete heritability maps are shown on the right. Data are based on a study in 258 monozygotic and dizygotic twin pairs and their siblings from 112 Dutch families. For genetic analyses, structural equation modeling and voxel-based morphometry was used (Hulshoff Pol et al, 2006a). For a colour version of this illustration see Appendix, Figure 2.1

However, this finding does not necessarily mean that genes influence focal brain structures in the same manner throughout the brain. Moreover, it does not necessarily mean that a common genetic origin with general intelligence is shared by all structures throughout the brain. In a study involving monozygotic and dizygotic twin pairs frontal gray matter volume and intelligence were positively correlated (Thompson et al., 2001). Since this correlation was more prominent in monozygotic as compared to dizygotic twins this finding suggested that frontal lobe volume and intelligence share genetic factors, although it was unresolved

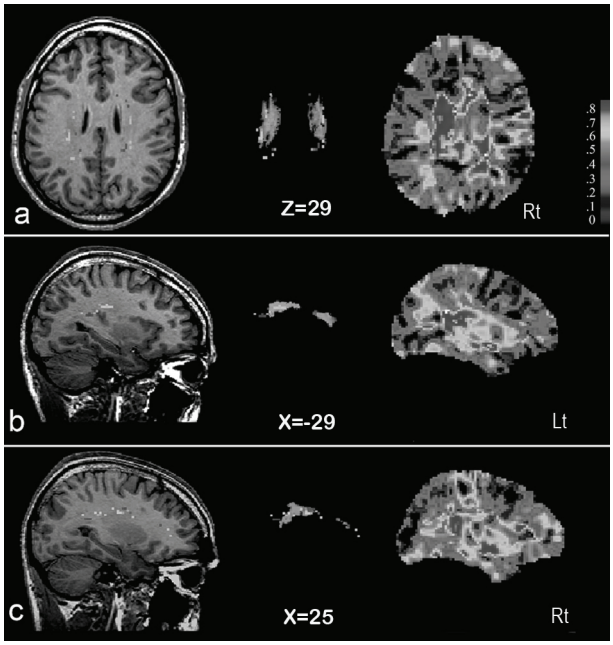

Figure 2.2 Genetically influenced focal white matter density brain areas in adult twins. Significant heritability maps are superimposed on axial and sagittal sections in the left (Lt) and right (Rt) hemisphere through the magnetic resonance image of the standardized reference brain (left) and superimposed on the histologically defined map of the occipitofrontal superior fascicle (middle). The complete heritability maps are shown on the right (for details see Hulshoff Pol et al, 2006a, reprinted with permission of the Journal of Neuroscience). For a colour version of this illustration see Appendix, Figure 2.2.

whether this association shares a common genetic origin (Thompson et al., 2001; Toga and Thompson, 2004). In a study involving monozygotic and dizygotic twin pairs and their singleton siblings using structural equation modelling, verbal and performal intelligence were found to share a common genetic origin with an anatomical neural network involving the frontal, occipital, and parahippocampal gray matter and connecting white matter of the superior occipitofrontal fascicle, and the corpus callosum (Hulshoff Pol et al., 2006a).

2.4 Discussion

Based on twin and family studies it can be inferred that variation in overall human brain volume is highly heritable. The large influence of genes on human brain volume seems to be already present in childhood. Moreover, variation in brain volumes remains to be largely explained by genetic factors, even in old age. Adult twin studies showed high heritability estimates for volumes of specific structures and for overall brain size in adulthood (between 66 and 97%). Also, both variations in volumes of global gray and global white matter are largely determined by genes. However, individual differences in lateral ventricle volume are mainly explained by environmental factors, suggesting that its surrounding brain tissue is at least partly influenced by environmental factors. Genetic effects were shown to vary regionally within the brain, with high heritabilities of frontal lobe volumes (90-95%), moderate estimates in the hippocampus (40-69%), and environmental factors influencing several medial brain areas. Heritability estimates of focal brain structures based on voxel-based morphometry and cortical thickness studies revealed high heritabilities for densities of medial frontal cortex, Heschl's gyrus, and postcentral gyrus. In addition, moderate heritabilities for densities of hippocampus, amygdala, gray matter of the parahippocampal gyrus and white matter of the superior occipitofrontal fasciculus were reported in one or more studies. Areas which show a high heritability for volume emphasize the relevance of these brain areas when searching for genes influencing brain structure.

Significant heritabilities for total brain volume and gray and white matter volumes were consistently found across studies, with most studies finding high heritabilities. Estimates varied between 65 and 97%. Focally, moderate to high heritabilities of the bilateral medial frontal cortex, bilateral Heschl's gyrus, and left postcentral gyrus were consistently found across studies (Thompson et al., 2001; Wright et al., 2002; Hulshoff Pol et al., 2006a). Furthermore, the posterior cingulate was found to be heritable in two studies (Wright et al., 2002; Hulshoff Pol et al., 2006a), underscoring the relevance of these brain areas when searching for genes influencing brain structure and function. Broca's language area, ventrolateral prefrontal cortex, and anterior cingulate gyrus, superior frontal cortex, occipital

(striate and extrastriate) cortex, the anterior cingulate, amygdala, and parahippocampal gyrus were found to be significantly heritable in single studies. These single findings were at least partly due to differences in methodology using between studies in healthy adult twin samples (for a discussion see Hulshoff Pol et al., 2006a). Notwithstanding differences in findings, the overlapping results are quite promising. It seems that the individual variation in morphology of areas involved in attention, language, visual, and possibly emotional processing, as well as in sensorimotor processing are strongly genetically influenced. Unique environmental factors influenced the lateral ventricles in the majority of studies (but see Pfefferbaum for high heritabilities) and brain tissue surrounding the lateral ventricles (up to 50% in Hulshoff Pol et al., 2006a). This suggests that medially, some focal brain regions are probably largely influenced by (unique) environmental influences.

To which extent genetic and environmental factors influence the age-related changes in brain structure is an important question. So far, only one study measured the stability of genetic influences onto changes in brain structure over time. In elderly twin-subjects, high heritability estimates were found to remain stable after a 4-year interval (Pfefferbaum et al., 2004). Thus, at this point, it can only be inferred from separate twin-cohorts with different age-ranges that a high heritability for global brain volumes seems to be present throughout life, including the intracranium, total brain, cerebral gray and white matter. A possible exception may have to be made for the cerebellum, which revealed high heritability estimates in adult twin-samples, but was found to have a low heritability estimate in a childhood twin-sample. Whether the extent of genetic and environmental influences changes with age remains to be investigated. The only study to date suggests that in the elderly the relative influences of genes and environmental factors is quite stable and largely determined by genes (80%).

Importantly, the high heritability of brain volume is functionally relevant. For instance, the association between brain volumes and intelligence was found to be of genetic origin (Posthuma et al., 2002). Moreover, the association between frontal gray matter volume and intelligence was suggested to be due to genetic factors (Thompson et al., 2001; Toga & Thompson, 2004). Recently, the

association of intelligence with gray matter of the frontal and occipital lobes, the parahippocampus and connecting white matter was found to be influenced by genes common to brain structure and intelligence (Hulshoff Pol et al., 2006a). These findings demonstrate that a common set of genes may cause the association between brain structure and cognitive functions. However, in elderly twins, the associations between fronto-temporal brain volumes and executive function were found to be due to common environmental influences shared by twins from the same family (Carmelli et al., 2002). These results point to the possibility that overlapping sets of genes or common environmental influences cause variation in two distinct phenotypes. However, other causal models are also consistent with the findings. It might be, for example, that a higher level of cognitive functioning leads a person to select an environment that also increases brain size. The genetic influence on brain size then simply reflects the genetic influences on cognition. Thus, the specific mechanism, pathways and genes that are involved in human brain morphology and its association with cognitive functions remain elusive.

Considering the high heritabilities for global brain volumes and particular focal brain densities and thicknesses, the search for genes that are involved in brain growth, aging and brain structure maintenance is important. Such knowledge can help us understand normal developmental and age-associated changes in individual variation in brain functioning. Moreover, it enhances our knowledge of individual variation in brain functioning and facilitates the interpretation of the morphological changes found in psychiatric disorders such as schizophrenia. Also, it allows future efforts to find particular genes responsible for brain structures to be concentrated in areas that are under considerable genetic influence (Hulshoff Pol et al., 2006a). A genetic approach to find genes involved in brain structure that has been applied in several studies is that of diseases with a clear genetic etiology. A review of brain imaging studies in Huntington's disease, Down syndrome, Williams syndrome, and Velocardiofacial syndrome, revealed, besides disease specific brain changes, decreases in total brain, white matter and hippocampus volumes, irrespective of the genes and/or chromosomes involved. This suggests that many genes are probably involved in the individual variation of these measures (Peper et al., in press (a)). Another genetic approach that may aid us in our quest to find genes involved in

individual brain variation is the study of polymorphisms of specific genes in healthy subjects. A polymorphism is defined as the existence of multiple alleles of a gene within a population. It is a naturally occurring variation in the sequence of genetic information on a segment of DNA among individuals. Those variations are considered normal and should not be confused with true mutations, which are alterations of the original genetic material, often being harmful.

The few studies on polymorphisms in healthy subjects have revealed associations with brain volumes or densities. For example, Val/met (i.e. valine/methionine amino acids) variant carriers of the Brain Derived Neurotrophic Factor (BDNF)-gene (a gene involved in reducing the amount of naturally occurring neuronal cell death) were found to have a reduced size of the prefrontal cortex (Pezawas et al., 2004) and hippocampus compared to val/val carriers (Bueller et al., 2006; Pezawas et al., 2004; Szeszko et al., 2005). In addition, in met-BDNF carriers a negative relation was found between volume of the dorsolateral prefrontal cortex and age, which was not present in the val-BDNF carriers (Nemoto et al., 2006). A study of allelic variants of the Apolipoprotein (ApoE)-gene - thought to be involved in cell growth and regeneration of nerves – showed that healthy elderly subjects who were homozygous for the Epsilon4 allele, i.e. (e4-e4 genotype) had smaller hippocampal volumes than subjects heterozygous for that allele and than e4 non-carriers (Lemaitre et al., 2005; Lind et al., 2006). Also, the presence of a single ApoE-epsilon4 allele was associated with an increased rate of hippocampal volume loss in healthy women (Cohen et al., 2001). Two variants of the X-linked monoamine oxidase A-gene (MAOA) were recently associated with brain volumes in healthy subjects. The low expression variant predicted volume reductions in cingulate gyrus, amygdala, insula and hypothalamus, whereas the high expression variant was associated with changes in orbitofrontal volume (Meyer-Lindenberg et al., 2006). Overall, studying polymorphisms in healthy subjects yields valuable information on specific genes that may be involved in brain volume. However, as it is a newly developing area of research, the robustness of the findings needs to be pointed out and therefore replication is warranted.

One has to keep in mind that there are certain limitations with respect to the currently reviewed twin studies. First, due to small sample sizes of a number of

studies, assumptions on the contribution of genetic effects in these studies have to be interpreted with some caution. Although the high heritabilities in themselves require smaller sample sizes than for traits which are characterized by a smaller contribution of genetic effects, the main drawback is the lack of power to test for the influence of common environment. Testing an AE model versus a CE model depends on larger sample sizes. Unfortunately, in MRI-research, due to costs and complexity, this is currently not feasible. Also, models testing for interaction between genes or between genetic and environmental factors are complicated because of a lack of power. One might consider, for these situations that aim to go beyond simply establishing heritability, to include other relatives and to use for example an "extended twin design" in which siblings of twins are also tested (Posthuma and Boomsma, 2000). Another limitation is that in some studies there is a large variability in age of the sample. This complicates generalizability of findings, as with age brain volumes undergo dynamic changes. Also, with age the influences of genes and environmental factors on human brain structure may change. Therefore, in homogeneous age groups more accurate heritabilities can be provided. In addition, there are some limitations of the twin-design in general. First, in the classical twin design it is generally assumed that with respect to the trait under study, MZ and DZ twin pairs are treated alike, i.e. the 'Equal environment Assumption (EEA)'. It seems unlikely that with respect to the development of the brain, there are environmental treatments that make MZ twins more alike than DZ twins. In studies in which the EEA has been tested for other traits, by using for example the influence of actual and perceived zygosity on trait similarity, no violation of EEA was found for a range of psychological traits (Kendler et al., 1993, see also Martin et al., 1997).

Finally, it has been argued that the twin method may yield an inflated estimation of heritabilities compared to family and/or adoption studies. On the other hand, family studies might give lower heritability estimations as subjects with different ages within families are compared. Thus, when taking these factors into careful consideration, estimates of genetic influences are probably quite accurate (Martin et al., 1997). Indeed, findings on brain volume in twins could be generalized to the general (singleton) population in a study comparing twins and singleton siblings,

birth order, and zygosity - particularly after correcting for head size or intracranial volume (Hulshoff Pol et al., 2002b).

Without specific knowledge of candidate genes, linkage studies are now employed with the goal to localize a gene that influences a phenotype. This approach can be used when genetic marker data (based on DNA polymorphisms of known location in the genome) are available in extended families or in sibling pairs. Linkage studies are often called a-theoretical ("blind" search for genes) in contrast to association studies which require knowledge of candidate genes (Vink and Boomsma, 2002). Linkage studies require data collection in related individuals (e.g. siblings or large pedigrees). A newly emerging field of genetic research is the study of epigenetics. Epigenetics comprises mechanisms of inheritance, which are not the consequences of changes in DNA-structure. They affect gene transcription, with environmental factors acting as modulators or inducers of epigenetic factors. One such (important) factor is DNA-methylation (see Santos et al., 2005). The genome-wide pattern of DNA-methylation was found to be more alike within monozygotic young than in monozygotic adult and elderly twin pairs (Fraga et al., 2005), although recent studies of DNA methylation profiling did not observe an association with age (Eckhart et al. 2006; Heijmans et al., 2007). It is important to investigate which environmental factors have an influence on the expression of genes (as found in DNA-methylation). Additionally, the study of interaction between genes and environmental factors is warranted. Furthermore, the simultaneous effects of multiple genes and possibly the interaction among genes, also needs investigation as the high heritability of a complex quantitative phenotype such as brain volume cannot be explained by a single gene polymorphism.

Taken together, MRI studies in twins indicate that, given the basic additive genetic model, overall brain volume in adulthood is highly heritable. Twin studies carried out in large and more homogenous samples, analyzed with advanced quantitative genetic methods are needed to test for influences of genetic, common and unique environmental factors or interactions between genetic and environmental influences. Since brain volume changes dynamically throughout life, longitudinal twin studies in childhood as well as in adulthood are needed to investigate the

stability of genetic (and environmental) influences onto functional neural networks in human brain. New brain imaging methods, such as Diffusion Tensor Imaging (DTI)-fiber tracking and (resting state) functional MRI allow study of the heritability of neural networks underlying brain functioning. Such new MRI methods, in coherence with new genetic approaches, will enable us to further disentangle which genes and environmental factors and interactions therein influence human brain structure throughout life.

Chapter 3

Heritability of regional and global brain structure at the onset of puberty: a Magnetic Resonance Imaging study in 9-year old twin pairs

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Abstract

Puberty represents the phase of sexual maturity, signalling the change from childhood into adulthood. During childhood and adolescence, prominent changes take place in the brain. Recently, variation in frontal, temporal-and parietal areas was found to be under varying genetic control between 5 and 19 years of age. However, at the onset of puberty, the extent to which variation in brain structures is influenced by genetic factors (heritability) is not known. Moreover, whether a direct link between human pubertal development and brain structure exists has not been studied. Here we studied the heritability of brain structures at 9-years of age in 107 monozygotic and dizygotic twin pairs (N=210 individuals) using volumetric MRI and voxel-based morphometry. Children showing the first signs of secondary sexual characteristics (N=47 individuals) were compared with children without these signs, based on Tanner-stages. High heritabilities of intracranial, total brain, cerebellum and gray and white matter volumes (up to 91%) were found. Regionally, the posterior fronto-occipital, corpus callosum and superior longitudinal fascicles (up to 93%), and the amygdala, superior frontal and middle temporal cortices (up to 83%) were significantly heritable. The onset of secondary sexual characteristics of puberty was associated with decreased frontal and parietal gray matter densities. Thus, in 9-year-old children global brain volumes, white matter density in fronto-occipital and superior longitudinal fascicles and gray matter density of (pre-) frontal and temporal areas are highly heritable. Pubertal development may be directly involved in the decreases in gray matter areas that accompany the transition of our brains from childhood into adulthood.

3.1 Introduction

Puberty is an important period during development. It represents the phase of sexual maturity marking the change from childhood into adulthood (Tanner, 1978). During puberty, increased availability of sex steroids results in the development of secondary sexual characteristics. The pubertal period partly overlaps with adolescence, but is usually referred to as the period during which behavioural, cognitive and emotional changes arise as well (Sisk and Zehr, 2005). During the course of childhood and adolescence, prominent brain changes take place in the proportion of gray and white matter within the cerebrum: region-specific gray matter decreases have been reported (Jernigan et al., 1991; Giedd et al., 1999; Thompson et al, 2000; Sowell et al., 2002; 2004; Gogtay et al., 2004; O'Donnell et al., 2005; Wilke et al., 2007), as well as white matter increases (Giedd et al., 1999; Paus et al., 1999; Thompson et al, 2000; Barnea-Goraly et al., 2005). Especially, around the onset of puberty, global gray matter (Giedd et al., 1999) as well as frontal and parietal gray matter (Jernigan et al., 1991; Giedd et al., 1999) start to decrease.

Knowledge on the aetiology of variation in brain structures at the onset of puberty is important for our understanding of both healthy and pathological brain development. Brain areas that are strongly influenced by genes in healthy subjects, may allow a more powerful search for candidate genes that are predominantly expressed in these brain regions. This in turn facilitates the interpretation of morphological changes found in neuropsychiatric disorders with an origin in puberty or early adolescence, such as schizophrenia (Andreasen, 1995) and anxiety or mood disorders (Kessler et al., 2005). Also, puberty is a period during which a child's environment changes from parent-dependent to more self- or peer-dependent (Steinberg, 2005). As the developing brain exhibits increased plasticity during this period (Huttenlocher, 1994), it might be hypothesized that the brain is particularly vulnerable to a shift in stimulating or activity-dependent environmental effects.

To estimate the relative importance of genetic and environmental factors on variation of brain structure, twin studies yield valuable information. They are based

on the fact that monozygotic (MZ) twins are (nearly) genetically identical, whereas dizygotic (DZ) twins share on average 50% of their segregating genes (Boomsma et al., 2002). Both types of twins also share their familial environment. Therefore, if the difference in relatedness between MZ and DZ pairs corresponds with differences in trait resemblance, this gives information on the relative influence of genetic factors on brain volumes (Posthuma and Boomsma, 2002). Twin studies in adults measuring gray matter density using voxel-based morphometry and cortical thickness, showed a high heritability (i.e. the proportion of phenotypic variance due to genetic factors) most consistently of frontal, temporal cortices (Thompson et al., 2001; Wright et al., 2002; Hulshoff Pol et al., 2006a). White matter density showed a high heritability in the occipitofrontal fascicles, corpus callosum, optic radiation, and corticospinal tract (Hulshoff Pol et al 2006a). Recently, in a sample of twins between 5 and 19 years of age, a high heritability of most global brain volumes (Wallace et al., 2006; Schmitt et al., 2007) and a high heritability of frontal, temporal and parietal cortical thickness was found (Lenroot et al., 2007b). Both studies also demonstrated that heritability of brain structures changed with age.

So far, at the crucial period of maturational transition, i.e. the onset of puberty, the extent to which individual variation in global and regional brain morphology is influenced by genetic and environmental factors remains unknown. Importantly, heritability of regional white matter has never been investigated in children. Besides genetic and environmental influences on brain structure, other (interrelated) factors could be mediating cerebral composition. Recently, it has been reported that the first endocrinological marker of puberty, luteinizing hormone, is associated with white matter increases in early puberty (Peper et al., 2008). Also, around the onset of puberty overall gray matter starts to decrease (Giedd et al., 1999). However, a direct link between gray matter structure and pubertal development is currently lacking.

In this paper, we address these issues by studying a sample of twins within a very narrow age-range (between 9 years and 9 years and 8 months) on the brink of puberty. The aims of the present study were to quantify the relative contribution of genetic and environmental influences on individual variation in global and regional brain structure in the specific age group of 9-year old twin pairs. Moreover, we

explored possible effects of pubertal development on gray and white matter structure, based on physical examination of secondary sexual characteristics.

3.2 Methods and materials

3.2.1 Participants

The cohort consisted of 214 healthy children coming from 107 Dutch twin-families. Participants were recruited from the Netherlands Twin Registry (Boomsma et al., 2006; Van Leeuwen et al., 2008; Peper et al., 2008) and included 45 monozygotic (MZ) (23 female and 22 male), 62 dizygotic (DZ) (21 female, 22 male and 19 opposite sex (DOS)) twin pairs between 9 years, 0 months and 9 years, 8 months old. Four individuals (coming from 4 DZ pairs) dropped out during the MR scanning, leading to a total number of 210 children who successfully completed the protocol. Exclusion criteria consisted of having a pacemaker, any metal materials in the head -including dental braces-, chronic use of medication, any known major medical or psychiatric history, participation in special education or an IQ < 70. Physical health and mental health were assessed with a medical history inventory. The sample contained a normal distribution of IQ-scores (range 70 to 140, mean 101 (*s.d.* 13) and the percentage right-handed subject was 84%. Zygosity of the twins was determined based on DNA polymorphisms, using 8-11 highly polymorphic di-, tri- and tetranucleotide genetic markers. Parents and the participants themselves gave written informed consent to participate in the study. The study was approved by the Central Committee on Research involving Human Subjects (CCMO) of the Netherlands and was in agreement with the Declaration of Helsinki (Edinburgh amendments).

3.2.2 Image acquisition and processing

Structural magnetic resonance imaging (MRI) scans of the whole brain were made on a 1.5 T Achieva scanner (Philips, Best, the Netherlands).

Volumetric MRI processing

A three-dimensional T1-weighted coronal spoiled-gradient echo scan of the whole head (256 × 256 matrix, TE = 4.6 ms, TR = 30 ms, flip angle = 30^{o}, 160–180 contiguous slices; 1 × 1 × 1.2 mm^{3} voxels, Field-of-View = 256 mm / 70%) was acquired. Furthermore, a single-shot EPI (Echo Planar Imaging) scan was made as part of a diffusion tensor imaging (DTI)-series (SENSE factor 2.5; flip angle 90^{o}; 60 transverse slices of 2.5 mm; no gap; 128 x 96 acquisition matrix; FOV 240 mm; TE=78 ms) together with a magnetization transfer imaging (MTI) scan (60 transverse slices of 2.5 mm; no gap;128 x 96 acquisition matrix; FOV 240 mm; flip angle 8^{o}; TE=4.5 ms; TR=37.5 ms), which were used for segmentation of the intracranial volume. Our imaging protocol made use of T2-weighted contrast of the DTI-B0 and MTI-series for segmentation of the intracranial volume. The DTI-B0 and MTI images were superimposed onto the T1-weighted image to remove non-brain tissue voxels, as described previously (Peper et al., 2008). DTI and MTI data are currently being analyzed for white matter tract characteristics.

The scans were coded to ensure blindness for subject and zygosity identification. The T1-weighted images were automatically put into Talairach orientation (Talairach and Tournoux, 1988) without scaling, by registering them to a model brain in Talairach orientation. The translation and rotation parameters of this registration were then applied to the images (Maes et al., 1997). After linear registration to the T1-weighted image, the intracranial segment served as a mask for all further segmentation steps. The T1-weighted images were corrected for field inhomogeneities using the N3 algorithm (Sled et al., 1998). Our automatic image processing pipeline was used for segmentation of total brain, gray and white matter of the cerebrum, cerebellum and lateral ventricle volumes. The software included histogram analysis, mathematical morphology operations, and anatomical knowledge based rules to connect all voxels of interest, as was validated before (Schnack et al., 2001a, 2001b). The intracranial, total brain, ventricles and cerebellum segments were all visually checked and edited if necessary. Ten brains from the cohort were randomly selected and analyzed by two independent raters to estimate inter-rater reliability. Intra-class Correlation Coefficients (ICC) were all above 0.97.

A Kolmogorov-Smirnov test showed that brain volumes were normally distributed, except for lateral ventricle volumes ($p<0.01$). Therefore, lateral ventricle volumes were logarithmically transformed. After transformation and removal of one extreme outlier (> 3SD) lateral ventricles were normally distributed. Due to motion artifacts, separation of gray and white matter tissue was not possible in 14 subjects (6 MZ, 8 DZ). These subjects were included in the analyses of the other volumes, i.e. intracranial volume, total brain, cerebellum and lateral ventricle volumes. Consequently, the total number of individuals included in global gray and white matter analyses was 195 (84 MZ, 111 DZ), whereas for other global brain volumes the total number of participants was 209 (90 MZ, 119 DZ).

Voxel-based morphometry.

Regional measures of gray and white matter concentration ("density") were generated using voxel-based morphometry (VBM) in a similar manner as was done previously (Hulshoff Pol et al., 2006a). VBM involved the following steps. First, a model brain was created on a sample of 298 children aged 9 to 14 (including the 210 children discussed in this report), similar to the method used in (Grabner et al., 2006). The use of a model brain specifically created from children's brains ensures an optimal warping from the individual brains to the model. The model brain was created by subsequent linear and nonlinear warpings of the original brains (in Talairach space and corrected for nonuniformity) with ANIMAL (Collins et al., 1995). For a detailed description of the creation of the modelbrain, see supplementary material.

After the creation of the model brain, in the following step the binary gray matter (GM) and white matter (WM) masks with voxels of 1 x 1 x 1.2 mm^3 were blurred by a 3D Gaussian kernel (FWHM = 8 mm), in order to gain statistical power. The voxel values of these blurred GM and WM segments (between 0 and 1) reflect the local presence, or density, of GM or WM, respectively. These images are referred to as "density maps". To compare brain tissue at the same anatomical location in all subjects, the GM and WM segments were transformed into a standardized coordinate system (the model space). These transformations were calculated in two steps. First, the T1-weighted images were linearly transformed to the model brain.

In this linear step a mutual information metric was optimized (Maes et al., 1997). In the second step nonlinear (elastic) transformations were calculated to register the linearly transformed images to the model brain up to a scale of 4 mm (FWHM), thus removing global shape differences between the brains, but retaining local differences. For this step the program ANIMAL (Collins et al., 1995) was used. The GM and WM density maps were now transformed to the model space by applying the concatenated linear and nonlinear transformations. Finally, the maps were resampled to voxels of size 2 x 2 x 2.4 mm^3. Voxels with an average GM density below 0.1 were excluded from the GM density voxel-based analysis. Similarly, voxels with an average WM density below 0.1 were excluded from the WM density voxel-based analysis. Heritable white matter voxels were overlaid on probabilistic maps of post-mortem white matter fiber-bundles for identification (Bürgel et al., 2006; Hulshoff Pol et al., 2006a).
The total number of individuals included in the VBM analyses was 195 (84 MZ, 111 DZ).

3.2.3 Pubertal assessment

Secondary sexual characteristics of puberty were physically determined by a trained researcher (no self-report) using the stages of development devised by Tanner (Marshall and Tanner, 1969; Marshall and Tanner, 1970). The training of the researcher (J.S.P.) consisted of a complete instruction by a medical doctor and practical sessions at the Utrecht University Medical Center. No intra- or inter-rater reliability statistics are available due to ethical restrictions, i.e. children were not allowed to undress twice and had to be rated by one person only. However, our data on Tanner-development (i.e. frequency of the first symptoms across the sexes) have probably been reliably measured since they were comparable with earlier studies (Herman-Giddens, 1997; 2001). The Tanner questionnaire consists of a six-category measurement of pubic hair in both boys (induced by androgens mainly from the testes) and girls (induced by androgens from both the adrenal gland and the ovaries). In girls, breast development, induced by estrogen secretion from the ovaries, is measured using five different stages ranging from 1 to 5. In boys, penis

and testicle size (under androgenic control) are assessed based on scales divided in 6 (ranging from 1 to 6) and 4 (ranging from 1 to 4) stages respectively. On all these scales, the lowest stage (1) represents pre-puberty and the highest stage corresponds to fully matured. As in the current sample of 9- year old children only stages 1 and 2 were present, a binary variable 'Tanner-status' was created from all subscales together: "0" meaning no visible development on any of the scales, and "1" meaning the first signs of development were visible on one or more scales. This variable thus captures both adrenal and gonadal maturation during puberty.

3.2.4 Genetic analyses

The phenotypic variance for each brain measure was decomposed into additive genetic (A), common environmental (C) and unique environmental variances (E) (with the total variance being equal to A + C + E).The expected covariance between MZ twins is A + C, and the expected covariance between DZ twins is ½ A + C, which is based on the fact that MZ twins are genetically identical, whereas DZ twins share on average 50% of their segregating genes. Genetic analyses were carried out with structural equation modeling in Mx (Neale et al., 2003).

All global brain volumes were corrected for the effects of sex and Tanner-status with a (linear) regression analysis. Variances and covariances of the residuals from the regression analyses were simultaneously decomposed into A, C and E components. Equality of variances and mean structures for MZ and DZ twins and for first and second born twins were established before genetic models were fitted to the data. Model selection among hierarchically nested models was based on likelihood-ratio tests. The test statistic has a chi-square (χ^2) distribution with degrees of freedom (*df*) equal to the difference in *df* between a full and a more constrained model. A full ACE model was compared with an AE-model (excluding common environmental factors), a CE-model (excluding additive genetic effects) and an E- model (excluding all familial resemblance). The selected model was the model with the fewest parameters that did not fit significantly worse than the more complex model. From the maximum likelihood estimates for parameters under the best model, heritability estimates (proportion of genetic

variance over the total variance, i.e. A/(A+C+E)) were calculated. The relative influences of C and E were estimated similarly.
Similar to the analysis of global brain volumes, genetic model-fitting was carried out in each voxel separately, to obtain A, C and E estimates for local GM and WM density (as was done previously (Hulshoff Pol et al., 2006a)). Even if the role of the shared environment is not statistically significant, calculating heritability estimates from an AE model will upwardly bias the estimates. Therefore, corresponding to the volumetric analysis, all heritability estimates in the VBM-analysis were calculated from ACE models. Handedness was added as additional covariate in the voxel-wise analysis (measured with the Edinburgh Handedness Inventory (Oldfield, 1971)), since handedness can have subtle effects on, for example, asymmetry of the motor cortex (Amunts et al., 2000; Hervé et al., 2005) and sylvian fissure (Witelson and Kigar, 1992). The critical χ^2-value for $p<0.05$ after correction for multiple comparisons was 17.7 for WM density and 20.5 for GM density ($df=1$), according to the false discovery rate (Genovese et al., 2002).

3.2.5 Analysis of pubertal effects on brain measures

A (linear) regression analysis was carried out in Mx to estimate the linear effect of Tanner-status on the means of global and regional gray and white matter, correcting for sex and handedness in the voxel-wise analysis. Likelihood-ratio χ^2 -tests were performed to test for significance.
In addition, in a post-hoc analysis it was explored whether the impact of the variance components A, C and/or E on regional brain density changed with the transition into puberty. To that end, the fit of a model in which variance components A, C or E were allowed to be different within the pre-pubertal and pubertal group, was tested against a model in which these variance components were constrained to be equal within both groups (Purcell, 2000).
Analyses were done on the 5 highest peaks within gray matter that showed a substantial effect of Tanner-status and had a heritability higher than 50%.

3.3 Results

3.3.1 Mean brain volumes and Tanner-status: influences of zygosity and sex

MZ and DZ twins and youngest and oldest member of a twin pair demonstrated similar means and variances of global brain volumes. MZ and DZ twins did not significantly differ in age and Tanner-status (**Table 3.1**). In total, 30 twin pairs were discordant for handedness (28%). This percentage was equally distributed among MZ and DZ twin pairs. According to the Tanner questionnaire, 47 (24%) of the 9-year old twins showed the first signs of secondary sexual characteristics (i.e. Tanner-status=1), including a larger part of females: 32.3% versus 15.6% males (χ^2=7.5 (*df*=1), *p*=.006). Tanner-stage (0/1) was not significantly correlated with age (r=.06, *p*=.89). Furthermore, mean age of children with Tanner-status 0 and 1 was 9.21 versus 9.23 respectively (i.e. a difference of 7 days, difference not significant). When females were investigated separately (they obviously showed more pubertal development than males), the age difference between Tanner status 0 and 1 was 11 days (9.22 versus 9.25 years), also not significantly different. 62% of children with Tanner-status 1 showed both signs of gonadarche (breast development in girls, genital growth in boys) and adrenarche (pubic hair development in both boys and girls), the remaining 38% had adrenarche development only. These frequencies were equally distributed in boys and girls. Within the MZ twins, 7 pairs were concordant for Tanner-status 1 (16%) and 34 pairs for Tanner-status 0 (76%). Within the DZ twins, 6 pairs were concordant for Tanner-status 1 (11%) and 35 pairs for Tanner-status 0 (61%). The rate of discordance for Tanner-status in MZ twins was 9% (4 pairs out of 57), versus 28% in DZ twins (16 pairs out of 57). The heritability of Tanner-status was estimated at 92%. Overall, boys had on average a 9% larger total brain volume (1427 ml, SD 91 ml) as compared to girls (1285 ml, SD 87 ml) (*p*<.05). Also, gray and white matter, lateral ventricles and cerebellar volumes were significantly larger in boys than in girls (*p*<.01). After correction for intracranial volume, these sex differences in brain volumes were no longer present.

Table 3.1 Demographics and brain volumes of the twin-sample.

	MZM	MZF	DZM	DZF	DOS-M	DOS-F
N (individuals)	44	46	43	41	17	19
Age	9.19 (.11)	9.22 (.11)	9.19 (.10)	9.23 (.08)	9.25 (.17)	9.25 (.16)
Tanner-status 0/1	36/8 (81.8/18.2)	36/10 (78.3/21.7)	38/5 (88.4/11.6)	26/15 (63.4/36.6)	13/4 (76.5/23.5)	12/7 (63.2/36.8)
Hand (R/NR)	37/7	38/8	37/6	37/4	13/4	15/4
IQ	101 (16)	96 (10)	101 (13)	107 (12)	104 (15)	103 (10)
Height	139.5 (5.9)	136.6 (4.3)	138.7 (5.3)	138.8 (4.7)	140.2 (5.2)	140.6 (5.0)
Intracranium	1539.4 (113.6)	1368.6 (100.3)	1559.2 (106.6)	1399.9 (92.8)	1526.0 (63.3)	1422.9 (75.2)
Total Brain	1417.6 (94.9)	1267.8 (96.5)	1440.9 (96.9)	1290.1 (82.3)	1414.8 (55.7)	1317.2 (66.2)
Gray Matter	748.6 (50.2)	668.7 (53.4)	758.4 (55.1)	678.9 (52.2)	782.6 (36.5)	718.5 (45.0)
White Matter	499.8 (44.7)	448.9 (45.5)	509.7 (49.3)	455.3 (40.9)	460.5 (25.4)	440.1 (26.1)
Cerebellum	157.7 (14.9)	145.7 (10.3)	164.0 (11.1)	145.8 (10.8)	155.1 (15.8)	148.6 (11.2)
Lat. Ventricles	13.0 (12.0)	7.9 (4.2)	10.3 (6.3)	8.1 (4.4)	9.7 (4.5)	6.9 (3.2)

MZM=monozygotic male; MZF=monozygotic female; DZM=dizygotic male; DZF=dizygotic female; DOS-M=dizygotic opposite sex male; DOS-F=dizygotic opposite sex female N=number of individuals, Age in mean (sd) years, Tanner-status 0 and 1 in absolute numbers (%), Hand= number of right/non-right handed, IQ=mean full scale IQ (sd) measured with WISC-III, Height in mean (sd) centimeters, Brain volumes in mean (sd) milliliters.

3.3.2 Influences of genes on variation in brain volumes

Model fitting analyses revealed that an AE-model best fitted intracranial volume, total brain volume, gray and white matter, cerebellum (**Table 3.2**). For lateral ventricle volume, specific sources of A or C could not be distinguished, but since the confidence interval (CI) of E did not reach up to 100%, familial influences seem present. The heritability estimates (with 95% CI) were: 91% (56%-94%) for intracranial volume, 94% (62%-96%) for total brain volume, 77% (40%-90%) for gray matter volume, 84% (50%-90%) for white matter volume and 88% (56%-97%) for cerebellum volume. The heritability of lateral ventricles was estimated at 35% (0%-78%, not significant). It might be argued that additive effects on a logarithmically transformed trait, such as lateral ventricle volume, are associated with multiplicative effects (Khambanonda, 1950; Khoury et al., 1993; Yang et al., 2005). In other words, the individual effects of genes and environmental factors influencing lateral ventricles might act as the product instead of the sum of the individual effects.

To allow a straightforward comparison with other studies (Baaré et al., 2001; Wright et al., 2002; Wallace et al., 2006) non-transformed ventricle volumes were analyzed as well. Consistent with these earlier studies, substantial influences of C (64%) and E (36%) were found for untransformed ventricle volumes, whereas additive genetic effects were estimated at 0%. The influence of common environmental factors on intracranial volume, total brain volume, gray and white matter volumes and cerebellum volume was not significant.

Table 3.2 Relative proportions of a^2, c^2 and e^2 estimates of global brain volumes (95% confidence intervals).

				ACE *vs.* CE		ACE *vs.* AE	
Absolute volumes	**a^2**	**c^2**	**e^2**	**χ^2 (df=1)**	***p***	**χ^2 (df=1)**	***p***
Intracranial volume	.91 (.56-.94)	.00 (.00-.04)	.09 (.06-.14)	36.3	**<0.001**	0.00	1.000
Total Brain	.94 (.62-.96)	.00 (.00-.32)	.06 (.04-.09)	51.7	**<0.001**	0.00	1.000
Lateral ventricles*	.35 (.00-.78)	.35 (.00-.69)	.30 (.19-.44)	2.9	0.08	2.02	0.29
Gray matter	.77 (.40-.90)	.08 (.00-.43)	.15 (.09-.25)	16.9	**<0.001**	0.12	0.92
White matter	.84 (.50-.90)	.00 (.00-.31)	.16 (.10-.27)	19.2	**<0.001**	0.00	1.000
Cerebellum	.88 (.56-.97)	.07 (.00-.39)	.05 (.03-.08)	54.5	**<0.001**	0.11	0.93

* Logarithmically transformed. A= additive genetic factors, C= Common environmental factors, E= Unique environmental factors. The 4 right columns display the fit of models with and without an A or without a C component respectively, using chi-square tests. All model-fitting analyses were corrected for sex and secondary sexual characteristics (Tanner-status). Untransformed lateral ventricle volumes yielded an a^2 of .00 (.00-.64), c^2 of .64 (.20-.74) and e^2 of .36 (.25-.49).

3.3.3 Influences of genes on variation in white and gray matter density

VBM analyses revealed that within regional white matter, genetic effects were significant in areas along the right superior and inferior fronto-occipital fascicles, superior longitudinal fascicles bilaterally (**Figure 3.1, Table 3.3**), genu of the corpus callosum and left cingulum. Heritability estimates in these areas ranged from 67% to 93%. Regional gray matter density revealed significant additive genetic effects in the left superior frontal gyrus, right middle temporal lobe and left amygdala (**Figure 3.2, Table 3.3**). Heritability in these areas was estimated between 81% and 83%. Relaxing the FDR-value to α=.10 or .15 did not result in novel significantly heritable brain areas, but resulted in a growth of areas which were already detected as significantly heritable at an FDR-value of α=.05. When examining the heritability maps, uncorrected for multiple comparisons at p<.05 (corresponding to a χ^2-value of 3.84), gray matter density of the middle and inferior frontal gyrus could be included as highly heritable (>70%).

Common environmental effects on gray and white matter density were estimated up to 77%, but failed to reach statistical significance in any region of the brain. By definition, variance in density of the remaining gray and white matter areas was significantly influenced by unique environmental factors and other components within E, such as measurement error. Areas of white matter density with an E-model as the best-fitting model were located in the orbitofrontal cortex, anterior cingulate and (parts of) the cingulum. In addition, regional gray matter areas in which an E-model fitted best mainly included frontal and temporal areas, with E-estimates > 80%.

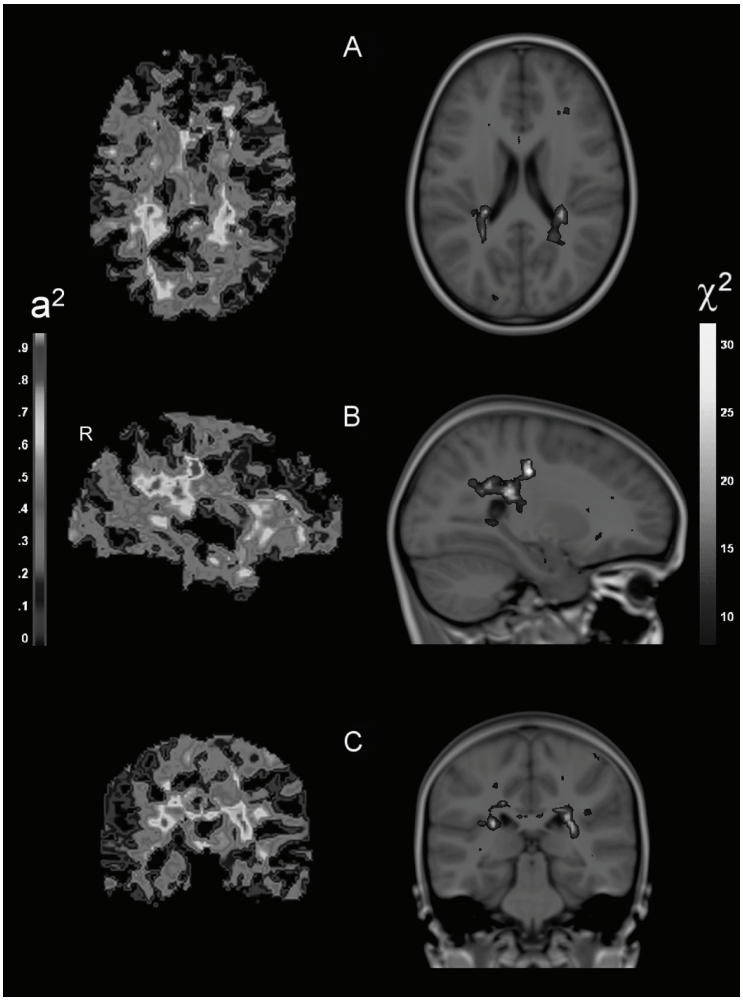

Figure 3.1 Genetically influenced regional white matter density.
A) Superior occipitofrontal fascicle (Z=36), B) Superior occipitofrontal fascicle, superior longitudinal fascicle (X=27), C) Superior longitudinal fascicle (Y=-22). Images are according to neurological convention (left=left). The left side displays heritability-estimates. The right side displays significant genetic effects in χ^2-values, overlaid on the model brain: CE versus ACE model (critical level of significance is 17.7, corrected for multiple comparisons according to the False Discovery rate, α=0.05). For visualization purposes, this threshold is relaxed to an FDR-rate of α=0.15 (corresponding to a χ^2-value of 10.5). χ^2-maps are resampled to anatomical resolution for overlap with anatomical boundaries. For a colour version of this illustration see Appendix, Figure 3.1

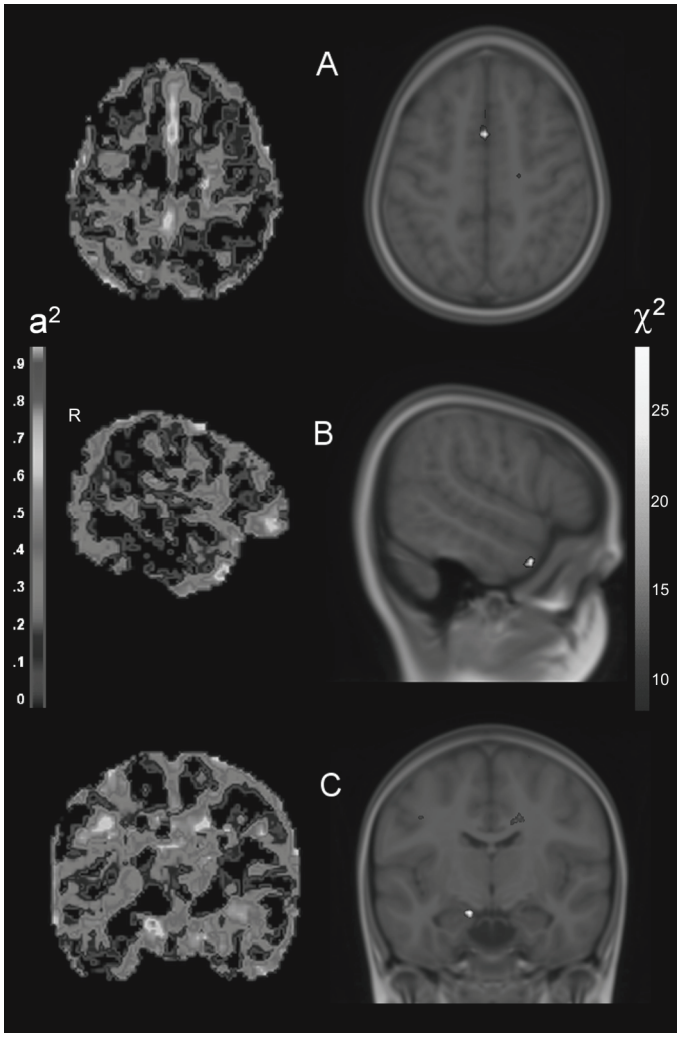

Figure 3.2 Genetically influenced regional gray matter density.
A) Superior frontal gyrus (Z=57) B) Middle temporal gyrus (X=55) C) Amygdala (Y=-9). Images are according to neurological convention (left=left). The left side displays heritability-estimates. The right side displays significant genetic effects in χ^2-values, overlaid on the model brain: CE versus ACE model (critical level of significance is 20.5, corrected for multiple comparisons according to the False Discovery rate, α=0.05). For visualization purposes, this threshold is relaxed to an FDR-rate of α=0.15 (corresponding to a χ^2-value of 12.2). χ^2-maps are resampled to anatomical resolution for overlap with anatomical boundaries. For a colour version of this illustration see Appendix, Figure 3.2

Table 3.3 Significantly heritable regional gray and white matter density with voxel-based morphometry.

				Talairach Coordinates		
Area (Gray matter)	**Heritability**	**χ^2 (ACE vs. CE)**	**# voxels**	**X**	**Y**	**Z**
Mid. Temporal Gyrus (R)	.83	20.9	1	55	5	-26
Sup. Frontal Gyrus (L)	.82	20.8	1	1	9	57
Amygdala (L)	.83	23.2	2	-13	-9	-15
Area (White matter)						
Sup. Fronto-Occipital Fascicle (R)	.67	19.5	1	31	30	20
Sup. Fronto-Occipital Fascicle (R)	.93	46.0	11	27	-22	36
Inf. Fronto-Occipital Fascicle (R)	.82	18.4	2	23	-77	12
Sup. Longitudinal Fascicle (R)	.91	29.3	6	27	-49	27
Sup. Longitudinal Fascicle (R)	.76	17.7	16	26	-45	27
Sup. Longitudinal Fascicle (L)	.88	28.7	8	-27	-32	21
Genu Corpus Callosum (R)	.86	22.1	6	13	26	-2
Genu Corpus Callosum (L)	.80	17.9	1	-3	23	11
Posterior Cingulum (L)	.86	26.3	8	-22	-29	36

Shown are voxels with the highest χ^2-value within a significant region. Mid.=Middle, Sup.=Superior, Inf.=Inferior, L=Left hemisphere, R=Right hemisphere. CI=Confidence interval, A=Additive genetic, C=Common environment, E=Unique environment. # voxels=number of voxels within a significant cluster. Analyses were corrected for sex, handedness and Tanner-status. The critical χ^2-difference between an ACE and a CE –model (excluding A) was 20.5 for GM and 17.7 for WM, after correcting for multiple comparisons according to the false discovery rate (α=0.05). Heritability estimates are drawn from ACE-models.

3.3.4 Influence of pubertal status on gray and white matter

Due to the stringent correction for multiple comparisons, Tanner-status did not predict gray and white matter density significantly (although χ^2-values ranged up to 19.3 (p<.00005 uncorrected)). When an exploratory χ^2-threshold of 3.84 was adopted (df=1; p<0.05 uncorrected), a reduction of (pre)frontal and parietal gray matter density could be observed in children already showing secondary sexual characteristics compared to children without any secondary pubertal signs (**Figure 3.3**). In addition, an increase in occipital white matter density was seen in the pubertal children. Tanner-status did not significantly correlate with global gray or white matter volume. Since only 15.6% of the pubertal group consisted of boys (N=7), leaving them out of the volumetric and VBM analysis did not substantially affect the results.

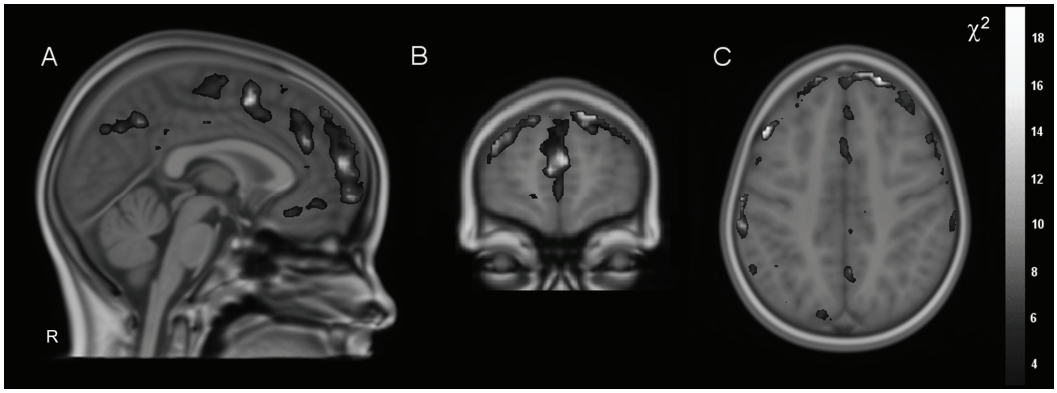

Figure 3.3 Decreases in (pre)frontal and parietal gray matter density in pubertal children, compared to non-pubertal children at 9 years.
Shown here are χ^2-values of the worsening in fit of a (means) model with Tanner-status versus without Tanner-status, corrected for sex and handedness. Level of significance: $\chi^2 > 3.84$ (α<0.05, uncorrected for exploratory purpose). To measure decreases or increases in density, χ^2-maps were multiplied by negative and positive regression maps respectively. Significant reductions in gray matter density are mainly located in the (pre)frontal cortex (A (X=2), B (Y=60) & C (Z=41)) and parietal cortex (A & C) bilaterally with χ^2-values up to 18.5. Images are according to neurological convention (left=left) χ^2-maps are resampled to anatomical resolution for overlap with anatomical boundaries. For a colour version of this illustration see Appendix, Figure 3.3.

In addition, possible confounding effects of age were explored, by adding age as a covariate. This did not affect the results in the whole sample or in the girls separately (excluding the 7 boys with Tanner-development). In the post-hoc analysis it was found that with the transition into puberty, total variance in superior-, middle- and medial frontal gray matter density increased significantly (**Table 3.4**). A trend towards an increase of both genetic and unique environmental factors was observed; therefore it can be argued that the increase in total variance was not completely due to an increased unique environmental or error variance. However, a significant difference in contribution of genetic and/or environment factors between the pre-pubertal and pubertal group to gray matter variation could not be demonstrated.

3.4 Discussion

We investigated the influence of genetic and environmental effects on variation in brain structure in 107 nine-year old twin pairs. Also, we explored possible effects of pubertal development on cerebral gray and white matter. To our knowledge, this is the first study focusing on the relative influence of genes and environment (in percentages) on regional white matter density in children. Results showed high heritability estimates for global brain volumes, i.e., the intracranium (91%), total brain (94%), gray (77%) and white matter (84%) of the cerebrum and cerebellum (88%). Lateral ventricle volume was moderately heritable (35%). Regionally, gray matter densities within the left amygdala, left superior frontal and middle temporal gyrus were significantly heritable. Regional white matter densities of the bilateral superior fronto-occipital fascicle, bilateral superior longitudinal fascicle in posterior parts of the brain and areas within the cingulum and corpus callosum were found to be significantly heritable, with estimates ranging from 67% to 93%. Our results indicate that the onset of secondary sexual characteristics of puberty is associated with a lower mean and larger variation in frontal and lower mean in parietal gray matter densities. Importantly, these reductions in gray matter density could not be explained by age effects, as all children in our sample were 9 years of age.

Table 3.4 Puberty-related decreases in gray matter density (Δ GM) with total variance in pre-puberty and puberty of 195 nine-year old twins.

	Δ GM density		Total Variance GM density					Talairach Coordinates		
Area	**Pub vs. Pre-pub**	**χ^2 Δ GM (*df*=1)**	**Pre-pub**	**Pub**	**χ^2 (*df*=3)**	**Overall heritability**	**# voxels**	**X**	**Y**	**Z**
Sup. Frontal G (R)	- 1.9%	5.63*	4.87×10^{-3}	9.82×10^{-3}	9.65*	.56	26	12	27	59
Med. Frontal G (R)	- 4.1%	13.26**	2.97×10^{-3}	7.22×10^{-3}	16.10**	.64	32	6	58	12
Sup. Frontal G (L)	- 4.3%	9.90**	5.04×10^{-3}	9.83×10^{-3}	9.58*	.76	23	-2	8	53
Mid. Frontal G (L)	- 7.3%	14.94**	10.11×10^{-3}	15.65×10^{-3}	8.90*	.63	27	-45	21	41
Precentral G (R)	- 3.9%	4.26*	12.36×10^{-3}	9.54×10^{-3}	1.78	.70	13	50	0	49

*) $p<.05$, **) $p<.01$.The third column represents the fit of a model when excluding an effect of Tanner-status, in this case: whether the GM density decrease with Tanner-status 1 versus status 0 is significant. The sixth column represents the fit of a model when excluding all variance components (A, C and E). Overall heritability estimates are derived from the univariate analyses of the total sample. The heritability of Tanner-status was estimated at 92%. Sup.=superior, Med.=medial, Mid.=middle, G=gyrus, Pre-pub=pre-puberty, Pub=puberty, R=right hemisphere, L=left hemisphere. # of voxels=number of voxels within a significant cluster (with$\chi^2 > 3.84$).

We showed that at 9 years of age, when pubertal development was not yet visible in 76% of the children, the main proportion of individual variation in overall brain volume is influenced by genetic factors. Our approach of studying children within a very narrow age-range, allowed an estimation of heritability which is unaffected by age x genotype interactions or age-dependent gene expression (Plomin et al., 1997). Our data are in line with heritability estimates of global brain volumes in adults (Carmelli et al., 1998; Pfefferbaum et al., 2000; Posthuma et al., 2000; Baaré et al., 2001; Geschwind et al. 2002; Wright et al., 2002) and with a paediatric sample in which a considerable part of the children had already entered adolescence (Wallace et al., 2006). When the findings of these studies are combined, one might speculate that the genetic factors contributing to overall brain volume remain stable throughout life. This suggestion is also underscored by the fact that total brain volume has reached 95% of its final adult size by the age of 6 years (Giedd et al., 1999).

White matter densities of the bilateral superior fronto-occipital fascicle, bilateral superior longitudinal fascicle in the posterior part of the brain and areas within the cingulum and corpus callosum were found to be significantly heritable, with estimates up to 93%. So far, heritability of regional white matter densities in pediatric twins was unknown. The fronto-occipital fascicle (FOF) and superior longitudinal fascicle (SLF) have been suggested to be involved in visuospatial processing (FOF) and language processing (SLF) (Makris et al., 2005; 2007). Interestingly, in an earlier study in adult twin pairs the FOF and parts of the corpus callosum were also found to be highly heritable (Hulshoff Pol et al, 2006a). However, in contrast to this study in adults, we did not observe significant heritability within the corticospinal tract or optic radiation. It is noteworthy that the white matter areas with a high heritability estimate in the 9-year old twins were mostly posteriorly oriented whereas in the adult twins they extended towards the frontal cortex. Therefore, it may be speculated that the “posterior-to-anterior” pattern of heritable brain areas throughout development (Gogtay et al., 2004) can be extended to white matter maturation. Our reported heritable white matter voxels within the superior FOF, SLF, cingulum and genu of the corpus callosum largely overlapped with the post-mortem maps of fiber bundles (Bürgel et al., 2006;

Hulshoff Pol et al., 2006a), thus supporting that indeed these fiber tracts were involved. However, since no actual fiber-bundles could be traced on the T1-weighted brain images we have to interpret the specific tracts with some caution. The analysis of DTI and MTI-data of this twin sample is currently in progress.

The heritable gray matter densities in the 9 year old twins were located in the left amygdala, left superior frontal and middle temporal gyri. That the frontal cortex is partly driven by genetic factors in humans was suggested recently in a large pediatric twin sample between 5-19 years of age (Lenroot et al, 2007b) as well as in adult samples (Thompson et al., 2001, Wright et al, 2002; Hulshoff Pol et al, 2006a). Also in line with our findings are high heritabilities of the (middle) temporal gyrus (Lenroot et al., 2007b) and amygdala (Hulshoff Pol et al., 2006a). Furthermore, our findings support data on cortical thickness suggesting that the prefrontal cortex has a lower heritability in younger as compared to older children and becomes more heritable with age (Lenroot et al., 2007b).

We also found differences between our study and previous findings across childhood and adolescence. We did not find significant heritabilities for dorsolateral prefrontal cortex, orbitofrontal cortex, angular-, superior temporal and superior parietal gyri, as reported earlier (Lenroot et al. 2007b). Moreover, there are some differences between our study and findings in adults. We did not observe significant heritabilities for ventrolateral prefrontal- and anterior cingulate cortices (Wright et al., 2002), Broca's language area (Thompson et al., 2001), postcentral-, medial frontal-, and Heschl's gyri (Hulshoff Pol et al., 2006a; Thompson et al., 2001; Wright et al., 2002) and parahippocampal-, or occipital gyri (Hulshoff Pol et al., 2006a).

The differences in findings between our study and previous studies might be due to a number of factors. Differences in age of the samples may have influenced the heritable areas between studies. Our study consisted of 9-year old children, whereas others investigated children and adolescents between 5 and 19 years (Lenroot et al 2007b), and adults with a mean age of 31 years (Hulshoff Pol et al, 2006a), 48 years (Thompson et al, 2001) and between 18 and 54 years (Wright et al. 2002).

Furthermore, gray matter density as measured with VBM (our study; Hulshoff Pol et al., 2006a) versus cortical thickness measures (Lenroot et al., 2007b), cortical brain mapping (Thompson et al., 2001) and linear warping (Wright et al., 2002) might have yielded different heritability estimates. In addition, a correction for multiple comparisons on cortical gray matter only (in cortical thickness measures) allows for a lower statistical threshold than when it is applied on both cortical and subcortical gray matter (as is the case with VBM). A study comparable to our study with respect to the applied VBM-technique (Hulshoff Pol et al., 2006a) reported larger heritable (anterior) gray matter areas than in the 9-year olds. Considering that in (young) adult twins the focal heritable gray matter areas were larger than in the 9-year olds, it seems reasonable to suggest that heritability of (anterior) gray matter brain areas increases with age during puberty, as suggested before (Lenroot et al, 2007b).

We found hemispheric asymmetry in significantly heritable gray matter areas, i.e. the left amygdala, left superior frontal gyrus and right middle temporal gyrus. However, these differences in heritability were gradual: homologous areas within the contralateral hemisphere also showed a substantial heritability (**Figure 3.2 A & C**), but did not reach statistical significance. More specifically, the heritability estimates within the homologous contralateral brain areas were 50% (right amygdala), 69% (right superior frontal gyrus) and 55% (left middle temporal gyrus). In line with our findings, a higher heritability was found within the left as compared to the right amygdala in adults (Hulshoff Pol et al., 2006a). The superior frontal gyrus was found to be significantly heritable on the left and right side, both in children and adolescents (Lenroot et al., 2007b) as well as in adults (Hulshoff Pol et al., 2006a). However, Thompson et al. (2001) demonstrated a significantly heritable left frontal lobe as opposed to the right frontal lobe, which supports our results in 9 year-olds. Finally, in contrast to our data, cortical thickness of the left and right middle temporal lobe was reported to be heritable in children and adolescents (Lenroot et al., 2007b). However, this was not found in adults (Thompson et al., 2001; Wright et al., 2002; Hulshoff Pol et al., 2006a). A possible explanation for hemispheric asymmetry in genetically influenced brain areas could be that certain genes are predominantly expressed in one hemisphere. For example,

it was reported that the LM04-gene (essential for cortical development in mice) was more highly expressed in the right hemisphere than in the left (Sun et al., 2005).

Besides heritability of gray and white matter, we explored the influence of pubertal stage upon brain structure. This was possible since a quarter of our sample (24%) showed the first secondary sex characteristics of puberty, a number which corresponds to American children rated at 9 years of age (Herman-Giddens et al., 1997; 2001). Our results indicate that children who already showed secondary characteristics of puberty as compared to those who did not have decreased gray matter density mainly in (pre-) frontal and parietal areas. Indeed, in earlier studies frontal and parietal gray matter decreases have been shown around the onset of puberty (although direct measures of puberty were not reported) (Jernigan et al., 1991; Giedd et al., 1999). It has been suggested that the decrease of gray matter represents the process of synaptic pruning, i.e. elimination of neuronal connections which are infrequently used (Paus, 2005). Our results thus provide a first lead that the possible process of pruning in frontal and parietal regions might be initiated by the onset of pubertal characteristics. We tried to shed light on the etiology of these gray matter decreases. We found that with the transition into puberty total variance in (pre-) frontal gray matter areas increases. This enlarged variance seems to be driven by both genetic and unique environmental factors although a significant contribution of these factors could not be demonstrated. This is possibly due to little variation in pubertal development at this relatively young age. A direct underlying mechanism of secondary sexual development is increased gonadal hormone secretion. Gonadal hormones can alter brain morphological processes (McEwen, 1994) via receptors which are found in various areas of the brain, including the prefrontal cortex (Finley and Kritzer, 1999). Whether testosterone and estradiol are implicated in frontal and parietal gray matter decreases needs further investigation.

As to the genes that might be involved in the heritable brain areas in this critical period of puberty-onset we can merely speculate. However, the identified areas may aid in the identification of genes related to the (development) of specific brain areas. These candidate genes, given the evidence for abnormal brain development

in most (severe) psychiatric disorders, are likely to be involved in the brain pathology of neuropsychiatric disorders with an onset during puberty. A number of studies have been published in which particular genetic polymorphisms, i.e. BDNF (Pezawas et al., 2004; Bueller et al., 2006; Nemoto et al., 2006), DISC1 (Szeszko et al., 2008), COMT (Cerasa et al., 2008) and AKT1 (Tan et al., 2008) are associated with variation in brain structure in the healthy population. It can be argued that certain genes are typically expressed or exert an effect in puberty (Barnett et al., 2007). However, studies of polymorphisms associated with brain volumes in healthy pubertal children are presently lacking.

There are some limitations on the current study. Our study with a sample size of 107 twin pairs has sufficient statistical power (87%) to detect a heritability of global brain volumes of at least 70% (based on a MZ-correlation of .75 and a DZ-correlation of .40). However, within VBM analyses, a correction for multiple comparisons needs to be carried out to minimize false positive results. This strict correction may have prevented areas from reaching significant heritability. Thus, one has to keep in mind that there could be an under-representation of the actual genetically influenced gray or white matter areas. For example, to detect a heritability of 70% within a gray matter voxel, 317 twin pairs are needed to obtain a power of 80% (based on the corrected p-value corresponding to a critical χ^2-value (20.5), applying an FDR of .05 in our sample). One could also argue that the areas that we did find to be significantly heritable can be considered highly reliable.

Second, although our results indicate that with the transition into puberty total variance in frontal gray matter areas increases significantly, we were unable to investigate whether genetic and/or environmental factors contribute to this process. Furthermore, at this relatively young age, we could measure first pubertal signs in 24% of the children, leading to an unequal distribution of this variable, thus decreasing power to detect significant Tanner-related decreases in gray matter. Indeed, to be able to detect a decrease of 10% within density of a single voxel, 1600 pairs are needed to obtain a statistical power of 80% (α=.05, uncorrected). Therefore, our results should be interpreted with caution, i.e. other brain areas that are associated with Tanner-development could stay undetected. Larger samples are

needed with a more equal distribution of children showing Tanner-development versus children without signs of Tanner-development.
A third limitation concerns gene-environment interactions. For example, it has been demonstrated that within different environmental conditions, gene expression in the brain is altered (Bhansali et al., 2007; McNair et al., 2007). Elaborate models testing for gene-environment interactions require larger samples. In addition, other designs are needed to focus on interaction between genes, as a complex phenotype like brain structure is unlikely to be influenced by a single-gene polymorphism. Finally, increased morphological similarities in MZ relative to DZ twins could potentially contribute to increased similarities in gray and white matter density, leading to a higher heritability estimate that is more due to global and not local brain structure characteristics. However, since we non-linearly registered each individual brain separately to the model brain, it seems unlikely that a bias occurred that behaved differently in MZ and DZ twins other than the similarities that we expected to find attributable to differences in the overlap of their genetic makeup.

3.5 Conclusions

At 9 years of age global brain volumes are already strikingly heritable. Particularly, individual variation in posterior white matter (posterior parts of the superior longitudinal and superior/inferior fronto-occipital fascicles) as well as anterior (pre-) frontal and temporal gray matter areas is primarily influenced by genes. The high heritability of posterior white matter overlaps with findings in adults, suggesting stable heritable brain areas across development. Importantly, our study also provides a first lead that with the emergence of the first secondary pubertal characteristics prefrontal and parietal gray matter densities decrease. Future studies should focus on longitudinal measurements in twins to reveal possible changing influences of puberty-related (hormonal) factors, genes and environment upon the dynamically developing adolescent brain.

Chapter 4

Cerebral white matter in early puberty is associated with luteinizing hormone concentrations

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Abstract

Puberty is a period in which cerebral white matter grows considerably, whereas gray matter decreases. The first endocrinological marker of puberty in both boys and girls is an increased secretion of luteinizing hormone (LH). Here we investigated the phenotypic association between LH, global and focal gray and white matter in 104 healthy nine-year old monozygotic and dizygotic twins. Volumetric MRI and voxel-based morphometry were applied to measure global gray and white matter and to estimate relative concentrations of regional cerebral gray and white matter respectively. A possible common genetic origin of this association (genetic correlation) was examined. Results showed that higher LH levels are associated with a larger global white matter proportion and with higher regional white matter density. Areas of increased white matter density included the cingulum, middle temporal gyrus and splenium of the corpus callosum. No association between LH and global gray matter proportion or regional gray matter density was found. Our data indicate that a common genetic factor underlies the association between LH level and regional white matter density. We suggest that the increase of white matter growth during puberty reported earlier might be directly or indirectly mediated by LH production. In addition, genes involved in LH production may be promising candidate genes in (neuro-) psychiatric illnesses with an onset in early adolescence.

4.1 Introduction

Puberty represents the process of physical change towards adulthood leading to the capacity to reproduce. During puberty, global and regional gray matter volumes in the brain decrease (Giedd et al., 1999) and white matter volume increases (Giedd et al.,1999; Paus et al., 1999). Regional white matter increases have been found in the splenium of the corpus callosum and the temporal and parietal lobe (Thompson et al., 2000). Currently, the mechanisms underlying these brain changes during puberty are unclear, although genes are likely to play an important role (Peper et al., 2007). Endocrinologically, puberty is characterized by the activation of the hypothalamic-pituitary-gonadal (HPG) axis. The first measurable endocrinological marker of puberty is a nocturnal rise in luteinizing hormone (LH) from the pituitary gland (Delemarre-Van de Waal et al., 1991). In both boys and girls this is reflected in a nightly increase in LH-pulse frequency and amplitude detectable in first morning urine samples (Demir et al., 1996). The nightly LH-pulses are detectable several years before secondary sexual characteristics become apparent (Boyar et al., 1972; Demir et al., 1996) and are an augmentation of the existing prepubertal circadian LH-secretion (Apter et al., 1989). LH, together with the gonadotropin follicle stimulating hormone (FSH), stimulates the production of sex steroids which leads to the production of testosterone in boys and estrogens in girls. From animal studies, it has been established that sex steroids have organizational properties onto the brain that include neurogenesis and neurite outgrowth (McEwen and Alves, 1999), myelination of axons (Yates and Juraska, 2008) and growth of astrocytic processes in white matter (Chowen et al., 2000). In adult humans, pharmacologically-induced changes in levels of testosterone and estrogens have been shown to alter total brain and hypothalamus volumes (Hulshoff Pol et al., 2006b). Although it has been shown that LH can cross the blood-brain barrier (Lukacs et al., 1995) and LH receptors have been found in various brain areas (Lei et al., 1993), the relation between LH and brain morphology remains unclear. In the current study, we explored the association between LH and gray and white matter structure in a cohort of nine year-old twin pairs, using both volumetric MRI and a voxel-based morphometry approach. In addition, the extent to which a

possible association between LH and gray or white matter structure is determined by genetic or environmental factors was explored. This can be accomplished by comparing cross-trait/cross-twin correlations of monozygotic (MZ) twins, who share 100% of their DNA, with dizygotic (DZ) twin pairs, who share on average 50% of their segregating genes. If cross-trait/cross-twin correlations between LH level and white matter (i.e. the LH level in one twin predicts white matter volume in the co-twin) are larger in MZ twins than in DZ twins, this suggests genetic mediation of the association.

4.2 Methods and materials

4.2.1 Participants

Participants were 104 healthy Dutch twins, recruited from the Netherlands Twin Registry (Boomsma et al., 2006; Van Leeuwen et al., 2008) and included 24 monozygotic (MZ) pairs (11 female and 13 male, 1 male pair incomplete) and 29 dizygotic (DZ) pairs (9 female, 12 male and 8 opposite sex, 1 female pair incomplete) between 9 years, 0 months and 9 years, 8 months old **(Table 4.1)**. Physical and mental health was assessed with a medical history inventory. Children with any mental or physical (endocrinological) illness were excluded from the study. Zygosity of the twins was determined based on DNA polymorphisms, using 8-11 highly polymorphic di-, tri- and tetranucleotide genetic markers. Parents of subjects gave written informed consent to participate in the study. The study was approved by the Central Committee on Research involving Human Subjects (CCMO) of the Netherlands and experiments were in accordance with the Declaration of Helsinki.

	Monozygotic	Dizygotic
N (Individuals)	47	57
Age *(s.d.)*	9.20 (0.10)	9.21 (0.12)
Sex (F/M)	22/25	25/32
Handedness (R/NR)	40/7	47/10
Tanner 0/1 (%)	39/8 (83/17)	44/13 (77/23)
Birth weight *(s.d.)*	2476.0 (461)	2699.8 (530)
Gestational age *(s.d)*	36.7 (1.8)	36.8 (1.8)
LH (U/l) *(s.d.)*	.22 (.18)	.22 (.19)
Prop. Gray *(s.d.)*	.51 (.02)	.51 (.02)
Prop. White *(s.d.)*	.31 (.01)	.31 (.02)

Table 4.1 Demographics of the twin-sample.
Age in mean (*s.d*.) years, F=female, M=male, R=right-handed, NR=non-right handed, Tanner 0/1: number with no development/with development (%). LH: Luteinizing hormone in mean Units per Litre (U/l) *(s.d.),* Prop. Gray= mean proportion gray matter of intracranial volume *(s.d.)*. Prop. white= mean proportion white matter of intracranial volume *(s.d.)*.

4.2.2 MRI acquisition and processing

MRI scans were acquired from a 1.5 Tesla scanner (Philips, The Netherlands). A three-dimensional T1-weighted coronal spoiled-gradient echo scan of the whole head (256 × 256 matrix, TE = 4.6 ms, TR = 30 ms, flip angle = 30^{o}, 160-180 contiguous slices; 1 × 1 × 1.2 mm3 voxels, Field-of-View = 256 mm / 70%) was acquired. Furthermore, a single-shot EPI (Echo Planar Imaging) scan was made as part of a diffusion tensor imaging (DTI)-series (SENSE factor 2.5; flip angle 90^{o}; 60 transverse slices of 2.5 mm; no gap; 128 x 96 acquisition matrix; FOV 240 mm; TE=78 ms) together with a magnetization transfer imaging (MTI) scan (60 transverse slices of 2.5 mm; no gap;128 x 96 acquisition matrix; FOV 240 mm; flip angle 8^{o}; TE=4.5 ms; TR=37.5 ms). Our automatic image processing pipeline was used for segmentation of intracranial volume and global gray (GM) and white matter (WM) of the cerebrum. The T1-weighted images were automatically put into Talairach orientation (Talairach and Tournoux, 1988) without scaling, by registering them to a model brain in Talairach orientation. The two other scans

were registered to the T1-weighted image by minimizing a mutual information joint entropy function (Maes et al., 1997). The co-registered scans were used for automatic segmentation of the intracranial volume, based on histogram analysis and morphology operations. The intracranial volume was subsequently checked visually and edited where necessary. The intracranial segment served as a mask for global gray and white matter segmentation. The software included histogram analysis, mathematical morphology operations, and anatomical knowledge based rules to connect all voxels of interest, as was validated before (Schnack et al., 2001b).

Regional measures of GM and WM concentration ("density") were generated using voxel-based morphometry (VBM) in a similar manner as was done previously (Hulshoff Pol et al., 2006a). VBM included the following steps. First, a model brain was created on a sample of 298 children aged 9 to 14 (including the 104 children discussed in this report), similar to the method used in Grabner et al (2006). The use of a model brain specifically created from children's brains ensures an optimal warping from the individual brains to the model. Second, the binary gray matter (GM) and white matter (WM) masks with voxels of 1 x 1 x 1.2 mm^3 were blurred by a 3D Gaussian kernel (FWHM = 8 mm), in order to gain statistical power. The voxel values of these blurred GM and WM segments (between 0 and 1) reflect the local presence, or concentration, of GM or WM, respectively, and these images are referred to as 'density maps.' Third, in order to compare brain tissue at the same anatomical location in all subjects, the GM and WM segments were transformed into a standardized coordinate system (i.e. the model-brain). These transformations were calculated in two steps. A) The T1-weighted images were linearly transformed to the model-brain. In this linear step a joint entropy mutual information metric was optimized (Maes et al., 1997). B) Nonlinear (elastic) transformations were calculated to register the linearly transformed images to the model-brain up to a scale of 4 mm (FWHM), thus removing global shape differences between the brains, but retaining local differences. For this step the program ANIMAL (Collins et al., 1995) was used. Fourth, the GM and WM density maps were transformed to the model space by applying the concatenated

linear and nonlinear transformations. Finally, the maps were resampled to voxels of size 2 x 2 x 2.4 mm^3.
Voxels with an average GM density below 0.1 were excluded from the GM density voxel-based analysis. Similarly, voxels with an average WM density below 0.1 were excluded from the WM density voxel-based analysis.

4.2.3 LH measurements

LH was determined in morning urine using highly sensitive immunometric assays (Luminiscention), carried out by the endocrinological laboratory of clinical chemistry of the VU Medical Center in Amsterdam (Architect, Abbott Laboratories, Abbott Park, Illinois USA). Samples were collected on two consecutive mornings directly after waking up. It has been demonstrated that first morning urine samples, using highly sensitive immunometric assays, are able to detect nocturnal rises in LH level at the beginning of puberty, even 1-2 years before serum levels of LH increase or secondary sexual signs of puberty are visible (Demir et al., 1996). The detection limit was 0.1 U/l. Urinary LH levels were divided by creatinine level to correct for variations in urine excretion rate. A creatinine correction has been demonstrated to enhance the detection of LH-surges (Kesner et al., 1998).
Secondary sexual characteristics of puberty were measured by a trained researcher (no self-report) using the Tanner-staging questionnaire (Marshall and Tanner, 1969; 1970).

4.2.4 Statistical analysis

The two urinary LH measurements were significantly correlated (r=.52, $p<.0001$). In the analyses, the average of the two LH measures was used. The LH means were not normally distributed (Kolmogorov-Smirnov (K-S) test (Chakravarti et al., 1967): $D = .24$, $p< .05$), therefore a log-transformation was applied leading to a normal distribution of LH ($D =.06$, $p>.20$). Brain volumetric data were normally distributed (p's>.20). In the volumetric analysis of global gray and white matter, a correction for intracranial volume within each person was carried out, by calculating GM and WM proportions of intracranial volume.

Phenotypic correlations (r_p) with 95% confidence intervals (CI) between global GM and WM proportion and LH level were estimated with maximum likelihood using the structural equation modeling software package Mx (Neale et al., 2003). The within-person correlation between LH level and brain structure (r_p), which is independent of zygosity, was estimated while taking into account the dependency of the twin data. Within the applied genetic twin model, phenotypic correlations of MZ and DZ pairs are by definition constrained to be equal. Analyses were done in the entire sample correcting for sex effects on mean gray and white matter or mean LH-level. Furthermore, r_p was estimated for boys and girls separately. To test whether LH level and GM and WM proportion share a common origin, r_p was decomposed into a genetic and environmental part using a standard bivariate twin-model (Neale and Cardon, 1992, or see http://www.psy.vu.nl/mxbib/). This decomposition was based on cross-trait cross-twin correlations in MZ and DZ twins: for example, when the correlation between WM proportion in twin 1 and LH level in twin 2 is higher in MZ twins than in DZ twins, this indicates that a common genetic factor influences LH level and WM proportion. The amount of overlap is reflected by the genetic correlation (r_g). r_g gives the correlation between genetic factors influencing both phenotypes.

The same statistical analyses as applied on global GM and WM proportions, were carried out on regional GM and WM densities throughout the brain, but with a covariate for handedness (right vs. non-right), as handedness has been associated with subtle changes in brain structure (Amunts et al, 2000). In addition, given the number of voxels in the brain, a correction for multiple comparisons was carried out according to the false discovery rate ($\alpha<.05$, two-tailed), allowing for an overall 5% chance of false positives (Genovese et al., 2002). To that end, r_p-maps needed transformation into Z-values via a Fisher-transformation. The critical z-value was 3.39 and the corresponding uncorrected *p*-value was .0007.

4.3 Results

A larger proportion of overall white matter volume was associated with a higher level of LH (r_p=.31 (CI=.11 – .47)) (**Table 4.2**), in the total sample and in boys and girls separately. No significant correlation was found between LH and gray matter proportion. The genetic correlation between LH-level and overall white matter proportion did not reach statistical significance (r_g=.36 (CI=-.02 – .74)).
With respect to the voxel-wise analysis, a higher level of LH was significantly correlated with higher white matter density in parts of the left cingulum (**Table 4.2, Figures 4.1 & 4.2)** (r_p from .35 (posterior) to .46 (medial)), in the middle temporal gyrus bilaterally (.40 (left) to .44 (right)), right superior frontal gyrus (.38) and splenium of the corpus callosum (.32). These positive associations between LH and white matter density were shown in the total sample. However, r_p estimates in boys and girls separately failed to reach significance due to smaller sample sizes.
Genetic correlations ($r_{g)}$ were significant and ranged from .52 in the posterior cingulum, to .67 in the middle temporal gyrus and .76 in the splenium (**Table 4.2**). LH level and gray matter density were not significantly correlated, although some prefrontal and temporal areas suggested a trend for a negative association. Twenty percent of the current sample (N=21) was already showing the first signs of secondary sexual development (8 boys, 13 girls), indicating that our measurements were carried out at the onset of puberty. LH levels of children showing secondary sexual development were equal to LH levels of children without secondary sexual characteristics. In addition, both groups consisted of an equal number of MZ and DZ twin pairs. Leaving the children with secondary sexual development out of the analyses did not change the results.

4.4 Discussion

In a sample of nine-year old twins, we demonstrated that an elevation of the first endocrinological marker of puberty, LH production, is associated with an overall larger cerebral white matter proportion of intracranial volume.

Table 4.2 Phenotypic (r_p) and genetic (r_g) correlations between LH level and white matter.

	Talairach Coordinates						
Area	**X**	**Y**	**Z**	**r_p**	**z-value**	**r_g**	**Heritability**
Total white matter proportion*	-	-	-	.31 (.11 - .47)	-	.36 (-.02 - .74) NS	82%
Cingulum (L anterior)	-13	23	36	.44 (.26 - .58)	4.49	.95 (.20 - 1.00)	18%
Cingulum (L medial)	-18	44	8	.46 (.28 - .60)	4.79	.52 (.12 - .83)	43%
Cingulum (L posterior)	-22	-21	41	.35 (.15 - .51)	3.40	.61 (.25 - 1.00)	65%
Splenium	-6	-38	17	.32 (.13 - .48)	3.44	.76 (.27 - 1.00)	39%
Mid Temporal Gyrus (R posterior)	55	-31	-8	.38 (.19 - .54)	3.77	.37 (-.12 - .73) NS	46%
Mid Temporal Gyrus (R medial)	54	-59	11	.44 (.27 - .57)	4.69	.67 (.26 - 1.00)	34%
Mid Temporal Gyrus (L posterior)	-47	-73	12	.40 (.22 - .55)	4.11	.80 (.35 - 1.00)	37%
Sup Frontal Gyrus (R)	17	-31	51	.38 (.18 - .55)	3.51	.90 (.40 - 1.00)	36%

* White matter volume was calculated as a proportion of intracranial volume.
R=right hemisphere, L=left hemisphere, Mid=middle, Sup=superior, r_p=observed phenotypic correlation (95% confidence interval), r_g=genetic correlation (95% CI), NS=not significant. Correlations were transformed to z-values (Fisher transformation). The critical z-value was 3.39 (corrected for multiple comparisons). Heritability is the proportion genetic variance over the total variance. The heritability of LH was estimated at 76% (28 - 97%).

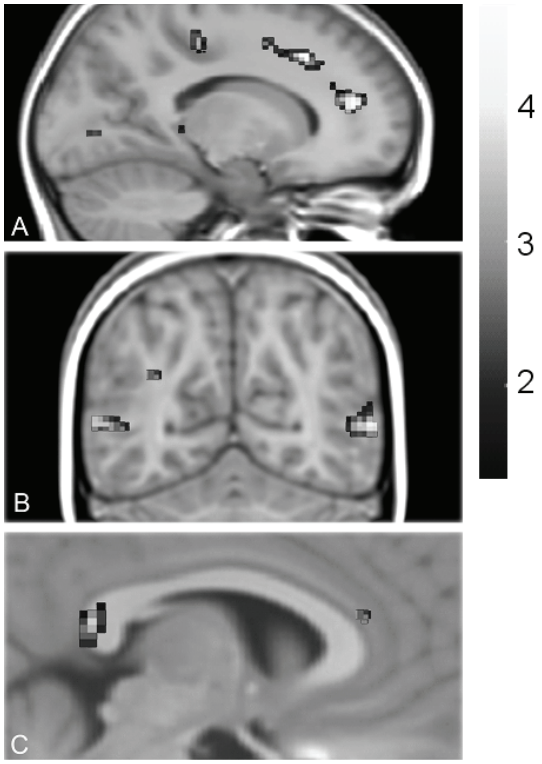

Figure 4.1 Positive phenotypic associations between LH level and regional white matter density in 104 9-year old twins. A) Left cingulum B) Bilateral middle temporal gyrus C) Splenium of the corpus callosum. Displayed are z-values. The critical z-value was 3.39 (corrected for multiple comparisons according to the False Discovery Rate α=.05. For a colour version of this illustration see Appendix, Figure 4.1

In addition, we found that a higher LH level was correlated with higher regional white matter density in the left cingulum, the middle temporal gyrus bilaterally, superior frontal gyrus and the splenium of the corpus callosum. A common genetic factor underlies the association between LH level and regional white matter density. The current findings could not be explained by age variation (i.e. all children were 9 years of age) and largely preceded the development of secondary sexual characteristics. The splenium of the corpus callosum as well as the temporal areas were found to develop most rapidly between 9-13 years of age as compared to younger and older children (Thompson et al., 2000).

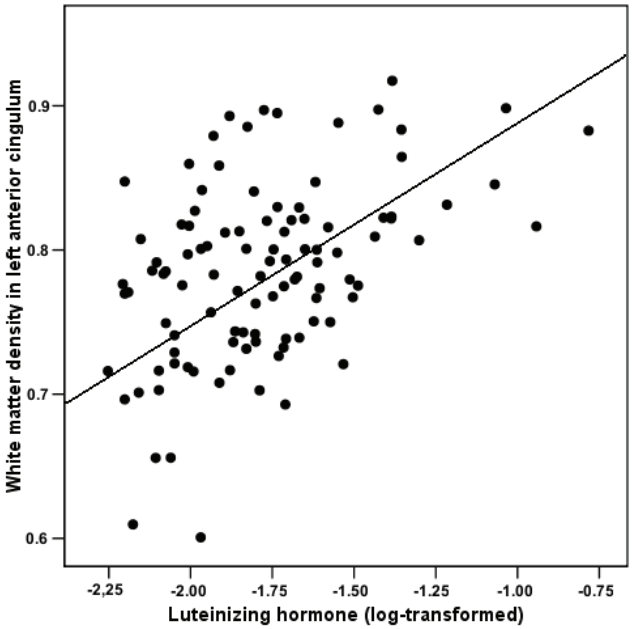

Figure 4.2 Scatterplot of phenotypic association between LH level and white matter density in the left anterior cingulum**.** Depicted are LH levels versus white matter densities in the left anterior cingulum (Talairach x, y & z coordinates -13, 23, 36). The phenotypic correlation coefficient is 0.44 (95% CI=0.26-0.58). The LH-values are log-transformed and ranged between -2.25 and -.78, corresponding to LH values ranging between 0.056 and 0.167 U/l (devided by creatinine-level). The actual raw uncorrected LH values range between 0.1 and 1.1 U/l

Strikingly, the areas that we found to be positively associated with LH level at 9 years are in fact those that develop fastest in children between 9 and 13 years (Thompson et al., 2000). Since parts of the cingulum bundle and splenium both project to the temporal lobes (Wakana et al., 2004), these areas might present a neural network susceptible to the influence of increased LH production in early puberty. Evidently, from our data a causal relation between LH-level and white matter can not be inferred and studies into LH and its effects on brain morphology have been limited. Therefore, we can merely speculate on the underlying mechanism(s) of the associations we report here. Possibly, LH-production is directly associated with morphological processes in the brain. For example, it has

been found that astrocyte plasticity in the hypothalamus affects LH-surges in rats (Cashion et al., 2003). Alternatively, the observed effect of LH might be an indirect result of sex steroids, as sex steroids are the end-products of the HPG-axis. Indeed, myelination of axons in the splenium is affected by manipulating levels of estrogen as demonstrated in pubertal rats (Yates and Juraska, 2008). Consequently, we are not able to exclude the contribution of other (sex steroid) hormones to the association between LH and white matter structure. However, in the early stages of puberty it has been found that the pulsatile production of LH during the night precedes the secretion of gonadal steroids by 1 to 2 years (Demir et al., 1996). It might be argued that at this age there is little gonadal hormone production, indirectly shown by the small number of children demonstrating secondary sexual characteristics of puberty. Furthermore, leaving these children with signs of secondary sexual characteristics out of the analyses did not change the results.

LH is a sensitive index of early pubertal development (Demir et al, 1996). However, it has also been suggested that the LH/FSH-ratio is a sensitive index of early pubertal development (Demir et al 1995). Therefore, in a posthoc analysis, the ratio between urinary LH and FSH was calculated. FSH was determined in first morning urine samples using immunometric assays (Luminiscention). The ratio between LH and FSH was not significantly correlated with global gray or white matter proportion or with regional gray or white matter densities. We could therefore argue that LH, and not FSH, is underlying the associations with white matter structure. Another pituitary hormone interrelated with LH-release (Dunger et al., 1991; Rosenfield, 1994) is prolactin. Interestingly, in the murine central nervous system, prolactin treatment was capable of increasing myelination of axons, by oligodendrocyte regeneration (Gregg et al., 2007). Around the onset of puberty, there might be involvement of prolactin production in the observed increased white matter density. However, in early puberty it has been demonstrated that there is a substantial sex difference in the production of prolactin (Dunger et al., 1991). In the current study, we found similar associations between LH level and white matter density in both sexes. It therefore seems unlikely that in this early pubertal period, prolactin is involved in the observed associations.

Our results indicate that the association between LH-level and white matter density is driven by a common genetic factor, reflected by a significant genetic correlation between the two traits. It must be noted that the 95% confidence intervals corresponding to these genetic correlations are wide (due to the sample size). Recent studies point to the importance of the KiSS1-gene in the initiation of puberty. More specifically, a peptide-product of the KiSS1-gene, kisspeptine, is expressed throughout the brain (for review see Smith and Clarke, 2007). During puberty, mRNA expression of the KiSS1-gene was reported to be upregulated in the hypothalamus (Navarro et al., 2004). Kisspeptine, together with its G-coupled receptor GPR54, was found to stimulate LH secretion in both mice (Dungan et al., 2007) and humans (Dhillo et al., 2005). The KiSS1 gene might be a member of a network of genes that contributes to integrating glia-to-neuron communication into a functional unit capable of initiating puberty (Ojeda et al., 2006b). It may be speculated that integration of glia-to-neuron communication could be reflected in increased white matter density as we found in MRI scans with increasing stage of puberty. However, this requires further investigation.

Human studies that examine the effects of LH on brain structure are currently lacking. However, several neuropsychiatric illnesses such as schizophrenia and Alzheimer disease have been associated with abnormal levels of LH (Ferrier et al., 1983; Bowen et al., 2000) together with white matter abnormalities (Hulshoff Pol et al., 2004; Xie et al., 2006; Sydykova et al., 2007). Interestingly, schizophrenia has a typical onset around puberty (Sham et al., 1994). One may therefore hypothesize that the onset of mental disorders like schizophrenia is related to altered hormonal (LH) influences, leading to abnormal brain development. Our finding that there is a common genetic factor to LH level and regional white matter densities, suggest that genes involved in LH production may be promising candidate genes in psychiatric illnesses with an onset in early adolescence. Among these potential candidate genes are for example the earlier mentioned KiSS1-gene, the LH-receptor gene (Wu et al., 2000), and the ErbB-1 and ErbB-4 genes (belonging to the family of epidermal growth factors). The ErbB-1 and ErbB-4 receptors are located on astrocytes, and both genes have been implicated in LH secretion and pubertal development (Prevot et al., 2005).

In the current study we were unable to demonstrate an association between global gray matter proportion or regional gray matter density and LH. A possible explanation for this might be that the gray matter decrease around puberty-onset observed in earlier studies (Giedd et al., 1999; Sowell et al., 2002) might not have begun in the main part of our sample at 9 years of age. Also, the possible underlying neural substrates including nuclear size, dendrite length or number of synapses might be less sensitive (if at all) to the first endocrinological marker of puberty. Alternatively, one can argue that endogenous LH levels were currently too low to be able to affect gray matter.

The narrow age range of the subjects can both be considered as an advantage (one can measure associations between LH levels and brain structure without being affected by age-related factors) as well as a limitation. Since all subjects were 9 years of age, we cannot state that the observed association between LH and white matter is specific for this age period.

In conclusion, our results suggest that the earlier reported white matter growth during puberty might be directly or indirectly mediated by LH production. In addition, genes involved in LH production may be promising candidate genes in (neuro-) psychiatric illnesses with an onset in early adolescence. The results of this study provide important new leads into the complex interplay between pubertal hormones and the developing human brain.

Chapter 5

Sex steroids and brain structure in pubertal boys and girls

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Abstract

Sex steroids exert important organizational effects on brain structure. Early in life, they are involved in brain sexual differentiation. During puberty, sex steroid levels increase considerably. However, to which extent sex steroid production is involved in structural brain development during human puberty remains unknown. The relationship between pubertal rises in testosterone and estradiol levels and brain structure was assessed in 37 boys and 41 girls (10-15 years). Global brain volumes were measured using volumetric-MRI. Regional gray and white matter was quantified with voxel-based morphometry (VBM), a technique which measures relative concentrations ('density') of gray and white matter after individual differences in global size and shape of brains have been removed.

Results showed that, corrected for age, global gray matter volume was negatively associated with estradiol levels in girls, and positively with testosterone levels in boys. Regionally, a higher estradiol level in girls was associated with decreases within prefrontal, parietal and middle temporal areas (corrected for age), and with increases in middle frontal-, inferior temporal -and middle occipital gyri. In boys, estradiol and testosterone levels were not related to regional brain structures, nor were testosterone levels in girls. Pubertal sex steroid levels could not explain regional sex differences in regional gray matter density. Boys were younger than girls, which may explain part of the results.

In conclusion, in girls, with the progression of puberty, gray matter development is at least in part directly associated with increased levels of estradiol, whereas in boys, who are in a less advanced pubertal stage, such steroid-related development could not (yet) be observed. We suggest that in pubertal girls, estradiol may be implicated in neuronal changes in the cerebral cortex during this important period of brain development.

5.1 Introduction

Puberty is an episode in life which is characterized by hormonal fluctuations. During this period, there is a marked increase in sex steroids testosterone and estradiol, the end products of the maturing hypothalamus-pituitary-gonadal (HPG)-axis (Grumbach and Styne, 2003).

During puberty, sex steroids lead to the maturation of secondary sexual characteristics in a sex-specific manner (Grumbach and Styne, 2003). However, sex steroids also exert a wide range of effects on brain morphology, as the brain is an important target tissue for steroid hormone receptors (McEwen et al., 1982; McEwen, 1984). Among the important organizing effects of sex steroids is their influence on neurogenesis, receptor expression and neurite outgrowth (Romeo and McEwen, 2004). Most of these organizational effects take place during the prenatal period; however, these effects are subject to changing hormonal fluctuations throughout life and are not always irreversible (Pilgrim and Hutchison, 1994). Indeed, in adults, pharmacologically-induced changes in levels of testosterone and estradiol have been shown to alter total brain and hypothalamus volumes (Hulshoff Pol et al., 2006b).

During puberty, global gray matter volume within the cerebrum decreases (Giedd et al., 1999) and white matter volume increases (Giedd et al.,1999; Paus et al., 1999). Also, region-specific gray matter decreases have been reported in frontal, parietal and temporal areas (Jernigan et al., 1991; Giedd et al., 1999; Sowell et al., 2002; 2004; Gogtay et al., 2004; Wilke et al., 2007). It has been suggested that these neuro-anatomical changes during puberty and adolescence reflect the refinement of neuronal connections which could be related to cognitive and emotional development (Paus, 2005; Blakemore, 2008).

In keeping with the notion that puberty is a period of steroid-dependent brain organization (Romeo, 2003), the idea has been put forward that around the onset of puberty hormonal changes trigger selective neuro-anatomical alterations, as indirectly shown by gray matter decreases in frontal and parietal lobes (Giedd et al., 1999). Recently, in the early phase of puberty (at 9 years of age), elevated levels of the precursor of sex hormones, luteinizing hormone (LH) were found to

be associated with cerebral white matter increases, whereas LH was not associated with gray matter (Peper et al., 2008). With the emergence of secondary characteristics of puberty (a result of sex steroid production), gray matter density decreases were found in frontal and parietal areas in 9-year olds (Peper et al., in press (b)). Thus, during early puberty, variation in pubertal mechanisms is accompanied by distinct structural brain changes. Interestingly, it was recently argued that the adolescent brain might respond differentially to changing steroid hormones levels over time (Sisk and Zehr, 2005) as brain development during puberty and adolescence is a dynamic and protracted process characterized by region specific gray matter decreases and global white matter increases (Giedd et al., 2006). Consequently, it can be suggested that sex steroids testosterone and estradiol play a more prominent role in brain development in an advanced stage of puberty, compared to LH which is a marker of early puberty (Demir et al., 1996). In a first recent study on the interrelations between brain organization and sex steroid hormones in 30 children between 8 and 15 years of age, sex steroid levels were associated with sexual dimorphic gray matter areas (Neufang et al., 2008).

The aim of the current study was to explore the interrelations between naturally occurring pubertal rises in testosterone and estradiol and brain structure in 10 to 15-year-old boys (37 subjects) and girls (41 subjects). As the progression of puberty is associated with global and regional gray matter decreases and white matter increases, it was expected that sex steroids are involved in these processes. The production of sex steroids occurs in a sex-specific manner. Also, the exposure to sex steroids has been implicated in the development and/or maintenance of sex differences in brain structure and these organizational effects of steroids are subject to changing hormonal fluctuations throughout life (Pilgrim and Hutchison, 1994). Therefore, we assessed the influence of sex steroids in boys and girls separately. Moreover, we explored to what extent pubertal rises of testosterone and estradiol levels are associated with sexually dimorphic brain areas.

5.2 Methods and materials

5.2.1 Participants

The total sample consisted of 78 children between 10.0 and 14.9 years **(Table 5.1)**, including 37 boys and 41 girls. These children are older siblings of twin pairs which are described elsewhere (Peper et al., in press (b); Peper et al., 2008; Van Leeuwen et al., 2008). Exclusion criteria consisted of any major medical or psychiatric illness and participation in special education. Parents and the participants themselves gave written informed consent to participate in the study. The study was approved by the Central Committee on Research involving Human Subjects (CCMO) of the Netherlands and was in agreement with the Declaration of Helsinki (Edinburgh amendments).

5.2.2 MRI acquisition

MRI scans were acquired from a 1.5 Tesla scanner (Philips, The Netherlands). A three-dimensional T1-weighted coronal spoiled-gradient echo scan of the whole head (256 × 256 matrix, TE = 4.6 ms, TR = 30 ms, flip angle = 30^{o}, 160-180 contiguous slices; 1 × 1 × 1.2 mm^3 voxels, Field-of-View = 256 mm / 70%) was acquired. Furthermore, a single-shot EPI (Echo Planar Imaging) scan was made as part of a diffusion tensor imaging (DTI)-series (SENSE factor 2.5; flip angle 90^{o}; 60 transverse slices of 2.5 mm; no gap; 128 x 96 acquisition matrix; FOV 240 mm; TE=78 ms) together with a magnetization transfer imaging (MTI) scan (60 transverse slices of 2.5 mm; no gap;128 x 96 acquisition matrix; FOV 240 mm; flip angle 8^{o}; TE=4.5 ms; TR=37.5 ms).

5.2.3 Volumetric processing

Our automatic image processing pipeline was used for segmentation of intracranial volume, cerebellum and global gray (GM) and white matter (WM) of the cerebrum. The T1-weighted images were automatically put into Talairach orientation

	Boys	**Girls**
N (individuals)	37	41
Age *(mean, s.d.)* *	11.6 (1.0)	12.2 (1.2)
Mean Testosterone (pmol/l) (*s.d.*) (range)	70.8 (66.2) (15.4-285.6)	59.3 (35.7) (15.3-198.6)
Mean Estradiol (pmol/l) (*s.d.*) (range) *	1027.0 (698.8) (249.4-3829.9)	2371.6 (1332.2) (719.5-6157.6)
Mean Tanner A (*s.d.*)*	1.6 (0.8)	2.9 (0.9)
Mean Tanner B (*s.d.*)*	1.5 (0.6)	2.8 (1.3)
Mean Tanner C (*s.d.*)	1.6 (0.7)	-
Mean Height (*s.d.*)	152.7 (9.4)	156.0 (8.8)
Handedness (R/NR)	32/5	38/3

Table 5.1 Demographics of the sample
*) significant at $p<.05$ (corrected for age). Age in years (*s.d.*), Tanner A=penis growth in boys (1-5), breast development in girls (1-5), Tanner B=pubic hair development (1-6), Tanner C=testis size (1-4), Height in centimeters, Handedness: R= number of right-handed, NR=number of non-right handed. NB. Testosterone-levels were available in 29 boys and 38 girls; estradiol-levels were available in 37 boys and 35 girls.

(Talairach and Tournoux, 1988) without scaling, by registering them to a model brain in Talairach orientation. The two other scans were registered to the T1-weighted image by minimizing a mutual information joint entropy function (Maes et al., 1997).

The co-registered scans were used for automatic segmentation of the intracranial volume, based on histogram analysis and morphology operations. The intracranial segment was subsequently checked visually and edited where necessary. The intracranial segment served as a mask for global gray and white matter segmentation. The software included histogram analysis, mathematical morphology operations, and anatomical knowledge based rules to connect all voxels of interest, as was validated before (Schnack et al., 2001b).

5.2.4 Voxel-based morphometry

Regional measures of GM and WM concentration ("density") were generated using voxel-based morphometry (VBM) in a similar manner as was done previously (Peper et al., 2008). VBM included the following steps. First, a model brain was created on a sample of 298 children aged 9 to 14 (including the 78 children discussed in this study), similar to the method used in Grabner et al. (2006). The use of a model brain specifically created from children's brains ensures an optimal warping from the individual brains to the model. Second, the binary GM and WM masks with voxels of 1 x 1 x 1.2 mm^3 were blurred by a 3D Gaussian kernel (FWHM = 8 mm), in order to gain statistical power. The voxel values of these blurred GM and WM segments (between 0 and 1) reflect the local presence, or concentration, of GM or WM, respectively, and these images are referred to as "density maps". Third, in order to compare brain tissue at the same anatomical location in all subjects, the GM and WM segments were transformed into a standardized coordinate system (i.e. the model brain). These transformations were calculated in two steps. A) The T1-weighted images were linearly transformed to the model brain. In this linear step a joint entropy mutual information metric was optimized (Maes et al., 1997). B) Nonlinear (elastic) transformations were calculated to register the linearly transformed images to the model brain up to a scale of 4 mm (FWHM), thus removing global shape differences between the brains, but retaining local differences. For this step the program ANIMAL (Collins et al., 1995) was used. Fourth, the GM and WM density maps were transformed to the model space by applying the concatenated linear and nonlinear transformations. Finally, the maps were resampled to voxels of size 2 x 2 x 2.4 mm^3. Voxels with an average GM density below 0.1 were excluded from the GM density voxel-based analysis. Similarly, voxels with an average WM density below 0.1 were excluded from the WM density voxel-based analysis.

5.2.5 Hormonal measurements

Free (bioavailable) testosterone levels were determined in first saliva (Competitive immunoassay (luminiscention), IBL Hamburg). The intra-assay and inter-assay coefficients of variation (CVs) were below 12% at the lower limit of detection of 11 pmol/l. Total estradiol levels were determined in first morning urine (Competitive immunoassay (luminiscention), Architect, Abbott Laboratories, Abbott Park, Illinois USA). The intra-assay and inter-assay CVs were 5% and 10% respectively at levels > 150 pmol/l (lower limit of detection) and < 9000 pmol/l (upper limit of detection). Urinary estradiol levels were divided by creatinine level to correct for variations in urine excretion rate.

Both testosterone and estradiol data were collected on two consecutive days at consistent times directly after waking up. Analyses were carried out by the endocrinological laboratory of clinical chemistry of the VU Medical Center in Amsterdam, the Netherlands. Via the enzyme aromatase, testosterone is (partly) converted into estradiol (see Collaer and Hines, 1995); therefore testosterone and estradiol are analyzed in both boys and girls. The two consecutive testosterone and estradiol measurements were significantly correlated in both boys (r=.70, p<.00001 and r=.47, p<.003) and girls (r=.69, p<.00001 and r=.60, p<.00001). In the analyses, averages of the two measurements were used.

Secondary sexual characteristics of puberty were measured by a trained researcher using a Tanner questionnaire (no self-report) (Marshall and Tanner, 1969; 1970). A regular menstrual cycle was present in 5 girls (12%). None of the female participants used oral contraceptives.

Testosterone levels were available of 29 boys and 38 girls, and estradiol levels in 37 boys and 35 girls: estradiol and testosterone samples were not collected from 6 children, whereas in 5 other children testosterone levels were below the detection limit of 11 pmol/l.

5.2.6 Statistical analysis

Data were examined for normality in girls and boys separately. In boys, the averages of testosterone and estradiol were not normally distributed (Kolmogorov Smirnov (KS) test: $p<.006$ and $p<.002$ respectively); therefore a log-transformation was applied leading to a normal distribution of testosterone and estradiol levels (KS-test: $p=.36$ and $p=.10$). In girls, mean testosterone and estradiol levels were normally distributed (KS-test: $p=.34$ and $p=.43$). However, to consistently analyze the data within boys and girls, testosterone and estradiol data in girls were also log-transformed (leading to the following KS-test results: $p=.64$ and $p=.91$ respectively). A possible explanation for the difference in distributions of hormonal data in boys and girls could be due to the presence of a larger number of boys with relatively low hormonal levels as compared to boys with relatively high levels. Within the group of girls, the number of high and low hormonal levels was more equally distributed.The association between testosterone or estradiol and global brain volumes (total brain, cerebellum and gray and white matter) was investigated with a (linear) regression analysis within boys and girls separately, corrected for age. Furthermore, to investigate the relation between sex steroids and relative amount of gray and white matter and cerebellum volume, the association between sex steroids and cerebellum, gray and white matter was subsequently corrected for total brain volume.

In the voxel-based analysis, a (linear) regression was carried out to estimate the effect of hormone level on regional gray and white matter density within boys and girls separately, corrected for age and handedness. T-tests were performed to establish significance. A correction for multiple comparisons was carried out according to the false discovery rate (FDR; $\alpha=.05$, two-tailed) allowing for an average of false positives of 5% (Genovese et al., 2002). Sex differences in global brain volume and regional gray and white matter density were analyzed in boys and girls together. The effect of sex was estimated with a (linear) regression analysis, correcting for age. Also, the interaction between sex and age was investigated. In the voxel-based analysis, T-tests were performed to establish significance (FDR; $\alpha=.05$, two-tailed).

5.3 Results

5.3.1 Pubertal hormones and secondary sexual characteristics

On average, the girls were older than boys ($F_{(1,77)}$=5.45, p<.02) (**Table 5.1**). Moreover, girls were in more advanced stages of puberty (mean Tanner stage) ($F_{(1,77)}$=33.83, p<.0001). Indeed, all girls showed development of secondary sexual characteristics, compared to 62 % of the boys (N=23). After correction for age, testosterone levels were equal in boys and girls ($F_{(1,66)}$=1.06, p=.31). Estradiol levels were higher in girls as compared to boys ($F_{(1,71)}$=32.20, p<.0001). The difference in estradiol level between boys and girls and the equal level of testosterone in boys and girls remained unchanged without an age-correction ($F_{(1,71)}$=38.06, p<.0001) and ($F_{(1,66)}$=0.11, p=.74) respectively. Corrected for age, testosterone and estradiol levels were significantly correlated in girls (r=.49, p<.005), but not in boys (r=.28, p<.15). Without correcting for age, these correlations were .60 (p<.001) and .27 (p<.15) respectively. Except for estradiol in boys, age was highly correlated with steroid levels in both sexes (r's > .41) (**Table 5.2**).

	Estradiol		Testosterone	
	M	*F*	*M*	*F*
Tanner A	.13	.69*	.48*	.60*
Tanner B	-.07	.67*	.26	.63*
Tanner C	-.06	-	.32	-
Age	.18	.59*	.59*	.41*

Table 5.2 Correlations between pubertal hormones, Tanner-stages (Spearman's Rho) and age (Pearson's r). *) Significant at p<.01. M=male, F=female, Tanner A=penis growth in boys & breast development in girls, Tanner B=pubic hair development, Tanner C=testis size.

5.3.2 Associations between sex steroids and brain structures

Global brain volumes

Age was not significantly correlated with absolute brain volumes within both sexes. However, after controlling for total brain volume, in girls, gray matter decreased with age (r= -.36, p<.02) whereas white matter increased (r=.37, p<.02). In boys, age and gray or white matter volumes were not significantly associated after controlling for total brain volume. In girls, after correcting for age, a higher estradiol level was correlated with a smaller gray matter volume (r=-.39, p<.03) (**Table 5.3**). After correcting for total brain volume, this correlation was no longer significant. Estradiol level did not correlate with global brain volumes in boys. After correcting for age, a higher level of testosterone in boys was correlated with a larger gray matter volume (r=.41, p<.03) (**Table 5.3**). After correcting for total brain volume, this association was no longer significant. Testosterone was not associated with brain volumes in girls.

	Age		**Testosterone** [a)]		**Estradiol** [a)]	
Volume	*M*	*F*	*M*	*F*	*M*	*F*
Total brain	.22	-.12	.32[§]	-.06	.13	-.32[§]
Gray matter	.10	-.27[§]	.41[*]	-.11	.20	-.39*
White matter	.27	.11	.17	.04	-.04	-.17
Cerebellum	.22	-.09	-.10	.06	.12	-.21

Table 5.3 Associations between global brain volumes, age and sex steroids. [a)] Corrected for age; * Significant at p<.05, § p=.07; M=male, F=female. Depicted are (partial) correlations (Pearson's r).

Regional gray and white matter density

Significant main effects of age were found in girls only, comprising gray matter decreases within the precentral gyrus bilaterally, superior temporal gyrus bilaterally, left angular gyrus, left middle frontal and right inferior temporal gyri (with t-values up to 7.24 (critical t-value of significance was 3.35)). After controlling for age and handedness, in girls a higher estradiol level was associated

with a lower gray matter density in prefrontal (superior-, inferior- and orbitofrontal gyri), parietal (supramarginal and angular gyri) and middle temporal areas with t-values up to 5.77 (critical t-level of significance was 4.60) (**Table 5.4, Figures 5.1a & 5.2**). The reversed pattern (higher gray matter density with a higher estradiol level) could be observed in (parts of) the middle frontal-, inferior temporal -and middle occipital gyri with t-values up to 5.35 (critical t-level of significance was 4.60) (**Table 5.4, Figure 5.1b)**. Estradiol levels were not associated with white matter in girls. In boys, estradiol level was not associated with regional gray or white matter density. Testosterone levels were not significantly associated with gray or white matter density, in boys or in girls, although in girls a trend was found for gray matter decreases in the right fusiform gyrus, left inferior frontal gyrus and left middle temporal gyrus with increased levels of testosterone (t=4.90; at an FDR-value of α=.07).

To check for possible confounding effects of intra-individual variation of estradiol levels in the 5 (regularly) menstrual cycling girls, these subjects were removed from a subsequent analysis. Results showed no substantial change of the data: gray matter volume decreases remained significantly related to higher levels of estradiol and the same regional gray matter areas were significantly related to estradiol levels.

5.3.3 Sex differences in brain structures

Global brain volumes

After correcting for age, boys showed a larger (absolute) total brain volume ($F_{(1,77)}$=26.38, p<.000002), cerebellum ($F_{(1,77)}$=4.99, p<.03), white matter ($F_{(1,77)}$=22.39, p<.0001) and gray matter ($F_{(1,77)}$=21.92, p<.00001). There were no sex differences in cerebellum, global gray or white matter after correcting for total brain volume.

Table 5.4 Significant associations between estradiol level and gray matter density in girls (N=35).

Area	t	*r*	Talairach Coordinates		
Increases			*X*	*Y*	*Z*
Mid. Front Gyrus R	5.35	.69	35	19	47
Inf. Temp Gyrus R	4.65	.64	45	-7	-35
Mid. Occ Gyrus R	4.95	.66	39	-77	3
Decreases					
Inf. Front. Gyrus L- Ant.	5.77	-.72	-40	52	3
Inf. Front. Gyrus L- Post	5.25	-.65	-57	14	3
Sup. Front. Gyrus L (R)	5.77 (4.90)	-.72 (-.66)	-11 (15)	42 (46)	50 (47)
Orbitofrontal Gyrus- Post.(L)	4.90	-.66	-26	42	-10
Mid. Front. Gyrus L	4.81	-.66	-43	34	30
Supramarginal Gyrus R (L)	5.33 (4.70)	-.69 (-.64)	65 (-60)	-31 (-35)	36 (37)
Mid. Temp. Gyrus R	5.59	-.71	65	-29	-1
Angular gyrus L	5.59	-.71	-48	-56	47

Ant.=anterior, Front.=frontal, Inf.=inferior, L=left, Mid.=middle, Occ.=occipital, R=right, Sup.=superior, Temp.=temporal. Critical t-value according to FDR (α=.05) was 4.60. *r*=partial correlation (corrected for age/handedness). Voxels with the highest t-values within significant brain areas are depicted.

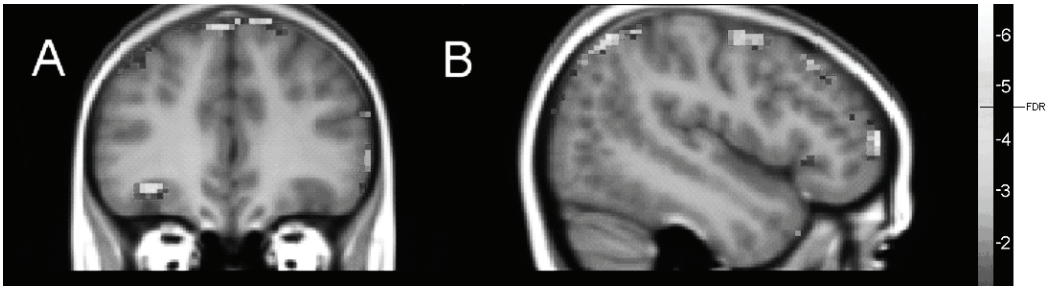

Figure 5.1a Estradiol associated with gray matter decreases (N=35 girls (10-15 years old)), corrected for age.
Bilateral superior- and left orbitofrontal gyri (A), Y=42, and right inferior frontal and angular gyri (B), X=-48. Images are according to neurological convention (left=left). Critical level of significance is -4.60, corrected for multiple comparisons according to the False Discovery rate, α=.05, two-tailed). Significant voxels are overlaid on our created model brain. For a colour version of this illustration see Appendix, Figure 5.1a.

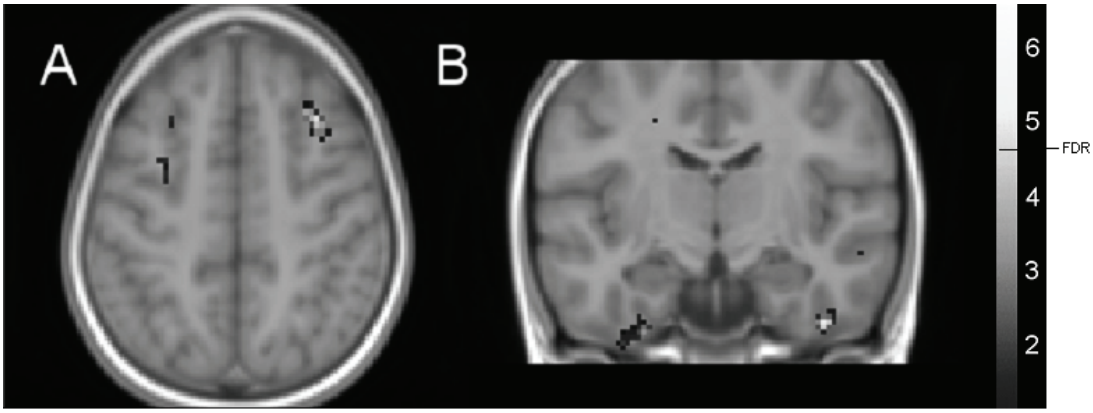

Figure 5.1b Estradiol associated with gray matter increases (N=35 girls (10-15 years old)), corrected for age.
Right middle frontal gyrus (A), Z=47 and right inferior temporal gyrus (B), Y=-7. Images are according to neurological convention (left=left). Critical level of significance is 4.60, corrected for multiple comparisons according to the False Discovery rate, α=.05, two-tailed. Significant voxels are overlaid on our created model brain. For a colour version of this illustration see Appendix, Figure 5.1b.

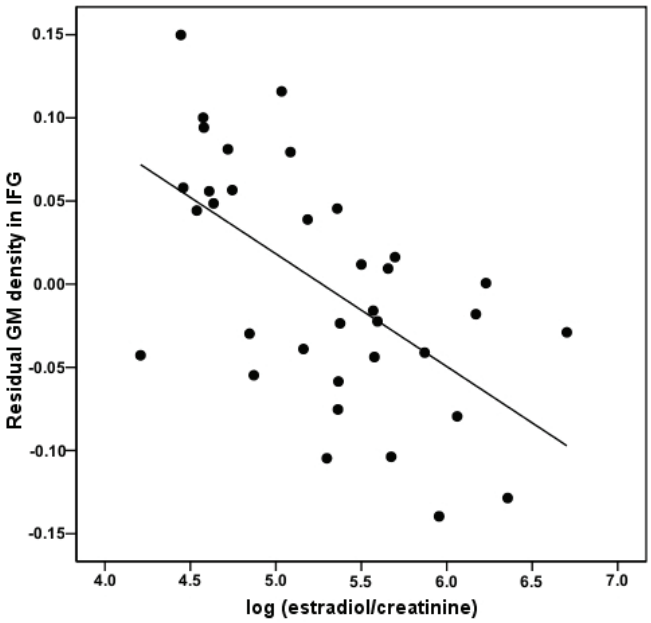

Figure 5.2 Estradiol associated with gray matter decrease within the inferior frontal gyrus (N=35 girls).
The graph represents the residual of gray matter density (corrected for age effect) within the left inferior frontal gyrus (IFG) (Talairach-coordinates: -40, 52, 3). The corresponding partial correlation is -.72.

Regional gray matter density

The critical t-value of significance, corrected for multiple comparisons (FDR, α=.05) was 2.88. After correcting for age and handedness, boys showed increased gray matter density mainly in the right middle temporal gyrus, right inferior frontal gyrus, insular gyrus bilaterally, putamen bilaterally, left rostral anterior cingulate gyrus, hypothalamus, thalamus, globus pallidus, amygdala and left middle occipital gyrus with t-values ranging from 3.85 to 7.27 (**Table 5.5, Figure 5.3a**). Girls had a higher gray matter density in small parts of the right posterior hippocampus, right insula, right anterior caudate nucleus, left inferior frontal gyrus and left caudal anterior cingulate cortex with t-values ranging from 2.94 to 5.38 (**Table 5.5, Figure 5.3b**). The most pronounced sexual dimorphic areas (M>F) included the

putamen, insula and amygdala with corresponding t-values of 6.85, 7.28 and 7.53 respectively. There were no significant interactions between age and sex for gray and white matter densities.

5.4 Discussion

To our knowledge, this is the first study investigating interrelations between naturally occurring pubertal rises in testosterone and estradiol and brain structure in 10-15 year old boys (37 subjects) and girls (41 subjects) separately. In girls, a higher level of estradiol was associated with a smaller global gray matter volume, after correcting for age-related decreases in global gray matter. In boys, no significant age-related changes in global gray or white matter could be observed. However, a higher level of testosterone in boys was associated with larger gray matter volumes. These effects were also in part driven by total brain volume. Regional age-related changes were observed in girls only, including gray matter decreases within the outside borders of middle frontal, angular gyrus of the parietal lobe, superior and inferior temporal gyri. Corrected for age, an increased level of estradiol in girls was associated with decreased gray matter densities in the superior-, inferior- middle- (left) and orbitofrontal gyri, supramarginal and angular gyri of the parietal lobe and middle temporal gyrus. In girls, estradiol-related increases were associated with gray matter densities of the middle frontal (right), inferior temporal and middle occipital gyri. These estradiol-related brain areas did not show sex differences in density. Prominent regional sex differences in several brain structures were found in the amygdala, putamen, thalamus, insula, rostral anterior cingulate, and superior temporal gyrus (larger in boys) and in the hippocampus, caudate nucleus, caudal anterior cingulate, middle temporal gyrus and inferior occipital gyrus (larger in girls). These areas did not show associations with pubertal sex steroid levels.

Table 5.5 Sexually dimorphic brain areas (gray matter density), measured in 78 children.

Area	t	Talairach Coordinates		
Boys > Girls		*X*	*Y*	*Z*
Inf. Front. Gyrus R	6.27	56	-9	12
Insula L (R)	7.27 (5.90)	-38 (39)	-3 (-5)	13 (11)
Ant. Cingulate - rostral L	6.53	-1	35	-6
Putamen L (R)	6.21 (4.58)	-28 (26)	-8 (7)	4 (3)
Amygdala L	7.58	-21	-10	-16
Thalamus L	5.21	-8	-27	5
Sup. Temp. Gyrus L	3.85	-60	-9	3
Post. Cingulate Gyrus L	4.52	-2	-38	26
Girls > Boys				
Inf. Front. Gyrus R	3.41	41	37	10
Ant. Cingulate -caudal L	4.48	-8	46	13
Hippocampus R	2.94	32	-29	-8
Caudate nucleus R	3.79	10	7	13
Mid. Temp. Gyrus R	4.63	54	-2	-23
Sup. Temp. Gyrus R	4.59	45	-28	15
Inf. Occ. Gyrus L	5.38	-47	28	-14

Ant.=anterior, Front.=frontal, Inf.=inferior, L=left, Mid.=middle, Occ.=occipital, R=right, Sup.=superior, Temp.=temporal. Critical t-value according to FDR (α=.05) was 2.88. Voxels with the highest t-values within significant brain areas are depicted. Analyses are corrected for age and handedness.

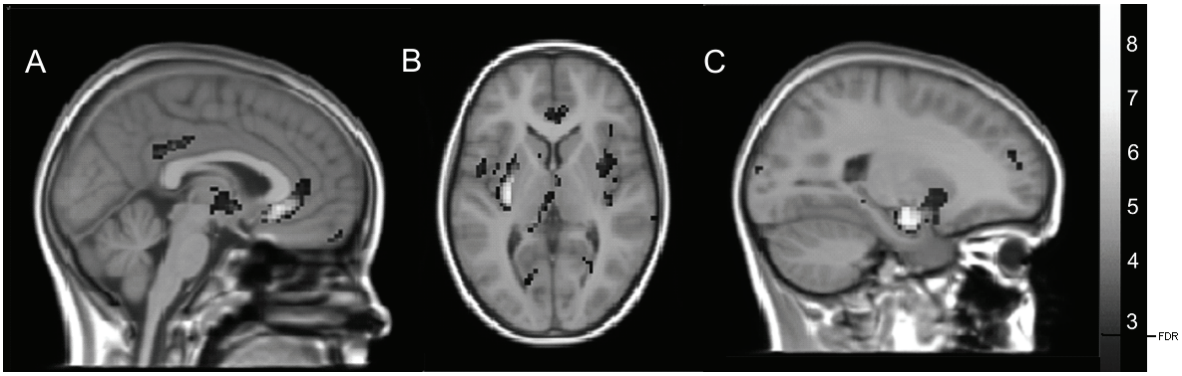

Figure 5.3a Larger gray matter density in boys compared to girls between 10 and 15 years (corrected for age).
A) left rostral anterior cingulate gyrus (X=-1), B) left putamen (Z=4) C) left amygdala (X=-21). Critical level of significance is 2.88, corrected for multiple comparisons according to the False Discovery rate, α=.05, two-tailed. Significant voxels are overlaid on our created model brain. For a colour version of this illustration see Appendix, Figure 5.3a.

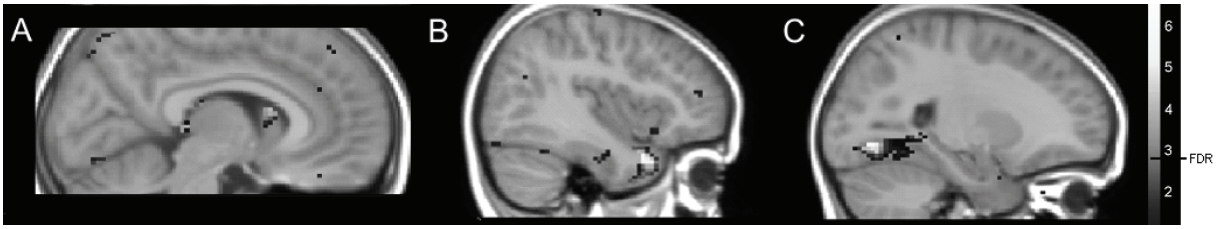

Figure 5.3b Larger gray matter density in girls compared to boys between 10 and 15 years (corrected for age).
A) Right caudate nucleus (X=10), B) right superior and middle temporal gyri (X=45) C) left inferior occipital gyrus (X=-47) Critical level of significance is 2.88, corrected for multiple comparisons according to the False Discovery rate, α=.05 (two-tailed). Significant voxels are overlaid on our created model brain. For a colour version of this illustration see Appendix, Figure 5.3b.

In earlier MRI studies it was reported that frontal and parietal gray matter decreases fastest over the course of adolescence contrary to other cortical brain areas (Jernigan et al., 1991; Giedd et al., 1999). Moreover, in the early pubertal stage, these areas were found to decrease with the emergence of secondary sexual characteristics (Peper et al., in press (b)). In this study, in girls, we could indeed replicate these age-related gray matter decreases reported earlier (Jernigan et al., 1991; Giedd et al., 1999), whereas in boys we could not demonstrate significant age-related brain changes. Thus, endocrinologically, girls advance into puberty 1 to 2 years before boys, a phenomenon which has also been demonstrated with respect to peak gray matter volume in frontal and parietal areas (Giedd et al., 1999; Lenroot et al., 2007a). Our data also indicate that at this age the process of gray matter decrease is already ongoing in the more advanced pubertal girls as opposed to boys. Importantly, increased production of estradiol in girls seems to be directly related to the negative association with frontal and parietal gray matter. We also found a positive association with estradiol and gray matter density in temporal and occipital areas in girls. This is in agreement with findings that gray matter in these areas increases well into adolescence (Giedd et al., 1999; Lenroot et al., 2007a).

In the present study, we used voxel-based morphometry to show gray matter density decreases that might indicate cortical thinning (Sowell et al., 2004). Decreases in gray matter during puberty have been suggested to reflect decreases in for example dendritic branching or number of synapses, however evidence from humans is currently lacking. In rats it was found that estrogen is involved in apoptotic processes, at least in the hippocampus (Barker and Galea, 2008). The first human neuroimaging study that has investigated the association between the precursor of sex hormones, luteinizing hormone (LH) and brain morphology in children at the onset of puberty (9 years of age), reported an association between increased LH-levels and cerebral white matter increases, whereas LH was not associated with gray matter (Peper et al., 2008). In the current study we found that in more advanced pubertal girls, estradiol is related to gray matter and not to white matter. Thus, it might be speculated that selective neuro-anatomical properties mature in conjunction with the secretion of distinct HPG-axis hormones.

Recently, in the first study on sex steroid levels and brain morphology in 8 to 15 year-old children, an estrogen-related increase in the parahippocampal gyrus was found when males and females were analyzed together (Neufang et al., 2008). Also, in that study a testosterone-related increase in diencephalic brain areas and a decrease in parietal gray matter density were reported (Neufang et al., 2008). This is in contrast to our findings as we could not demonstrate testosterone-related associations with regional gray (or white) matter density in boys or girls separately. The discrepancies between our study and Neufang et al. (2008), with respect to the reported associations between gray matter density and steroid levels, could be due to a number of factors. Age might contribute to differences between the two studies since we included a sample of 10 -15 years and Neufang et al. (2008) included also children between 8 and 9 years of age. Within the hormonal analyses, males and females were analyzed together, whereas we chose to analyze the sexes separately. Furthermore, we measured relative gray and white matter density (corrected for head size), whether Neufang et al. (2008) report on regional gray matter volumes. Finally, our sample size allowed for a correction for multiple comparisons on voxel-level, whereas Neufang et al. (2008) applied a correction on cluster-level, which is less conservative.

There are studies which indicate that throughout life, neural circuits remain responsive to changes in circulating sex steroids: for example, in adults, pharmacologically-induced changes in levels of testosterone and estrogen have been shown to alter total brain and hypothalamus volumes (Hulshoff Pol et al., 2006b). In addition, estrogen replacement therapy in postmenopausal women causes enlargement of neocortical areas (Boccardi et al., 2006) and hippocampal volumes (Eberling et al., 2003; Boccardi et al., 2006; Lord et al., 2008) compared to postmenopausal women not using estrogen therapy. Furthermore, animal studies point to a direct role for estrogens in positively affecting neuronal properties, such as synaptic spine density (reviewed by Woolley, 1999). Finally, it was reported that estrogen could change neuronal gene expression in the primate prefrontal cortex, causing increases or decreases depending on type of gene transcription factor (Wang et al., 2004).

We were not able to show associations between sex steroid levels and regional brain structures in boys. An explanation might be that males' and females' brains respond differentially to effects of sex steroids: it has been reported that female rats were more responsive to the effects of estradiol on neurogenesis and apoptosis than male rats (Barker and Galea, 2008). Moreover, it can be argued that at this age, circulating sex steroid levels might not be sufficient to induce an effect on regional brain structures. Indeed, peak levels of testosterone production in boys are found at (genital) Tanner-stage 4 (Butler et al., 1989), which is, on average, reached around 13 years (Marshall and Tanner, 1970). On the other hand, we did find that a higher level of testosterone in boys was associated with larger global gray matter volumes, which was no longer significant after correction for total brain volume. It seems unlikely that current testosterone levels are associated with a larger total brain, because by the age of 6, total brain volume has reached 95% of its whole size (Giedd et al., 1999). It has been found that higher prenatal testosterone exposure is associated with advanced pubertal development and higher pubertal steroid levels (Wood et al., 1991; Kosut et al., 1997; Herman et al., 2006). Moreover, prenatal testosterone exposure has been implicated in development of masculinization of brain volume (Collaer and Hines, 1995). It might be hypothesized that the association between total brain volume and current testosterone level has a fetal or neonatal origin. However, this requires further study. Future research should also focus on more advanced pubertal boys, in which rising testosterone levels might be related to brain development. Future research should focus also on more advanced pubertal boys, in whom rising testosterone levels might be related to development of specific brain areas. For example, in structures with a high density of androgen receptors such as the amygdala, hypothalamus and several cortical areas an association with testosterone can be expected.

The increased production of sex steroids at puberty was not related to any of the focal sexually dimorphic brain structures found at this age. However, the reported sexually dimorphic brain areas did overlap with areas found in adults (Chen et al., 2007; Goldstein et al., 2001, Good et al., 2001a) and in children or adolescents (Durston et al., 2001; Giedd et al., 1997; Lenroot et al., 2007a). Also, in various brain areas showing sexual dimorphisms at this age, androgen and estrogen

receptors are present, including the hypothalamus, thalamus, amygdala, hippocampus (Simerly et al., 1990) and throughout the cortex (Finley and Kritzer, 1999; Simerly et al., 1990). The fact that we could not demonstrate a significant relation between sex steroids and sex differences in brain structure could be due to a number of factors. The influence of sex hormone exposure leading to sex differences in brain structure is possibly most pronounced during the pre/perinatal period than during puberty (Schwartz and McCarthy, 2008). Accordingly, sex differences might have already developed at an earlier age. Indeed, neonatal boys already have larger total brain, cerebral gray and white matter volumes than neonatal girls (Gilmore et al., 2007). Another possible explanation why we failed to demonstrate an association between regional sex differences and steroid hormones is that beside sex hormones other factors can play a key role as well. For example, sex chromosome genes were found to contribute directly to the development of sex differences in the murine brain, independent of gonadal activity (De Vries et al., 2002; Büdefeld et al., 2008). It might also be argued that brain regions associated with estradiol levels in girls were sexually dimorphic when compared with boys prior to puberty and the change that occurs with increased estradiol levels eliminates this sex difference (De Vries, 2004). This would suggest that a lack of sex differences in these brain areas would persist after puberty despite any future potential changes in male testosterone or estradiol along pubertal development. On the other hand, as males advanced into puberty at a later age and testosterone levels increase, the sex difference might re-emerge. These hypotheses should be addressed in future research.

There are a number of limitations to this study which need to be taken into account when interpreting our findings. Girls in our sample were significantly older than boys, while it was demonstrated earlier that maximum gray matter volume in boys is reached at an older age than girls (Giedd et al., 1999). Thus, we may have sampled boys during the upward curve of gray matter development and girls during the downward curve. At this stage, it is therefore not possible to dissociate opposing actions of testosterone and estradiol from different hormone actions during different developmental stages. It might also be that at an earlier age, estradiol in females is related to gray matter increases. A further (related) limitation

is that in the analyses on sex differences in the brain we linearly corrected for age, but in longitudinal studies nonlinear age-related trajectories have been reported for global and focal brain areas (Giedd et al., 1999; Lenroot et al., 2007a). However, since our sample had a substantially smaller age range than the studies by Lenroot et al. (2007a) and Giedd et al. (1997; 1999) (10-15 years, versus 3-27 and 4-18 years), our applied age-correction may have been appropriate. Another limitation regarding our applied technique: as gonadal hormones likely affect the brain in very subtle ways (i.e. on a cellular level as can be measured with post-mortem and/or animal studies), 1.5-T MRI scans with 1x1x1.2 mm^3-voxels in general might not be sensitive enough to measure subtle brain changes due to hormonal development. Also, the relatively small sample size could have caused a lack of power to demonstrate any associations between testosterone and brain structure in either girls or boys.

In conclusion, in girls, with the progression of puberty, gray matter development is at least in part directly associated with increased levels of estradiol, whereas in boys, who are in a less advanced pubertal stage, such steroid-related development could not (yet) be observed. We suggest that in pubertal girls, estradiol may be implicated in the neural remodeling of heteromodal association areas in the cerebral cortex during this important period of brain development.

Chapter 6

Does a twin-brother make for a bigger brain?

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Abstract

Brain volume of boys augments that of girls by approximately 10%. Prenatal exposure to testosterone has been implicated in masculinization of the brain. Moreover, in litter-bearing mammals intrauterine position affects prenatal testosterone exposure through adjacent male fetuses. Here we studied the influence of the intrauterine presence of a male co-twin on masculinization of human brain volume. MRI brain scans, current testosterone and estradiol levels were acquired from four groups of dizygotic twins: boys from same sex twin-pairs (SSM), boys from opposite sex twin-pairs (OSM), girls from opposite sex twin-pairs (OSF) and girls from same-sex twin-pairs (SSF) at 9 years of age (N=119 individuals). Total brain, cerebellum, gray and white matter volumes were measured. Boys had significantly larger total brain, gray and white matter and cerebellum volumes than girls (on average +8.5%). Irrespective of their own sex, children with a male co-twin as compared to children with a female co-twin had larger total brain (+2.5%) and cerebellum (+5.5%) volumes. SSM, purportedly exposed to the highest prenatal testosterone levels, were found to have the largest volumes, followed by OSM, OSF and SSF children. Current testosterone and estradiol levels did not account for the volumetric brain differences. Sex of an older singleton brother or sister could not explain the findings. In conclusion, having shared the uterus with a brother seems to increase total brain volume as compared to having shared the uterus with a sister. This effect may be related to a higher level of prenatal testosterone exposure.

6.1 Introduction

In the human brain, sex differences have been well-studied in adults as well as in children and adolescents. The most consistent finding is a ~10% larger total brain volume in males versus females (Giedd et al., 1997; Durston et al., 2001; Raz et al., 2004). This volumetric difference is already present in neonates (Gilmore et al., 2007) and can not be accounted for by height (Witelson et al., 2006). Sexually dimorphic brain areas are thought to develop under the (early) influence of sex steroid exposure (in Collaer and Hines, 1995). Evidence from animal studies suggests that prenatal exposure to testosterone (or estrogen, converted from testosterone via the aromatase-enzyme (Pilgrim and Hutchison, 1994)) leads to 'masculinization' (McEwen, 1984; De Vries & Simerly, 2002) or 'defeminization' (Arnold and Gorski, 1984) of the brain, whereas preventing the exposure to testosterone leads to feminization of the brain (MacLusky and Naftolin, 1981).
Recently, we demonstrated that in healthy children, sex differences in brain volumes could not be explained by either pubertal testosterone or estradiol levels (Peper et al., in press (c)). According to these findings it was argued that prenatal exposure to sex steroids would have more pronounced effects on (the development of) sex differences in overall human brain size than the exposure to pubertal increases of steroid production.
Studies in litter-bearing mammals have shown that intrauterine position affects naturally occurring variations in sex hormones, which are not genetic in origin (for review see Ryan et al., 2002). More specifically, a male fetus has a higher blood level of testosterone than a female fetus, but irrespective of its own sex, a fetus located between male fetuses has a higher concentration of testosterone than a fetus positioned between females (Vom Saal, 1989). This phenomenon can in turn influence several anatomical and behavioral parameters, such as reproductive organs and aggressive behavior (Ryan et al., 2002). In humans the comparison of opposite sex with same sex twin pairs allows for exploration of masculinizing effects of prenatal testosterone exposure (Resnick et al., 1993; Miller, 1994). In human fetuses, hormone transfer may occur through two routes, i.e. the maternal–fetal transfer route (via maternal bloodstream), and the feto–fetal transfer route

(hormones diffusing through amniotic membranes) (Miller, 1994). Studies applying this 'same sex - opposite sex paradigm' confirm the masculinizing effect of a male co-twin on a female for less left hemispheric dominance in processing verbal stimuli (Cohen-Bendahan et al., 2004), more aggression (Cohen-Bendahan et al., 2005), less reproductive fitness (Lummaa et al., 2007) and disordered eating (Culbert et al., 2008). However, other studies did not demonstrate a masculinizing effect of a male co-twin, e.g. for birth weight (Orlebeke et al., 1993); aggression (Ligthart et al., 2005; Hudziak et al., 2003) or for handedness (Elkadi et al., 1999). The masculinizing pattern for disordered eating was also observed in males with a male co-twin as compared to a female co-twin (Culbert et al., 2008). Importantly, pubertal stage and circulating testosterone levels could not account for the differences in aggression between the group having a male co-twin versus the group having a female co-twin.(Cohen-Bendahan et al., 2005) and socialization from growing up with a male sibling did not account for the differences in disordered eating (Culbert et al., 2008).

The aim of the current study was to explore the effect of intrauterine presence of a male co-twin on masculinization of global brain volumes, possibly mediated by higher prenatal testosterone exposure. Brain volumes of boys from same sex twin-pairs (SSM), boys from opposite sex twin-pairs (OSM), girls from opposite sex twin-pairs (OSF) and girls from same sex twin pairs (SSF) were compared in order to establish whether brain volume differed by expected level of prenatal testosterone exposure. It was hypothesized that, based on their higher expected level of prenatal testosterone exposure, having a male co-twin would induce an enlargement of global brain volumes as opposed to having a female co-twin (on top of larger brain volumes in boys).

6.2. Methods and materials

6.2.1 Participants

The sample was drawn from a cohort of twin pairs in which MRI scans were acquired at the age of 9 years as described elsewhere (Peper et al., in press (b);

Peper et al., 2008; Van Leeuwen et al., 2008). The current sample consists of dizygotic twins, including: 43 SSM children (22 pairs, 1 incomplete), 16 OSM, 19 OSF (19 pairs, 3 incomplete) and 41 SSF (21 pairs, 1 incomplete), with a total number of 119 children (mean age was 9.2 years (**Table 6.1**)). Exclusion criteria consisted of any major medical or psychiatric illness and participation in special education. Physical health and mental health were assessed with a medical history inventory. Zygosity of the twins was determined based on DNA polymorphisms, using 8-11 highly polymorphic di-, tri- and tetranucleotide genetic markers. Parents and the participants themselves gave written informed consent to participate in the study. The study was approved by the Central Committee on Research involving Human Subjects (CCMO) of the Netherlands and was in agreement with the Declaration of Helsinki (Edinburgh amendments).

6.2.2 MRI acquisition and processing

Structural magnetic resonance imaging (MRI) scans of the whole brain were made on a 1.5 T Achieva scanner (Philips, Best, the Netherlands).

A three-dimensional T1-weighted coronal spoiled-gradient echo scan of the whole head (256 × 256 matrix, TE = 4.6 ms, TR = 30 ms, flip angle = 30^o, 160–180 contiguous slices; 1 × 1 × 1.2 mm^3 voxels, Field-of-View = 256 mm / 70%) was acquired. Furthermore, a single-shot EPI (Echo Planar Imaging) scan was made as part of a diffusion tensor imaging (DTI)-series (SENSE factor 2.5; flip angle 90^o; 60 transverse slices of 2.5 mm; no gap; 128 x 96 acquisition matrix; FOV 240 mm; TE=78 ms) together with a magnetization transfer imaging (MTI) scan (60 transverse slices of 2.5 mm; no gap;128 x 96 acquisition matrix; FOV 240 mm; flip angle 8^o; TE=4.5 ms; TR=37.5 ms), which were used for segmentation of the intracranial volume. Our imaging protocol made use of T2-weighted contrast of the DTI-B0 and MTI-series for segmentation of the intracranial volume. The intracranial volume segment was subsequently superimposed onto the T1-weighted image to remove non-brain tissue voxels, as described previously (Peper et al., 2008; Peper et al., in press (b)).

The scans were coded to ensure blindness for subject and zygosity identification. The T1-weighted images were automatically put into Talairach orientation (Talairach and Tournoux, 1988) without scaling, by registering them to a model brain in Talairach orientation. The translation and rotation parameters of this registration were then applied to the images (Maes et al., 1997). After registration to the T1-weighted image, the intracranial segment served as a mask for all further segmentation steps. The T1-weighted images were corrected for field inhomogeneities using the N3 algorithm (Sled et al., 1998). Our automatic image processing pipeline was used for segmentation of total brain, gray and white matter of the cerebrum and cerebellum. The software included histogram analysis, mathematical morphology operations, and anatomical knowledge based rules to connect all voxels of interest, as was validated before (Schnack et al., 2001a; Schnack et al., 2001b). The total brain and cerebellum segments were all visually checked and edited if necessary. Ten brains from the cohort were randomly selected and analyzed by two independent raters to estimate inter-rater reliability. Intra-class Correlation Coefficients (ICC) were all above 0.97.
Due to motion artifacts, separation of gray and white matter tissue was not possible in 8 subjects (2 SSM, 2 OSM and 4 SSF). These subjects were included in the analyses of the total brain and cerebellum only. Consequently, the total number of individuals included in global gray and white matter analyses was 111, whereas for total brain and cerebellum volumes the total number of participants was 119.

6.2.3 Hormonal measurements

Free testosterone levels were determined in first morning saliva (Competitive immunoassay (luminiscention), IBL Hamburg). The intra-assay and inter-assay coefficients of variation (CVs) were below 12% at levels > 11 pmol/l (lower limit of detection). Total estradiol levels as well as creatinine levels were determined in first morning urine (Competitive immunoassay (luminiscention), Architect, Abbott Laboratories, Abbott Park, Illinois USA). The intra-assay and inter-assay CVs were 5% and 10% respectively at levels > 150 pmol/l (lower) and < 9000 pmol/l (upper). Urinary estradiol levels were divided by creatinine level to correct for variations in

urine excretion rate. Both testosterone and estradiol data were collected on two consecutive days at consistent times directly after waking up, and the means of the two measurements were used in further analyses. Analyses were carried out by the endocrinological laboratory of clinical chemistry of the VU Medical Center in Amsterdam, the Netherlands. Measurable testosterone levels were available in 67% of the females (N=40; 12 OSF and 28 SSF), and in 64% of the males (N=38; 9 OSM and 29 SSM). Estradiol samples were measurable in all children.

6.2.4 Statistical analysis

A linear regression analysis was carried out to estimate the effect of co-twins' sex on brain volumes, corrected for the twin's own sex. Regression components were estimated using the software package Mx (Neale et al., 2003) which takes the dependency of the twin data into account. Although tests showed that there were no differences between the first and second born twin in mean brain volumes, birth order was taken into account by allowing mean brain volumes of the first and second born twin to be different.
Likelihood-ratio χ^2 -tests were performed to test for significance. To investigate a possible mediating role of current testosterone and estradiol levels or height, in additional analyses these variables were subsequently included as covariates. Interactions between the twin's own and co-twins' sex were investigated as well.

6.3 Results

A Kolmogorov-Smirnov test showed that all brain volumes were normally distributed. When comparing the four groups of twins, no differences in age, birth weight, gestational age, handedness, testosterone or estradiol level, weight or height were found (**Table 6.1**).

Table 6.1 Demographics of the sample.

	SSM	OSM	OSF	SSF
N (individuals)	43	16	19	41
Birth order (1^{st}/ 2^{nd})	21/22	8/8	10/9	21/20
Handedness (R/NR)	36/7	13/3	15/4	36/5
Age *(s.d.)*	9.20 (.11)	9.24 (.13)	9.22 (.14)	9.24 (.08)
Gestational age *(s.d.)*	36.8 (1.7)	37.0 (2.2)	37.2 (2.0)	36.7 (1.4)
Birth weight (*s.d.*)	2739.1 (531.7)	2642.5 (551.6)	2780.3 (531.9)	2493.7 (434.7)
Testosterone (pmol/l) (*s.d.*)*	30.8 (22.1)	23.9 (8.7)	30.9 (16.5)	38.6 (21.8)
Estradiol (pmol/l) /creatinine (*s.d.*)	115.9 (88.0)	120.0 (119.5)	119.3 (88.9)	94.9 (39.3)
Weight (*s.d.*)	31.2 (4.7)	32.3 (3.2)	32.0 (4.4)	31.8 (4.8)
Height (*s.d.*)	138.7 (5.5)	140.9 (4.8)	140.7 (5.2)	138.9 (4.8)

SSM=males from same sex pairs, OSM= males from opposite sex pairs, OSF=females from opposite sex pairs, SSF=females from same sex pairs
Age in years, Handedness: R=right handed, NR=non right-handed, Gestational age in weeks, Birth weight in grams, Height in centimeters, Weight in kilos. *) Testosterone levels were available in 29 SSM, 9 OSM, 12 OSF and 28 SSF children.

A main effect of the twins' own sex was found on global brain measures, i.e. a larger total brain (χ^2=57.9; p<.0001), cerebellum (χ^2=38.7; p<.0001), total cerebral gray (χ^2=48.5; p<.0001) and white matter (χ^2=24.0; p<.0001) in males, with a mean increase of 8.5% in male total brain volumes.

More importantly, results indicated that in both boys and girls, having a male co-twin is related to an increase in brain volumes (compared to having a female co-twin): a significant effect of co-twins' sex was found for total brain volume (χ^2=4.22; p<.04) (**Figure 6.1 & Table 6.2**), cerebellum (χ^2=8.06; p<.005) together with a trend for total white matter volume (χ^2=3.68; p<.06).

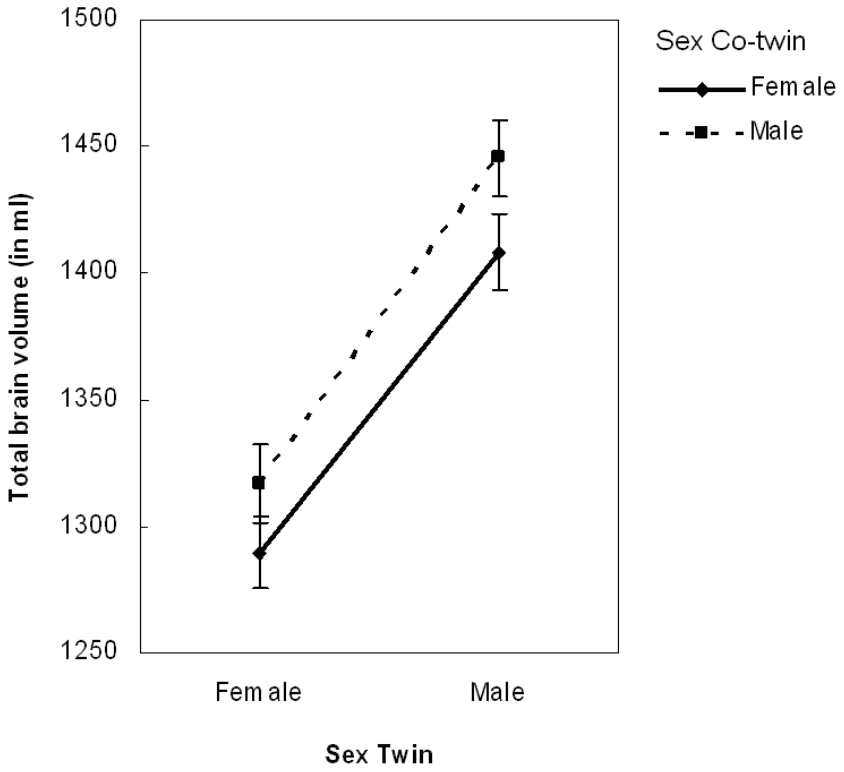

Figure 6.1 Effect of co-twins' sex on total brain volume.
Mean total brain volume (with standard error of mean) corrected for testosterone level, in male or female sex with a male co-twin (dashed line) or female co-twin (solid line). Both the main effect of the twins' own sex (p<.0001) and the main effect of co-twins' sex were significant (p<.04).

Table 6.2 Global brain volumes across same sex and opposite sex twins

Volume	Boys from Same sex	Boys from Opposite sex	Girls from Opposite sex	Girls from Same sex		Covariates Testos* Est & Hei
Total brain (*s.d.*) a	1445.7 (95.7)	1408.3 (56.7)	1317.2 (66.2)	1289.7 (85.4)	$p<.04$	$p<.02$
Cerebellum (*s.d.*) a	164.6 (10.9)	155.0 (16.7)	148.6 (11.2)	145.6 (11.0)	$p<.005$	$p<.003$
Gray matter (*s.d.*) b	788.3 (55.0)	782.6 (36.5)	718.5 (45.0)	704.3 (54.7)	*NS*	*NS*
White matter (*s.d.*) b	481.6 (48.9)	460.5 (25.4)	440.1 (26.1)	429.7 (40.2)	$p<.06$	$p<.02$

A significant effect represents an effect of co-twins' sex (comparing children with a brother to children with a sister, corrected for twins' own sex).
Brain volume in milliliters. NS=not significant, Testos.=testosterone, Est.=estradiol, Hei.=height. *) Current testosterone levels were available in 29 SSM, 9 OSM, 12 OSF and 28 SSF (65% of the total sample). Analyses are based on the full sample. a) Total brain and cerebellum volumes were available in 43 SSM, 16 OSM, 19 OSF and 41 SSF children. b) Gray and white matter volumes were available in 41 SSM, 14 OSM, 19 OSF and 37 SSF children.

When examining the means of total brain volume between the four groups of twins, the following pattern could be observed: SSM had the largest total brain volume, followed by OSM, OSF and SSF (**Figure 6.2**). An absolute increase in total brain volume of 27.4 ml could be observed in OSF compared to SSF (+2%), and a 37.3 ml increase in SSM compared to OSM (+3%). The same pattern could be observed for cerebellar volumes, and although not significant, for gray and white matter volumes (**Table 6.2**)

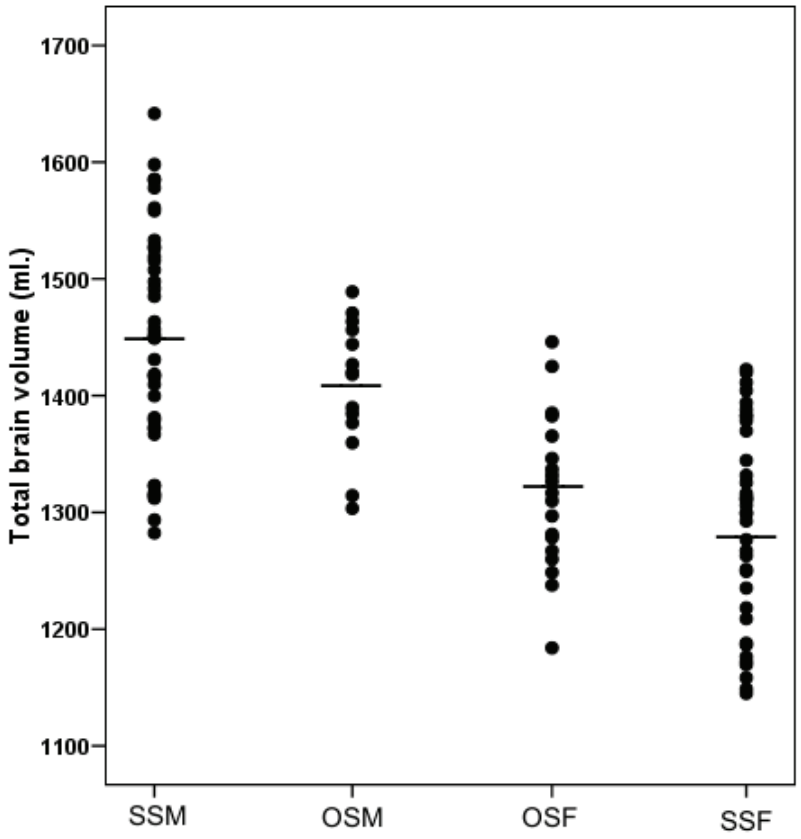

Figure 6.2 Total brain volume across the four groups of twins (N=119 individuals).
Individuals' total brain volume (with means) across same sex males (SSM), opposite sex males (OSM), opposite sex females (OSF) and same sex females (SSF).

There was no significant effect of co-twins' sex on gray matter volume (p=.33). Furthermore, no significant interaction between twins' own sex and co-twins' sex was found for total brain, gray or white matter volumes. However, for cerebellar volume, there was a significant interaction between the twins' own sex and co-twins' sex (χ^2=9.39; p<.009); data indicated that a male co-twin had a more pronounced (enlarging) effect on a males' cerebellum than on a females' (+9.4 ml, (6%) versus +3.0 ml (2%)).
When correcting the analyses for testosterone and estradiol levels or height, the effects on total brain volume, white matter and cerebellar volume remained significant (**Table 6.2**). However, after correction for birth weight, brain volumes in children with a male co-twin were no longer significantly larger than in children with a female co-twin.

6.4 Discussion

To our knowledge, this is the first study attempting to address the relation between the intrauterine presence of a male co-twin on masculinization of human brain volume, possibly mediated by higher prenatal testosterone exposure. Boys had significantly larger total brain, cerebellum and gray and white matter volumes than girls, irrespective of their co-twins' sex. Confirming our hypothesis, children with a male co-twin had larger total brain, cerebellum and white matter volumes than children with a female co-twin, on top of the overall larger brain volumes in boys. Boys from same sex twin-pairs (SSM) were found to have the largest volumes, followed by boys from opposite-sex twin-pairs (OSM), girls from opposite-sex twin-pairs (OSF), and finally girls from same sex twin-pairs (SSF). Importantly, current testosterone or estradiol level or height did not account for the observed volumetric differences in children with a male or female co-twin.
The observed larger brain volumes in boys versus girls is in agreement with earlier studies on sex differences in global brain volumes throughout development (Giedd et al., 1996; Sowell et al., 2002; Raz et al., 2004; Witelson et al., 2006; Gilmore et al., 2007; Leonard et al., 2008). Conform our expectations, having shared the

uterus with a brother as compared to a sister was related to a larger brain volume. Animal research suggests that the intrauterine presence of a male fetus leads to exposure to higher levels of prenatal testosterone within other fetuses, compared to the intrauterine presence of a female fetus (Vom Saal, 1989). Furthermore, prenatal exposure to sex steroids is implicated in the development of sex differences in brain structure (McEwen, 1984). Consequently, our findings might suggest that larger brain volumes in children with a male co-twin have resulted from higher prenatal testosterone exposure.

In animals it has been reported that prenatal testosterone-treatment increased head circumference (Steckler et al., 2005). It was argued that prenatal testosterone exposure, by influencing neuronal properties such as dendritic branching and synaptogenesis, could ultimately be reflected by an enlarged global brain volume. A probable mechanism of sexual differentiation by gonadal steroids could be apoptosis (cell death): androgens or their estrogenic metabolites can either prevent or induce apoptosis (Kawata, 1995). Interestingly, in humans, in a study on cross-sex hormone administration to transsexuals, it was found that androgen treatment in female-to-male subjects was capable of increasing total brain volume towards male proportions, whereas anti-androgen and estrogen treatment in male-to-female subjects decreased total brain volumes towards female proportions (Hulshoff Pol et al., 2006b). These data support the hypothesis that in humans, the exposure to large amounts of testosterone, similar to the prenatal surge, enlarges (masculinizes) total brain volume. After controlling for current testosterone or estradiol level, the masculinizing effects on global brain volumes remained unchanged. This indicates that (relatively low) levels of sex steroids at the age of nine cannot (fully) explain the global larger brain volumes in males, or in children with a male co-twin.

Our results are comparable to the earlier described masculinizing effects of a male co-twin on cerebral asymmetry (left hemispheric dominance in processing verbal stimuli) (Cohen-Bendahan et al., 2004) aggression (Cohen-Bendahan et al., 2005) and disordered eating (Culbert et al., 2008). Most studies applying the ‘same sex-opposite sex’ twin paradigm focussed on the masculinizing effects of a male co-twin on females only. However, our study suggests that an additional male in uterus might have a cumulative effect on prenatal testosterone exposure on a male

fetus as well. Indeed, in rats it was found that male fetuses located between other males displayed a larger (more masculine) sexually dimorphic nucleus of the pre-optic area (SDN-POA) than male fetuses located between females (Pei et al., 2006).

We were not able to directly measure hormonal levels and brain volumes during the prenatal or neonatal period (i.e. our measurements took place at approximately 9 years of age). After correcting the analyses for birth weight, brain volumes in children with a male co-twin were no longer significantly larger than in children with a female co-twin. This indicates that birth weight, which highly correlates with neonatal head circumference (Williams et al., 1997), is (at least) in part related to a larger brain volume at 9 years of age. On the other hand, it limits the specificity of our findings with respect to brain volume, i.e. other parts of the body may also be increased in children with a male co-twin versus a female co-twin.

We can therefore only speculate whether the enlarged brain volumes in children with a male co-twin are indeed a result of prenatal factors or in fact of postnatal factors, such as play –or socializing behavior. However, it seems unlikely that having shared the social environment with an opposite sex co-twin would have a substantial influence on neuroanatomical phenotypes. In a post-hoc analysis on part of the sample, we explored the possible effect of an older male or female singleton sibling (corrected for twins' own sex): N=37 girls: 18 with an older sister, 19 with an older brother; N=37 boys: 22 with an older sister, 15 with an older brother, all from same sex twin-pairs. Results of this post-hoc analysis showed no differences in brain volumes of children with an older singleton sister compared to children with an older singleton brother. These data support a role for prenatal rather than post-natal effects on masculinization of brain volumes. Of course, this post-hoc analysis requires the assumption that the influence of an older singleton brother or sister is the same as for a twin brother or sister. The unique influence of the presence of a - opposite sex - co-twin during childhood is obviously present and its influence cannot be excluded in this analysis.

We cannot state that the mechanism underlying prenatal masculinizing of brain volume is a specific result of testosterone, or might in fact result from other hormonal exposure. One such hormone is 17β-estradiol, an important metabolite of

testosterone which is converted through the aromatase enzyme in both sexes. For example, it is plausible that a higher rate of conversion into estradiol in females is responsible for 'demasculinization' or 'feminization' of brain volume, although evidence for this view is limited (Collaer and Hines, 1995). Moreover, there appears to be a dramatic sex difference in prenatal testosterone level in the human fetus between week 8 and 24 of gestation, a critical period of brain development (Hines, 2006). Prenatal estrogen levels do not show such a remarkable sex difference, making it likely that during critical periods of brain development, testosterone is implicated in producing sexual dimorphism.

To conclude, our results indicate that having shared the uterus with a brother does seem to increase total brain volume compared to having shared the uterus with a sister. This effect may be related to a higher level of prenatal testosterone exposure. Future studies in larger samples including neonatal subjects are required to further investigate this issue and replicate our findings.

Chapter 7

Summary and discussion

Summary and discussion

The aim of this final chapter is to provide a summary and discussion of the main findings of this thesis. Furthermore, possible implications and issues concerning some of the applied methods will be addressed and in the closing paragraph general conclusions will be presented.

The studies in this thesis as well as previous research have convincingly shown that the brain is still 'under construction' between nine to fifteen years of age. During this stage of life, the composition of gray and white matter within the cerebral cortex is undergoing rapid changes. Global as well as frontal and parietal gray matter starts to decrease (Jernigan et al., 1991; Giedd et al., 1999; Thompson et al., 2000; Sowell et al., 2002; Gogtay et al., 2004), whereas white matter continues to increase in a region-specific manner (Paus et al., 1999; Thompson et al., 2000; Barnea-Goraly et al., 2005). Furthermore, dramatic hormonal changes occur with the reactivation of the reproductive HPG-axis. It has been hypothesized that these developmental processes during puberty might be critical for optimal adult (brain) functioning (Giedd, 2008).

The main goal of this thesis was to explore the aetiology of individual differences in brain structure of healthy children, who are on the verge of undergoing the transition to adults. To that end, we focused on the following aspects:

(1) The relative importance of genetic and environmental influences on interindividual differences in global and regional brain volume (**Chapters 2 and 3**)

(2) The association between HPG-axis hormones and brain structure **(Chapter 4, 5 and 6**).

A total number of 107 twin pairs of 9 years of age with an additional older sibling was recruited from the Netherlands Twin Register for these studies. This is the largest sample to date of healthy twin pairs within a restricted age-range used in

structural MRI research. A direct link between pubertal hormones and human brain structure has never been investigated in such a large sample.

7.1 Summary

In **Chapter 2,** eighteen brain morphological twin studies were reviewed to investigate genetic and environmental influences on human brain development. Results showed that throughout development, individual differences in total brain volumes are strikingly heritable, with heritability estimates of total brain volume up to 97%. Variation in global gray and white matter volumes seems to be predominantly influenced by genetic factors. Variation in lateral ventricle volumes (i.e. spaces in the brain filled with cerebrospinal fluid) can almost be entirely explained by shared and unique environmental factors. On a regional level of the brain, however, genetic effects seem to be more variable. Studies using voxel-based morphometry (VBM) and cortical thickness measures have demonstrated high heritabilities for medial frontal cortex, Heschl's gyrus, and postcentral gyrus. Moderate heritabilities for gray matter have been found for the hippocampus, parahippocampal gyrus and amygdala, and white matter of the superior occipitofrontal fasciculus. Changing genetic influences with age have also been found. Specifically, heritability of global gray matter increases as a function of age and shows that gene-expression is a dynamical process throughout life.

Taken together it is recommended that in order to reliably test for genetic influences, common and unique environmental factors and their interactions, large and homogenous twin-samples are needed to be analyzed with advanced quantitative genetic methods, such as structural equation modeling (SEM). Since brain volume changes dynamically throughout life, longitudinal twin studies in childhood as well as in adulthood are needed to investigate the stability of genetic (and environmental) influences onto neural networks in the human brain.

In **chapter 3**, heritability of global and focal brain structures at the onset of puberty was investigated. In addition, the relation between the first secondary sexual characteristics of puberty and brain structure was explored. In a 9-year-old twin sample of 45 monozygotic and 62 dizygotic twin pairs (n=195 individuals),

intracranial volume, total brain volume, gray and white matter, cerebellum and lateral ventricle volume were measured. VBM was used to quantify regional gray and white matter densities. Results showed that in 9-year-olds, global brain volumes are already remarkably heritable, with estimates ranging from 77% (i.e., gray matter) to 94% (i.e., total brain volume). An exception was lateral ventricle volume, which was considerably lower heritable (35%). Regionally, substantial differences were found with respect to the relative importance of genetic influences: white matter density in posterior parts of the fronto-occipital and superior longitudinal fascicles, cingulum and corpus callosum were found to be significantly heritable with estimates ranging from 67% to 93%. Interestingly, the areas within gray matter density that were significantly heritable were substantially smaller. These areas included (pre-)frontal, middle temporal areas and the amygdala. It was suggested that there might be stable heritable white matter pathways across development, since the high heritability of posterior white matter overlaps with findings in adults. Moreover, the onset of secondary sexual characteristics of puberty was associated with decreased frontal and parietal gray matter densities and was mainly found in girls. With the transition into puberty total variance in these areas increased, but specific contributions of genetic or environmental variance could not be demonstrated, possibly due to the relatively small group of children showing secondary sexual characteristics.

Chapter 4 focused on the early endocrinological marker of puberty, luteinizing hormone (LH). LH was measured in first morning urine samples using highly sensitive immunometric assays. This method allows researchers to detect nocturnal rises in LH level that mark the beginning of puberty in both boys and girls, even 1 to 2 years before serum levels of LH increase or secondary sexual characteristics of puberty are present (Demir et al., 1996). The association between LH concentrations and brain structure was investigated within 104 nine-year-old twins (52 boys). In addition, the existence of a common genetic origin of this association was explored. We found that an increased production of LH was associated with larger global white matter relative to intracranial volume. Regionally, increased LH-levels were associated with white matter density increases within the splenium of the corpus callosum, middle temporal gyri and the cingulum. Interestingly,

results indicated that the association between LH-level and white matter density within these areas is driven by a common genetic factor, reflected by a significant genetic correlation between the two traits. LH-levels were however not related to global or regional gray matter. Compared to the findings in **chapter 3**, the earlier pubertal marker LH was found to be related to white matter increases, whereas a more advanced pubertal marker, i.e. the presence of secondary sexual characteristics (a result from sex steroid production), appears to be associated with gray matter decreases.

Following from these results, it was argued that the adolescent brain might respond differentially to changing hormone levels over time (Sisk and Zehr, 2005) as brain development during puberty and adolescence is a dynamic process characterized by region specific gray matter decreases and global white matter increases (Giedd et al., 2006). To further address this issue we investigated the possible association between sex steroids testosterone and estradiol and brain structure in **chapter 5**. This study included a more advanced pubertal sample consisting of the 78 older siblings of the twin pairs, aged between 10 and 15 years. As sex steroids are implicated in the development or maintenance of sex differences (Kawata, 1995), interrelations between testosterone, estradiol and sex-related differences in brain structures were also investigated. Results showed that, on top of overall age-related gray matter density decreases, higher levels of estradiol in girls were associated with decreased gray matter densities in the superior-, inferior- middle- (left) and orbitofrontal gyri, supramarginal and angular gyri of the parietal lobe and middle temporal gyrus. Estradiol-related increases in girls were associated with regional gray matter density increases of the middle frontal (right), inferior temporal and middle occipital gyri. In boys, estradiol and testosterone levels were not related to changes in brain structures, nor were testosterone levels in girls. Prominent regional sex differences in several brain structures were found. In boys increased gray matter densities were found in the amygdala, putamen, thalamus, insula, rostral anterior cingulate, and superior temporal gyrus. In girls, increased gray matter densities were found in the hippocampus, caudate nucleus, caudal anterior cingulate, middle temporal gyrus and inferior occipital gyrus. Gray or white matter densities in these areas did not show associations with pubertal sex steroid levels. It

was concluded that, at least in pubertal girls, estradiol seems to be implicated in the earlier described pubertal remodeling of heteromodal association areas in the cerebral cortex. Whereas in boys, at this age, no such associations could be demonstrated between sex steroid levels and brain structure.

Unexpectedly, we did not find an association between pubertal sex steroid levels and sexual dimorphic brain areas. We speculated that sex differences in the pubertal brain may be formed earlier in life, e.g. during the prenatal period. Indeed, in neonates, sex differences in global brain volumes are already present: boys have ~10% larger brain volumes than girls (Gilmore et al., 2007). In animal studies it was shown that prenatal testosterone exposure is implicated in 'masculinization' of the brain, whereas the absence of testosterone exposure is thought to lead to 'feminization' (Collaer and Hines, 1995). In addition, it was demonstrated that intrauterine position affects prenatal testosterone exposure, through adjacent male fetuses (Von Saal, 1989). Following from these findings, in the final chapter, **chapter 6**, we explored influences of the intrauterine presence of a male co-twin on masculinization of brain volume, possibly mediated by higher prenatal testosterone exposure. To that end, four groups of dizygotic twins were included: (i) boys from same sex twin pairs (SSM), (ii) boys from opposite sex twin pairs (OSM), (iii) girls from opposite sex twin pairs (OSF) and (iv) girls from same sex twin pairs (SSF) all 9 years of age (N=119 individuals). It was hypothesized that on the basis of higher prenatal exposure to testosterone, the intrauterine presence of a brother would result in larger global brain volumes, compared to the intrauterine presence of a sister. Results showed that, corrected for larger global brain volumes in boys, children with a male-co twin indeed showed a larger total brain and cerebellar volume versus children with a female-co-twin. SSM children, purportedly exposed to the highest level of prenatal testosterone, had on average the largest total brain volume, followed by OSM, OSF and SSF. Importantly, current testosterone or estradiol levels at age 9 years did not account for the brain volumetric differences. It should however be noted that in this design, it is difficult to disentangle prenatal from postnatal effects. However, brain volumes in children with an older singleton brother were not different from children with an older singleton sister, making a strong case for prenatal in stead of postnatal influences. The findings from chapter

6 suggest that the intrauterine presence of a brother is related to an increased brain volumes compared to the intrauterine presence of a sister. This effect may be associated with a higher level of prenatal testosterone exposure. For an overview of the main findings described in this thesis, see **Table 7.1**.

7.2 Discussion

Brains of children in puberty and adolescence are subject to complex and widespread changes. After an initial wave of synaptic overproduction which takes place in childhood, during puberty and adolescence selective synaptic elimination is initiated (Huttenlocher, 1994). This process most likely reflects the elimination of neuronal connections, rather than programmed cell death (Huttenlocher, 1990). In contrast, myelination of axons continues during this period (Yakovlev and Lecours, 1967). These findings from post-mortem research are supported by MRI-studies investigating gray and/or white matter volumes (Giedd et al., 1999; Sowell et al., 2002; Paus et al., 1999; Gogtay et al., 2004; Lenroot et al., 2007a). Based on these highly dynamic neuronal processes during puberty and adolescence it can be proposed that brain development in this phase of life is of critical importance to how the adult brain will ultimately function. A widely adopted view is that perhaps the 'blueprint' of synapses and neuronal connections created during pre/neonatal life is being fine-tuned in this period. In other words, connections which are not used will be eliminated (the so-called 'use it or lose it' principle) (see Blakemore, 2008). Evidence for this view comes from functional MRI studies in which it has been shown that developmental changes in brain activity appear to change from diffuse to more focal patterns of activation (for a review see Durston and Casey, 2006). Thus, cognitive capacity during childhood may result from a gradual loss of synapses together with a strengthening of the remaining synaptic connections (Casey et al., 2000).

Table 7.1 Main findings of the studies described in this thesis

Chapter	Aim	Main findings
2	Review of twin studies on heritability of brain structure.	1) High heritability of global brain volumes throughout development, lat. ventricles low heritable. 2) Regionally: high heritability of GM in frontal cortex, WM of SOF & SLF. 3) Somewhat lower heritability of (para-) hippocampus.
3	1) Study heritability of brain volume and structure in 9-year old twins. 2) Explore association between SSC of puberty (Tanner) and brain structure in 9-year-old twins.	1) High heritability of global brain volumes (between 77% for GM and 94% for TB volume). Heritability lat. ventricles 35%. 2) Regionally: GM density highly heritable in small areas (Sup. Frontal, Mid. Temporal and amygdala). Heritability of WM more widespread in genu of CC and post. areas of SOF, SLF, Cing. 3) Development of SSC associated with GM decrease in prefrontal and parietal areas.
4	1) Study association between LH and brain structure in 9-year-old twins. 2) Explore genetic etiology of association between LH and brain structure.	1) LH associated with global and focal WM increase: in Cing., mid temporal and splenium of CC (in both boys and girls). LH not associated with GM. 2) Association between LH and WM density is driven by common genetic factor (significant genetic correlation).
5	1) Investigate association between sex steroids T &E and brain structure/volume in 10-15-year old boys and girls. 2) Investigate relation with sex differences in brain structure.	1) In girls: higher E levels related to GM decrease in frontal and parietal areas, and to GM increases in temporal and occipital areas (not to WM). 2) In boys: no association between T or E and brain structure, nor between T and brain structure in girls. 3) Sex differences in GM density not related to T or E levels.
6	Explore effect of intrauterine presence of male co-twin on masculinization of brain volume, possibly through prenatal T exposure	1) Having a male co-twin associated with larger TB, CB and WM (corrected for own sex) compared to female co-twin. 2) Current T and E level could not explain the enlarged brain volumes. 3) Sex of older singleton sibling no effect on global brain volumes. Possible role for prenatal T exposure.

CB=cerebellum, CC=corpus callosum, Cing=cingulum, E=estradiol, GM=gray matter, Lat=lateral, Mid=middle, Post=posterior, SOF=superior occipitofrontal fascicle, SLF=superior longitudinal fascicle, SSC=secondary sexual characteristics, Sup=superior, T=testosterone, TB=total brain, WM=white matter.

An important question that comes to mind is to what extent these individual differences in brain structure are influenced by genetic and environmental factors, and what is the mediating role of hormones? In particular, what specific brain areas show high heritability and/or an association with hormonal factors? The findings from this thesis provide new insights into these issues.

7.2.1 Genetic influences

In **chapter 3** we have shown that when there is only limited production of sex steroids as indexed by the relatively small number of children showing signs of secondary sexual characteristics, 94% of the individual variation in global cerebral volume can be explained by genetic influences. Interestingly, heritability estimates at this young age are strikingly similar to heritability of (young) adult brain volume (Carmelli et al., 1998; Baaré et al., 2001, Wright et al., 2002) and even to elderly brain volumes (Pfefferbaum et al., 2000; 2004). These findings indicate that the magnitude of genetic factors influencing individual differences in brain volume remains relatively stable across the life-span. However, within certain critical time periods in life (i.e., puberty and adolescence), there might be a transient change in genetic factors or the magnitude of genetic factors that contribute to volumetric brain changes. This phenomenon may be directly linked to the timing of gene expression. For example, it has been suggested that the Catechol-*O*-methyltransferase (COMT)-gene contributes to normal variation in cognitive functioning. This gene appears to exert its largest effect on pubertal children as compared to pre-pubertal children (Barnett et al., 2007. Polymorphisms in COMT are also associated with adult brain volumes (Gothelf et al., 2005) and this gene might be involved in morphological brain changes around the onset of puberty.
Measuring the interaction between age and heritability of brain structure, during the course of adolescence heritability of white matter volume (Wallace et al., 2006) as well as gray matter of especially the prefrontal cortex (Lenroot et al., 2007b) increases. In an attempt to explore interactions between puberty and heritability of brain structures, we observed that with the onset of secondary sexual characteristics individual differences in gray matter density increased. However, whether this

increase in total variance was attributable to increased genetic or environmental variance could not be demonstrated.

To test the hypothesis of a temporary change in heritability of gray and white matter structure during pubertal development, further research using longitudinal studies that includes more pubertal variation is required. Notably, the sample described in this thesis is currently being scanned for the second time to investigate possible changes in heritability across the pubertal period.

In contrast to the high heritability of global brain volume, a more distinct pattern of genetic effects could be observed when investigating individual differences in regional brain structures at 9 years of age. Extensive areas of white matter density, including the fronto-occipital and superior longitudinal fascicles, genu and cingulum were found to be significantly heritable. In contrast, significantly heritable gray matter densities were less widespread than within white matter, and included relatively small areas within the amygdala, superior frontal -and middle temporal gyri. As opposed to the 9 year olds, adults show significant heritability of gray matter density in posterior as well as anterior parts of white (Hulshoff Pol et al., 2006a) and gray matter density (Hulshoff Pol et al., 2006a; Thompson et al., 2001; Wright et al., 2002). It can therefore be speculated that a "posterior-to-anterior" pattern of heritable brain areas throughout development is analogous to the back-to-front pattern of cortical thickness maturation (Gogtay et al., 2004) and myelination of axons (Yakovlev and Lecours, 1967) observed between childhood and adolescence.

7.2.2 Relationship between genetic and hormonal influences

Evidently, genetic influences cannot be viewed as independent factors. The magnitude of genetic effects depends on the relationship with internal and external environmental factors as well. For example, it has been demonstrated that within varying environmental conditions (external), such as stress or enriched environments, the expression of genes in the brain is also different (Englander et al., 2005; Bhansali et al., 2007; McNair et al., 2007; Fuchikami et al., 2008). Furthermore, a changing hormonal environment (internal), like during pubery,

affects the expression of genes also. For example, non-genomic actions of estrogens can interact with genomic actions in the brain (Vasudevan and Pfaff, 2008). We investigated a possible common genetic etiology of brain structure by examining an early pubertal marker, nocturnal LH production. Results showed an association between increased LH production and increased global and focal white matter. No relationship was found between LH production and changes in global and focal gray matter. Importantly, a common genetic factor was found for the association between LH and white matter. Thus, it is suggested that during the early stage of puberty, certain genes become active which are implicated in both white matter development as well as in the re-activation of the reproductive HPG axis or quantity of hormonal production. A possible candidate gene is the KiSS1-gene. Kisspeptide, the end-product of the KiSS1-gene is expressed throughout the brain (Smith and Clarke, 2007) and together with its G-coupled receptor GPR54, kisspeptide was found to stimulate LH secretion in humans (Dhillo et al., 2005). The KiSS1 gene might be a member of a network of genes that contributes to integrating glia-to-neuron communication into a functional unit capable of triggering puberty (Ojeda et al., 2006b). This would suggest that increased white matter density with increasing stage of puberty results from the integration of glia-to-neuron communication. Other candidate genes which are likely to contribute to early pubertal hormone production are the LH-receptor gene (Wu et al., 2000), and the ErbB-1 and ErbB-4 genes. The ErbB-1 and ErbB-4 receptors belong to the family of epidermal growth factors and are located on astrocytes (i.e., glial cells that facilitate myelinating activity through oligodendrocytes) (Ishibashi et al. 2006). Both ErB-1 and ErB-4 genes have been implicated in LH secretion and pubertal development (Prevot et al., 2005). Notably, astrocyte plasticity in the hypothalamus has been found to affect LH-peaks in rats (Cashion et al., 2003).

Results discussed in **chapter 4** showed that LH levels were not associated with changes in total gray matter volume or regional gray matter density. A possible explanation for the absent relation might be that the gray matter decreases around puberty-onset observed in earlier studies (Jernigan et al., 1991; Giedd et al., 1999; Sowell et al., 2002) might be associated with “more advanced” pubertal characteristics. Indeed, in the 9-year old children who already showed secondary

sexual characteristics which occurs on average 1-2 years after the first nocturnal LH rise, exploratory analyses indicated that this transition goes accompanied by decreases in frontal and parietal gray matter (**chapter 3**). Importantly, in more advanced pubertal girls between 10 and 15 years of age, we found that on top of overall age-related effects estradiol, a sex steroid and successor of LH within the HPG-axis, was mainly related to prefrontal and parietal gray matter decreases. It can be hypothesized that during early puberty, different hormones trigger selective neuro-anatomical alterations as evidenced by white matter increases in response to LH production and gray matter decreases with estradiol production. Since we did not measure hormonal or brain development directly (only one measurement in different age-groups), further research is needed to test this hypothesis within a longitudinal design.

7.2.3 Sex steroids and sex differences

In **chapter 5** we found age-related gray matter decreases in pubertal girls (mean Tanner-stage was 2.9 out of 6 stages) comparable to earlier studies (Giedd et al., 1999; Jernigan et al., 1991). Importantly, we observed that on top of these age-related gray matter decreases, higher estradiol levels in girls seemed directly related to decreases in prefrontal and parietal areas and increases in temporal and occipital areas. Animal research has shown that estrogen can affect neurogenesis as well as apoptosis (Barker and Galea, 2008). These mechanisms could account for the association between estradiol and gray matter density as we measured with MRI. Even though the etiology of the relations between estradiol and gray matter was not addressed in this thesis, a common genetic origin is nonetheless likely, because several sex-steroid related genes can alter various brain morphological parameters (reviewed by Westberg and Eriksson, 2008). In addition, it was reported that estrogen is able to change neuronal gene expression in the primate prefrontal cortex causing increases or decreases depending on type of gene transcription factor (Wang et al., 2004).

An association between age and gray matter was not seen in the 10 to 15 year-old boys, neither was there an association between sex steroids and focal gray or white

matter. In general, boys and girls show marked differences in steroid levels and girls enter puberty on average 2 years earlier than boys (Chapter 5; Delemarre-Van de Waal, 2002; Rosenfield, 1996; Styne, 1996). Interestingly, in previous studies it was found that maximal gray matter volume in frontal and parietal areas in girls is reached 1 to 2 years before boys (Giedd et al., 1999; Lenroot et al., 2007a). Thus, it might be argued that in our study the process of gray matter decrease is already ongoing in pubertal girls as compared to boys.

By measuring sex differences in the brain, the influence of sex steroids on brain structure can be investigated in an indirect way (Pilgrim and Hutchison, 1994; Kawata, 1995). It is therefore reasonable to suggest that due to changing steroid levels during puberty sex differences in steroid-responsive brain areas would either emerge or become more prominent. In **chapter 5**, we reported sexual dimorphic brain areas in the amygdala, hippocampus, hypothalamus, thalamus, basal ganglia and anterior cingulate gyrus, as reported earlier (e.g. Durston et al., 2001; Goldstein et al., 2001). Contrary to our expectations, we could not establish associations between sex steroid levels and sexual dimorphic brain areas. One possible explanation for this unexpected result might be that the influence of the sex hormones that presumably cause sex differences in the brain is most pronounced during the early pre/perinatal period (Schwartz and McCarthy, 2008). This is evidenced by the presence of a remarkable sex difference in prenatal testosterone level in the human fetus between week 8 and 24 of gestation, a critical period of brain development (Hines, 2006). Accordingly, sex differences might have already developed at an earlier age. Indeed, neonatal boys already have larger total brain, cerebral gray and white matter volumes than neonatal girls (Gilmore et al., 2007). Our final study described in **chapter 6** also supports a possible role for prenatal testosterone exposure being implicated in sex-related differences in global brain volumes. The intrauterine presence of a male co-twin (expected to cause a higher testosterone exposure within the other fetus) was related to more ‘masculine’ (i.e. larger) brain volumes compared to the intrauterine presence of a female co-twin. This effect was not due to current testosterone or estradiol levels, height or with being raised with an older singleton brother. These findings indicate that prenatal rather than postnatal factors play a role in enlargement of brain

volumes. It might be speculated that prenatal testosterone levels have a more pronounced effect on brain volume than early pubertal testosterone levels.
Whether the masculinizing effect of a male co-twin also affects regional brain areas awaits further study in larger samples (e.g. MRI data within the opposite sex twin-group were available of 14 boys and 18 girls) using voxel-based morphometry. Other factors besides sex hormones influence sexual differentiation of the brain as well and include genes located on sex chromosomes that show a sex-dependent difference in expression. These sex-linked genes can exert their effects even before the gonads are active (for review see Davies and Wilkinson, 2006).

7.3 Implications

In this thesis, we tried to shed light on the etiology of variation in brain structure in 9-year-old children. We set out to investigate the early pubertal period as this period represents a turning point in human development. Children undergo drastic changes with respect to brain structure, hormone levels and psychological function. It is of importance to investigate mechanisms underlying 'normal' development of these processes. Gaining knowledge on when certain brain structures are particularly sensitive to genetic or environmental influences during typical periods throughout development may contribute to a better understanding of neuropsychiatric diseases which often have their onset during this period (e.g., schizophrenia). Highly heritable brain morphometric measures provide biological markers for inherited traits (endophenotypes) and may serve as regions of interest for genetic linkage and association studies (Gottesman and Gould, 2003; De Geus et al., 2008).
We found that gray matter of the amygdala, superior frontal (SFG)-and middle temporal gyri (MTG) together with white matter within the fronto-occipital (FOF) and superior longitudinal fascicles (SLF), genu and cingulum are highly heritable at 9 years of age. These brain areas have been implicated in emotional processing (i.e., amygdala) (Sergerie et al., 2008), language processing (i.e., SFG/MTG) (Glasser and Rilling, 2008), visuospatial processing (i.e., FOF) (Makris et al., 2005), language processing (i.e., SLF) (Makris et al., 2007), inter-hemispheric

communication between anterior brain areas (genu) (Schmahmann and Pandya, 2006) and communication between limbic areas (i.e., cingulum) (Schmahmann and Pandya, 2006). Thus, when searching for genes possibly involved in illnesses wherein these areas or their implicated functions are affected, density within these brain areas appear to be good endophenotypes.

The cingulum, middle temporal lobes and splenium might comprise a neural network that is susceptible to the influence of increased LH production in early puberty. Our finding of common genes underlying LH level and white matter density in these areas might aid the search for candidate genes in illnesses in which both LH production and white matter integrity are affected. For example, schizophrenia and Alzheimer's disease have been associated with abnormal levels of LH (Bowen et al., 2000; Ferrier et al., 1983) and white matter abnormalities (Hulshoff Pol et al., 2004; Sydykova et al., 2007; Xie et al., 2006). Thus, early detection of (abnormal) LH-levels might be a useful marker for neuropsychiatric disorders in which white matter is affected. Interestingly, several sex steroid-related candidate genes have been suggested to be involved in neuropsychiatric disorders with sex-specific prevalence rates, age of onset and sex-specific course of the disorder, for instance, depression, Attention Deficit/ Hyperactivity Disorder (ADHD), autism, eating disorders and schizophrenia (recently reviewed by Westberg and Eriksson, 2008). Moreover, identifying brain areas which are related to sex hormones and which areas are not might also help to better understand the predisposition to neuropsychiatric disorders (Cahill, 2006).

Brain structure as measured by MRI and the dynamic changes therein, have functional relevance. For example, it was shown that the trajectory changes in cortical thickness throughout adolescence are associated with level of intelligence (Shaw et al., 2006). Moreover, we found that the relationship between brain volume and intelligence could be explained by a common genetic factor influencing both intelligence and brain volume (Van Leeuwen et al., in revision).

7.4 methodological considerations

There are some methodological considerations in this thesis which need to be addressed. Studying the association between hormonal levels in subjects with a narrow age range can both be considered as an advantage as well as a limitation. On the one hand, associations between hormonal levels and brain structure can be measured unconfounded by age-related factors. On the other hand, since all twins were 9 years of age, we cannot definitely state that, for instance, the observed association between LH and white matter is specific for this age or can be observed at another age as well. Although estradiol and testosterone levels in the 9-year old children were measured as well, their levels were very low with a substantial amount of samples below detection limits. We therefore chose to investigate steroid levels in the older sample of siblings. In a post-hoc analysis, we did not find evidence for an association between LH or FSH and brain structure in the older siblings, supporting the view that in more advanced pubertal children estradiol is the key player in gray matter decreases.

Endocrinological events preceding the development of secondary sexual characteristics such as nocturnal LH secretion, have already been ongoing 1-2 years before secondary sexual characteristics become apparent (Demir et al., 1996). Based on the measurement of secondary sexual characteristics, it is possible to make a clear distinction between pubertal and non-pubertal children (see **chapter 3**). However, due to considerable inter-individual variation in hormone levels and (some) prepubertal LH/FSH release (i.e., gonadotropin production at the onset of puberty is a gradual process), no normative values are available to classify the hormonal onset of puberty. Consequently, it was impossible to make a distinction between pubertal ('LH-producers') and pre-pubertal ('LH-non-producers') children on the basis of their LH-level. Therefore, in chapter 4, we needed to study the association between the gradual LH-increase and brain structure with a correlational design. This reasoning also applies to the relations between testosterone and estradiol and brain structure in the older siblings described in chapter 5, as no clear distinction could be made between producers and non-producers.

When combining both studies on puberty-onset and the heritability of brain structure, an important question would be whether genetic or environmental variance changes with the transition into puberty. Preliminary results have indicated that with the emergence of secondary sexual characteristics total variance in (pre-) frontal gray matter areas increases. This enlarged variance seemed to be driven by both genetic and unique environmental factors although a significant contribution of each of these factors could not be demonstrated. This is possibly due to little variation in pubertal development at this relatively young age. Follow-up studies with a more equal distribution of children showing secondary sexual characteristics versus children without these characteristics are needed to address this issue in further detail.

In **chapters 3, 4 and 5**, voxel-based morphometry (VBM) was used. VBM has several advantages over volumetric region-of-interest (ROI) segmentation. VBM provides a non-biased measure of localized brain regions which might have been overlooked within the often time-consuming ROI analyses (Ashburner and Friston, 2000). VBM results can be adjusted to account for the variable shape changes in nonlinear transformations ('optimized' VBM) (Good et al., 2001b), and thus preserves the volume of the particular tissue within a voxel. However, this method reintroduces the global differences in shape and scale. In our analyses we used the standard i.e. 'non-modulated' VBM, which means that we have measured relative regional differences in gray or white matter 'concentration'. An advantage of applying this method is that global effects of brain size have been removed, thus one can directly investigate regional differences in brain areas without being confounded by overall brain size. A disadvantage of this method is that our results in terms of regional densities or concentrations can not be readily translated into volumes within brain areas. Significant results in standard VBM analyses in gray or white matter density might point to a shift in the border between gray and white matter. ROI measurements and (standard) VBM measures yield comparable results (Allen et al., 2005b; Giuliani et al., 2005; Kennedy et al., 2007). However, since ROIs derived from manual segmentation have anatomical validity, these studies recommend to use VBM for first explorative purposes to provide guidance in choosing ROIs. For an elaborate discussion on the advantages and disadvantages of

the application of VBM see Ashburner and Friston (2001), Bookstein (2001) and Davatzikos (2004).

As discussed in **chapter 2**, one has to keep in mind that despite our large twin sample by MRI standards, by twin methodology standards our sample is relatively small and statistical power is limited to test for common environmental effects, interactions between genes or between genetic and environmental factors. Furthermore, in a VBM approach a correction for multiple comparisons is needed to prevent false positive results (i.e. Type-I errors). At the same time, false negatives (i.e., Type-II error) might increase due to this correction, and additional brain areas showing significant heritability may stay undetected (see also discussion in **chapter 3**).

Finally, it should also be noted that the term heritability is sometimes wrongly interpreted. For example, the reported heritability for global brain volume of 94% does not mean that the individual growth of total brain volume is 94% determined by genetic factors and the remaining 6% is due to environmental factors. The correct interpretation is that individual differences in total brain volume among individuals can for 94% be explained by genetic differences among them. In other words, to measure heritability of a certain trait, reliable variation within that trait is a prerequisite. Heritability is a descriptive measure which describes the contribution of genetic differences to individual differences in a particular population at a particular time (Plomin et al., 2001).

7.5 Concluding remarks

The series of studies in this thesis highlight that global brain volumes in 9-year old children are remarkably heritable. Genetic effects on regional variation in posterior white matter areas seem to be more prominent than anterior (frontal) gray and white matter. This observation is in agreement with the back-to-front pattern of brain maturation. Already at a relatively young age when secondary sexual characteristics of puberty are not yet visible in most children, the early pubertal marker LH is related to white matter growth. A common set of genes appears to be critically involved in this process. In more advanced pubertal girls, estradiol is

related to gray matter decreases, whereas in boys a relationship between sex steroids and brain structure could not (yet) be observed. Thus, selective neuro-anatomical properties appear to mature in conjunction with the secretion of distinct HPG-axis hormones. Prenatal testosterone levels may possibly explain differences in brain volume better at this age than pubertal testosterone levels, although this needs further study.

In conclusion, it has become clear that the pubertal brain is ‘a work in progress’. This thesis contains the first series of studies that has provided important new leads into the complex interplay between genetic and environmental factors, hormones and brain structure in this critical period of life. Longitudinal follow-up of this sample will examine whether age by genotype interactions are important during puberty and adolescence and further elucidate the role of sex steroids in the developing brain.

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Nederlandse samenvatting

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Het doel van dit proefschrift was om onderliggende factoren te onderzoeken die kunnen bijdragen aan het ontstaan van individuele verschillen in hersenstructuur in de (vroege) puberteit.

De puberteit is een belangrijke periode in de ontwikkeling tot volwassenheid. De meest bekende verandering die optreedt in de puberteit, is een sterke toename in de productie van geslachtshormonen door activering van de hypothalamus-hypofyse-gonadale as (HPG-as). Het gevolg is een toename in de afgifte van luteïniserend hormoon (LH) en follikel stimulerend hormoon (FSH) vanuit de hypofyse in zowel jongens als meisjes. Deze hormonen stimuleren op hun beurt de productie van testosteron in jongens en oestradiol in meisjes. In meisjes wordt echter ook een kleine hoeveelheid testosteron aangemaakt in de bijnierschors, terwijl in jongens ook oestradiol wordt geproduceerd als gevolg van omzettingen van testosteron door het enzym aromatase. Naast fysieke veranderingen, zoals secundaire geslachtskenmerken, laten kinderen een snelle vooruitgang zien in cognitieve en sociale vaardigheden. Deze vaardigheden zijn onder andere het vermogen om te plannen, problemen oplossen, het onderdrukken van impulsen, abstract redeneren en het begrijpen van iemand anders' emoties. Onderzoek met behulp van beeldvormingtechnieken zoals magnetische kernspinresonantie (MRI) wijst erop dat er na een sterke toename van grijze stof (i.e. neuronen en uitlopers van neuronen) in de hersenen van kinderen, er rond de puberteit een afname in grijze stof begint plaats te vinden in vooral de frontale en parietale hersengebieden. Witte stof (i.e. axonen), of te wel de bedrading tussen de neuronen, groeit nog door tot ver in de volwassenheid.

Het begin en de voortgang van deze puberteitsgerelateerde fenomenen kunnen van groot belang zijn voor het functioneren in de volwassenheid. Bijvoorbeeld, schizofrenie is een psychiatrisch ziektebeeld waarbij onderzoek in volwassenen heeft aangetoond dat de hersenen zijn aangedaan. Interessant in deze is het feit dat de eerste symptomen van de ziekte zich vaak rond de puberteit of vroege adolescentie manifesteren. Het is daarom van belang in eerste instantie meer

inzicht te krijgen in normale hersenontwikkeling om zodoende hersenafwijkingen op een vroege leeftijd adequaat te kunnen diagnosticeren en de voortgang van dergelijke ziektebeelden beter te kunnen begrijpen.

Met behulp van tweelingonderzoek kan de rol van genetische en omgevingsfactoren die bijdragen aan het ontstaan van individuele verschillen in een bepaalde eigenschap, zoals hersengrootte of structuur, in kaart worden gebracht. In het 'klassieke' tweelingdesign wordt de overeenkomst van een eigenschap (fenotype) binnen monozygote (eeneiige, MZ) tweelingparen vergeleken met de overeenkomst binnen twee-eiige (dizygote, DZ) tweelingparen. MZ tweelingen zijn ontstaan uit één eicel en delen grofweg 100% van hun genen, terwijl DZ tweelingen uit twee aparte eicellen zijn ontstaan en zij delen gemiddeld de helft van hun segregerende genen zoals 'gewone' broers en zussen (Martin et al., 1997). Omdat beide type tweelingen vaak samen opgroeien, delen ze ook hun gezinsomgeving, waarbij gedacht kan worden aan zorg, opvoeding, sociaal economische status en voedingspatroon. Daarnaast staan MZ en DZ tweelingen natuurlijk ook bloot aan omgevingsinvloeden die voor ieder individu uniek (kunnen) zijn, zoals ziektes, bepaalde hobby's, of het hebben van vriendjes en vriendinnetjes.

Binnen dit tweelingenmodel wordt gekeken naar de overeenkomsten binnen MZ paren versus DZ paren. Kort gezegd, indien een eigenschap binnen MZ tweelingen meer gelijkenis vertoont dan binnen DZ tweelingen (correlatie (r) MZ>rDZ), is dit een aanwijzing dat individuele verschillen binnen die eigenschap beïnvloed worden door erfelijke aanleg. De relatieve mate waarin individuele verschillen kan worden verklaard door genetische verschillen tussen individuen wordt ook wel erfelijkheid of 'heritability' genoemd. Deze maat wordt uitgedrukt in een percentage.

Om onderliggende factoren te onderzoeken die kunnen bijdragen aan het ontstaan van individuele verschillen in hersenstructuur in de (vroege) puberteit, werden in dit proefschrift twee onderzoekslijnen gevolgd: de eerste lijn was gericht op het in kaart brengen van genetische en omgevingsfactoren, zoals besproken in de hoofdstukken 2 en 3; de tweede lijn van onderzoek die is besproken in de hoofdstukken 4, 5, en 6 ging net name in op de relatie tussen HPG-as hormonen en hersenvolume en structuur.

De meeste hoofdstukken in dit proefschrift (3 tot en met 6) zijn gebaseerd op een speciaal voor dit onderzoek geworven groep gezinnen (107 in totaal), met een tweeling van 9 jaar oud en een oudere broer of zus tussen de 10 en 15 jaar oud. Voor zover wij weten, beschrijft dit proefschrift de grootste onderzoekspopulatie tot nu toe bestaande uit gezonde tweelingparen met dezelfde leeftijd op het gebied van MRI-hersenonderzoek. Daarnaast is de relatie tussen puberteitshormonen en hersenstructuur nooit eerder onderzocht in een groep kinderen met een dergelijke omvang.

In **hoofdstuk 2** werd een overzicht gepresenteerd van 18 tweelingstudies op het gebied van hersenvolume –en structuur. Een aantal van deze studies is gebaseerd op volumemetingen (structuren die manueel zijn gesegmenteerd), corticale dikte metingen, en een aantal studies waarbij gebruik werd gemaakt van *voxel-based morphometry* (VBM). VBM is een analysetechniek waarbij individuele verschillen in grootte en vorm van een brein worden verwijderd door middel van transformatie naar een model brein, maar waarbij lokale verschillen behouden blijven. De grijze en witte stof mappen worden 'uitgesmeerd', op een manier dat ieder voxel (i.e. een volume-element) de gemiddelde waarde van zichzelf representeert en van naburige voxels, oftewel relatieve dichtheden grijze of witte stof. Uiteindelijk kan men op voxel-niveau uitspraken doen over deze relatieve dichtheid binnen bepaalde groepen personen.

De resultaten van deze 18 tweelingstudies lieten zien dat gedurende het leven totaal hersenvolume erg hoog erfelijk is: erfelijkheidschattingen die gerapporteerd worden lopen uiteen van 89% tot wel 97%. Daarnaast lijken individuele verschillen in globaal grijze en witte stof ook te worden verklaard door genetische verschillen. Echter, voor de laterale ventrikels, de hersenkamers gevuld met cerebrospinale vloeistof, geldt dit niet. Het volume van deze ruimtes wordt vooral door (gedeelde en unieke) omgevingsfactoren beïnvloed. Indien erfelijkheden van kleinere structuren in het brein worden onderzocht, blijken er meer uiteenlopende resultaten te zijn: erfelijkheidschattingen variëren van 90-95% in de frontale hersenschors tot 40-69% in de hippocampus, versus meer prominente omgevingsinvloeden die bijdragen aan verschillen in hersengebieden rondom de ventrikels. Studies die gebruik maakten van corticale dikte metingen of VBM vonden een hoog

erfelijkheidspercentage in de mediale frontale cortex, Heschl's gyrus en de postcentrale gyrus. Matige erfelijkheidspercentages werden vooral gevonden in (para) hippocampus, amygdala en in de witte stof van de superior occipito-frontale fasciculus. Het blijkt dat de genetische invloed verandert met leeftijd: over een leeftijdspanne van de kindertijd tot late adolescentie is gevonden dat erfelijkheid van globaal grijze stof volume toeneemt. In dit hoofdstuk wordt veelal de nadruk gelegd op het belang van grote homogene (tweeling)populaties die geanalyseerd dienen te worden met geavanceerde statistische methoden, zoals structural equation modelling (SEM). Op deze manier kunnen betrouwbare uitspraken worden gedaan over genetische en omgevingsfactoren en de interactie tussen beide. Ook wordt in dit hoofdstuk het belang onderstreept van de bevindingen uit tweelingonderzoek indien men op zoek gaat naar specifieke genen en/of omgevingsfactoren die onze hersenontwikkeling gedurende het leven sturen.

In **hoofdstuk 3** werd de erfelijkheid van globale en regionale hersenmaten onderzocht in tweelingen rond de aanvang van de puberteit. Daarnaast werd de relatie tussen secundaire geslachtskenmerken (zoals borst-/penisgroei, schaamhaar ontwikkeling) en hersenstructuur in kaart gebracht door kinderen te vergelijken met en zonder deze kenmerken. In een groep kinderen bestaande uit 45 MZ en 62 DZ tweelingparen van 9 jaar oud werden globale hersenvolumes (intracranieel volume, totaal hersenvolume, grijze en witte stof, cerebellum en lateraal ventrikel volume) gemeten. Regionale grijze en witte stof-dichtheden werden gemeten met behulp van VBM. In overeenstemming met de resultaten besproken in hoofdstuk 2, lieten de resultaten van deze studie zien dat op 9-jarige leeftijd globale hersenvolumes zeer hoog erfelijk zijn: de erfelijkheidschattingen varieerden van 77% voor grijze stof volume tot 94% voor totaal hersenvolume. Een uitzondering vormde het ventrikelvolume dat maar voor 35% erfelijk werd geschat. De bevindingen waren meer variabel als het gaat om regionale schattingen. Omvangrijke witte stof gebieden gelegen met name achter in het brein (i.e. fronto-occipitale en superior longitudinale fasciculi) waren significant erfelijk. Een stuk minder omvangrijke grijze stof gebieden waren significant erfelijk, en werden gevonden in de amygdala, superior frontale gyrus en midden temporale gyrus. De resultaten impliceren dat er wellicht een stabiel genetische factor betrokken is bij de

ontwikkeling van witte stof dichtheid achter in het brein gedurende het leven: namelijk, achter in het brein gelegen witte stof is hoog erfelijk op 9 jaar wat overeenkomt met bevindingen in volwassenen. Daarnaast werd gevonden dat met het zichtbaar worden van de eerste secundaire geslachtskenmerken, grijze stof in frontale en pariëtale gebieden afneemt. Dit werd net name in meisjes gevonden en heeft hoogstwaarschijnlijk te maken met het feit dat relatief weinig jongens van 9 jaar secundaire geslachtskenmerken vertoonden.

Hoofdstuk 4 was gericht op de allereerste endocrinologische marker van de puberteit: luteïnizerend hormoon (LH). Dit hormoon werd bij de 9-jarige kinderen gemeten in ochtendurine en waardes werden bepaald met een zeer sensitieve meetmethode (immunometrisch assay). Eerder was al aangetoond dat met deze methode nachtelijke pieken in LH afgifte betrouwbaar kunnen worden vastgesteld en duiden op het begin van de puberteit (Demir et al., 1996). Deze nachtelijke LH pieken starten ongeveer 1 tot 2 jaar alvorens zich de secundaire geslachtskenmerken openbaren in zowel jongens als meisjes. In dit hoofdstuk werd de relatie tussen LH niveau en grijze en witte stof onderzocht en gekeken of er een mogelijke genetische basis ten grondslag ligt aan dit verband. Resultaten lieten zien dat hoe hoger het LH niveau is, hoe groter de proportie totale witte stof in de hersenen. Op regionaal niveau voorspelde een hogere LH spiegel meer witte stof dichtheid in het splenium van het corpus callosum, het cingulum en in de temporaal schors. De relatie tussen LH spiegels en deze witte stof gebieden lijkt te worden veroorzaakt door een gemeenschappelijk genetische factor. Doordat LH in geen enkel opzicht gerelateerd was aan grijze stof volume of dichtheid, suggereren deze data dat LH specifiek een invloed heeft op de ontwikkeling van witte stof.

Wanneer de resultaten van hoofdstukken 3 en 4 met elkaar worden vergeleken, dan valt op dat het vroege puberteitshormoon LH voornamelijk is geassocieerd met witte stof (toename), terwijl secundaire geslachtskenmerken, welke een resultaat zijn van de opvolgers van LH, namelijk oestradiol en testosteron, gerelateerd zijn aan grijze stof afnamen.

Omdat eerder onderzoek heeft aangetoond dat de hersenen tijdens de puberteit en adolescentie een regiospecifiek ontwikkelingspatroon laten zien (Giedd et al., 2006) is het wellicht mogelijk dat de hersenen verschillend reageren op

blootstelling aan fluctuerende hormoonspiegels gedurende deze turbulente periode (Sisk en Zehr, 2005). Om dit nader te onderzoeken werd in **hoofdstuk 5** de relatie tussen testosteron –en oestradiolspiegels en hersenstructuur onderzocht in een groep van 78 kinderen tussen de 10 en 15 jaar. Deze kinderen zijn de oudere broers en zussen van de eerder beschreven 9-jarige tweelingen en zij waren al wat verder in de puberteit. Op grond van het gegeven dat geslachtshormonen betrokken zijn bij de ontwikkeling van geslachtsverschillen, werd ook onderzocht of verhoogde puberteitsproductie van testosteron en oestradiol verband hield met geslachtsverschillen in het brein. Analyses wezen uit dat hogere oestradiol spiegels in meisjes waren geassocieerd met lagere grijze stof dichtheid in (pre-)frontale, parietale en temporale gebieden. Deze oestradiolgerelateerde afname bleef bestaan nadat er voor leeftijdsgerelateerde totale grijze stof afname was gecontroleerd. In andere delen van de hersenen werden juist oestradiol gerelateerde toenamen van de grijze stof gevonden, gelokaliseerd in occipitale, midden-temporale en midden-frontale gebieden van de cortex. Oestradiol spiegels in meisjes waren niet gerelateerd aan witte stof dichtheden. Oestradiol spiegels in jongens lieten geen verband zien met grijze als witte stof dichtheid. In geen van beide seksen konden testosteronniveaus worden gerelateerd aan grijze of witte stof. Conform eerdere studies werden er prominente geslachtsverschillen gevonden in grijze stof dichtheid, onder andere in de amygdala, putamen, thalamus, insula, rostrale anterior cingulate, en superior temporale gyrus (hogere dichtheid in jongens) en in de hippocampus, caudate nucleus, caudale anterior cingulate, midden temporale gyrus en inferior occipitale gyrus (hogere dichtheid in meisjes). Echter, deze seksueel dimorfe gebieden lieten tegen de verwachting in geen associatie zien met testosteron of oestradiol spiegels. Uit dit hoofdstuk kan worden geconcludeerd dat in meisjes die al wat verder in de puberteit zijn dan jongens, oestradiol betrokken lijkt te zijn bij de ontwikkeling van heteromodale gebieden in de cortex. De bevinding dat er in jongens geen dergelijk verband was tussen geslachtshormonen en breinstructuur, zou kunnen liggen aan het feit dat op deze leeftijd hormoonspiegels nog relatief laag zijn en/of dat de effecten zich mogelijk in een andere periode manifesteren.

Dus, tegen de verwachting in kon er geen associatie worden vastgesteld tussen geslachtshormoonspiegels in de (vroege) puberteit en geslachtsverschillen in het brein. Zodoende werd de hypothese opgesteld dat geslachtsverschillen in het brein wellicht al veel eerder dan de puberteit ontstaan, namelijk in de prenatale fase waarin het brein sterk gevoelig is voor de invloed van geslachtshormonen. Uit dierstudies kwam eerder al naar voren dat prenatale testosteron blootstelling een rol speelt bij het 'masculiniseren' van de hersenen, terwijl in de afwezigheid van testosteron blootstelling er juist 'feminisatie' optreedt (Collaer and Hines, 1995). Daarnaast werd in dieren gevonden dat de positie van een foetus in de baarmoeder ook prenatale testosteron blootstelling kan beïnvloeden, door middel van de nabijheid van een mannelijke foetus (Vom Saal, 1989). In mensen is er vlak na de geboorte al een duidelijk geslachtsverschil in breingrootte: pasgeboren jongens hebben grotere globale hersenvolumes (ongeveer 10% groter) dan pasgeboren meisjes (Gilmore et al., 2007). Ook na correctie voor lichaamslengte blijft dit opvallende verschil bestaan. Gegeven deze bevindingen, werd in het laatste **hoofdstuk 6** geprobeerd om een relatie te vinden tussen het delen van de baarmoeder met een broertje en masculinisatie van hersenvolumes ten opzichte van het delen van een baarmoeder met een zusje tijdens de zwangerschap. Vier groepen kinderen afkomstig van dizygote tweelingen werden onderzocht: jongens met een broertje (SSM), jongens met een zusje (OSM), meisjes met een broertje (OSF) en meisjes met een zusje (SSF), allemaal 9 jaar oud (119 individuen). Gebaseerd op hun vermoedelijke blootstelling aan prenatale testosteronspiegels, werd verwacht dat kinderen met een broertje (SSM en OSF) een groter hersenvolume zouden hebben dan kinderen met een zusje (OSM en SSF). Hierbij werd rekening gehouden met 'eigen' geslachtsverschillen en effecten van geboortevolgorde. Resultaten lieten zien dat kinderen met een broertje inderdaad een groter totaal breinvolume, witte stof volume en cerebellum hebben dan kinderen met een zusje, bovenop het "gewone" geslachtsverschil. SSM kinderen, van wie verwacht werd dat zij blootgesteld zijn aan de hoogste prenatale testosteronspiegels, vertoonden de meest 'mannelijke' (grootste) hersenvolumes gevolgd door OSM, OSF en SSF kinderen. Van belang is dat huidige testosteron (en/of oestradiol) spiegels of lichaamslengte deze verschillen niet konden verklaren. Deze resultaten suggereren

dat prenatale testosteron blootstelling mogelijk een rol kan spelen in 'masculinisatie' (vergroting) van totaal hersenvolume. Daarnaast bleek uit een post-hoc analyse dat het masculiniserende effect van een tweelingbroertje op de hersenen even sterk was in kinderen met een oudere broer en in kinderen met oudere zus. Deze bevinding maakt de rol van post-natale (sociale) effecten op totaal hersenvolume onwaarschijnlijker dan prenatale effecten.

Uit de resultaten van dit proefschrift blijkt dat erfelijke factoren ten grondslag liggen aan het tot stand komen van individuele verschillen in hersenvolume op 9 jaar. Echter, wanneer er op een meer regionaal niveau in het brein wordt gekeken, lijkt grijze stof in de voorste delen van de hersenen een stuk minder erfelijk dan de witte stof gelegen achter in het brein. Eerdere studies van andere onderzoeksgroepen toonden al aan dat het brein rond de puberteit en adolescentie nog niet is uitontwikkeld. Vooral de zogenoemde heteromodale cortex (prefrontale, parietale en temporale gebieden) ontwikkelt zich nog verder in die periode (e.g. Giedd et al., 1999; Sowell et al., 2002; Gogtay et al., 2004). De studies in dit proefschrift geven een eerste voorzichtige aanwijzing dat grijze stof in juist die gebieden meer gevoelig lijkt te zijn voor omgevingsinvloeden. Wat voor invloeden dat dan zijn moet nader worden onderzocht.

In de zojuist aangehaalde studies naar de hersenen in deze periode werd puberteitsontwikkeling nooit direct gemeten. Uit dit proefschrift wordt duidelijk dat in relatie met hormonale veranderingen in de (vroege) puberteit, grijze en witte stof zich verder lijken te ontwikkelen. Specifieker kan gesuggereerd worden dat voornamelijk in een allereerst stadium van de puberteit, LH productie witte stof groei zou kunnen moduleren in bepaalde gebieden. Het is goed wel mogelijk dat gemeenschappelijke genen die zowel LH productie als witte stof groei beïnvloeden hieraan een essentiële bijdrage leveren. In een meer gevorderd puberteitsstadium lijkt juist een verhoogde productie van oestradiol in meisjes gerelateerd te zijn aan grijze stof veranderingen (toe –evenals een afname). In jongens werd er geen verband gevonden tussen huidige geslachtshormoonspiegels en hersenstructuur. Een verklaring voor het feit dat in jongens (nog) geen dergelijk verband tussen geslachtshormonen en hersenstructuur kon worden gevonden, zou kunnen liggen aan een latere puberteitsontwikkeling in jongens dan meisjes. Echter, longitudinale

studies zijn noodzakelijk om daarover uitsluitsel te kunnen geven. Daarnaast kan het wellicht zo zijn dat prenatale testosteronspiegels hersenstructuur op deze leeftijd (tussen 9 en 14 jaar) beter voorspellen dan testosteronspiegels in de (vroege) puberteit.

Concluderend kan worden gezegd dat het puberbrein een ‘werk in uitvoering’ is. Dit proefschrift draagt bij aan een beter begrip van het samenspel tussen genetische en omgevingsfactoren, hormonen en hersenstructuur in deze belangrijke levensfase. Vervolgmetingen bij deze populatie zullen zich onder andere richten op de mogelijke interactie tussen leeftijd en genotype in relatie tot hersenontwikkeling. Ook zal de associatie tussen geslachtshormonen en hersenontwikkeling verder worden onderzocht.

List of publications

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Abstracts and conference proceedings

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Peper JS, Schnack HG, Brouwer RM, Van Baal GCM, Boomsma DI, Kahn RS, Hulshoff Pol HE . Genetic and hormonal influences on brain volume around the onset of puberty: An MRI study in healthy twin pairs and their siblings (2007). *J Neuroscience*. 729.12/OO25. *Poster presented at the 37th Annual Meeting of the Society for Neurocience. San Diego, USA. 3-7 november 2007.*

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Van Leeuwen M, **Peper JS**, Van den Berg SM, Kahn RS, Hulshoff Pol HE, Boomsma DI. The heritability of hormonal levels during puberty. *Poster presented at the 37th Annual Meeting of the International Society of Psychoneuroendocrinology (ISPNE). Leiden, The Netherlands, 23-26 August 2006.*

Van Honk J, Morgan BM, Thornton H, **Peper JS**, Pjetri E, Mandl RCW, Carey P, Spottiswoode B, Hulshoff Pol HE, Stein DJ. Neural degradation and growth in medial frontal structures after bilateral damage to the human amygdala. *Paper presented at the 39th Annual Meeting of the International Society of Psychoneuroendocrinology (ISPNE). Dresden, Germany. 17-20 July 2008.*

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Yiska

Curriculum Vitae

Jiska Peper was born on December 3, 1978, in Dordrecht, the Netherlands. In 1997, she graduated from the Sint Bonifatius College in Utrecht. In the same year, she started studying Psychology at Utrecht University. At the department of Psychonomics, she did an internship and carried out a research project on the influence of testosterone on brain activation and emotional processes, under supervision of Dr. Dennis Schutter and Dr. Jack van Honk. In 2003, she started a PhD-project at the Rudolf Magnus Institute of Neuroscience, Department of Psychiatry, at the University Medical Center Utrecht. Under supervision of Prof. Dr. Hilleke Hulshoff Pol, Prof. Dr. René Kahn and Prof. Dr. Dorret Boomsma, she investigated the influence of genetic and environmental factors and pubertal hormones on brain structure in early pubertal children, which resulted in the current thesis. Since September 2008, she has been working as a post-doc researcher at the departments of psychonomics (UU) and psychiatry (UMC) with Dr. Jack van Honk and Prof. Dr. Hilleke Hulshoff Pol.

Jiska Peper werd op 3 december 1978 geboren te Dordrecht. Ze behaalde in 1997 het VWO-diploma aan het Sint Bonifatius College te Utrecht. In datzelfde jaar ging zij psychologie studeren aan de Universiteit Utrecht. Zij heeft stage gelopen en een afstudeeronderzoek uitgevoerd bij de vakgroep Psychonomie, onder leiding van Dr. Dennis Schutter en Dr. Jack van Honk naar de effecten van testosteron op hersenactiviteit en emotionele processen. Zij begon in 2003 aan een promotietraject bij het Rudolf Magnus Instituut voor Neurowetenschappen, op de afdeling psychiatrie, in het Universitair Medisch Centrum Utrecht. Onder supervisie van Prof. Dr. Hilleke Hulshoff Pol, Prof. Dr. René Kahn en Prof. Dr. Dorret Boomsma heeft zij onderzoek gedaan naar genetische -en omgevingsfactoren en puberteitshormonen in relatie tot hersenstructuur in de vroege puberteit, dat heeft geleid tot dit proefschrift. Sinds september 2008 is zij werkzaam als post-doc onderzoeker aan de vakgroep psychonomie (UU) en psychiatrie (UMC) onder leiding van Dr. Jack van Honk en Prof. Dr. Hilleke Hulshoff Pol.

Appendix

Colour figures

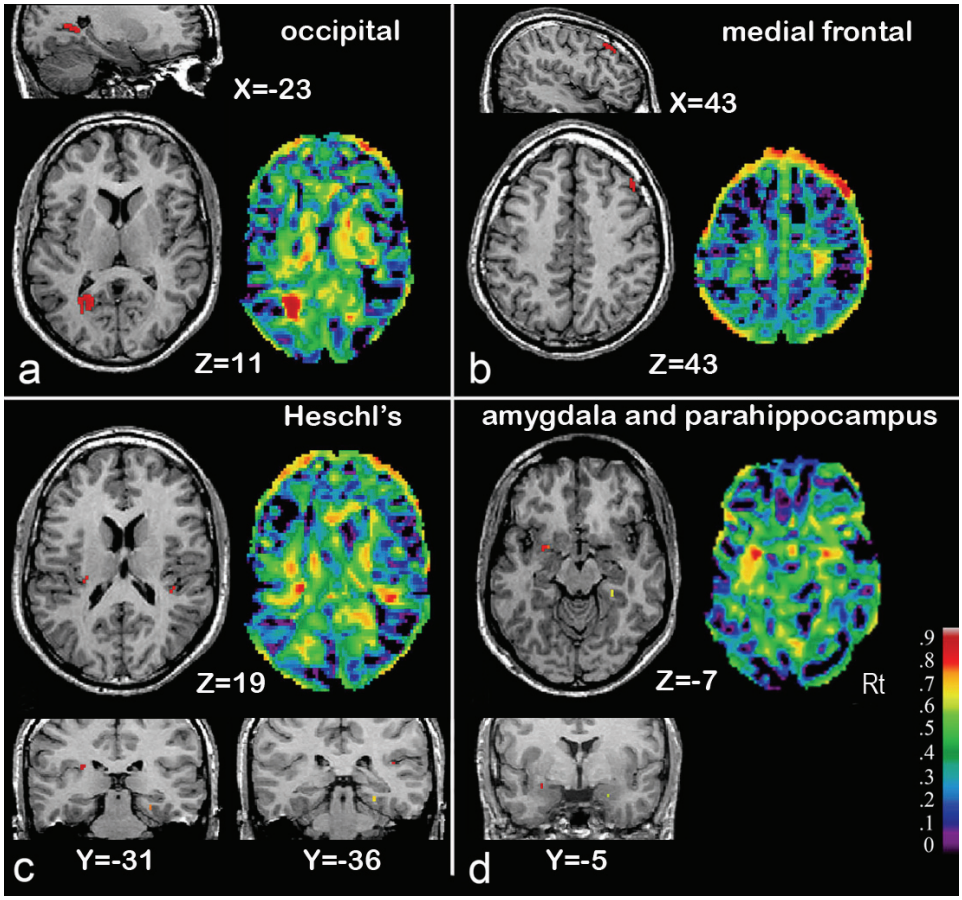

Figure 2.1 Genetically influenced focal gray matter density brain areas in adult twins.
Significant heritability maps are superimposed on axial and sagittal sections through the magnetic resonance image of the standardized reference brain (left). The complete heritability maps are shown on the right. Data are based on a study in 258 monozygotic and dizygotic twin pairs and their siblings from 112 Dutch families. For genetic analyses, structural equation modeling and voxel-based morphometry was used (Hulshoff Pol et al, 2006a, reprinted with permission of the Journal of Neuroscience).

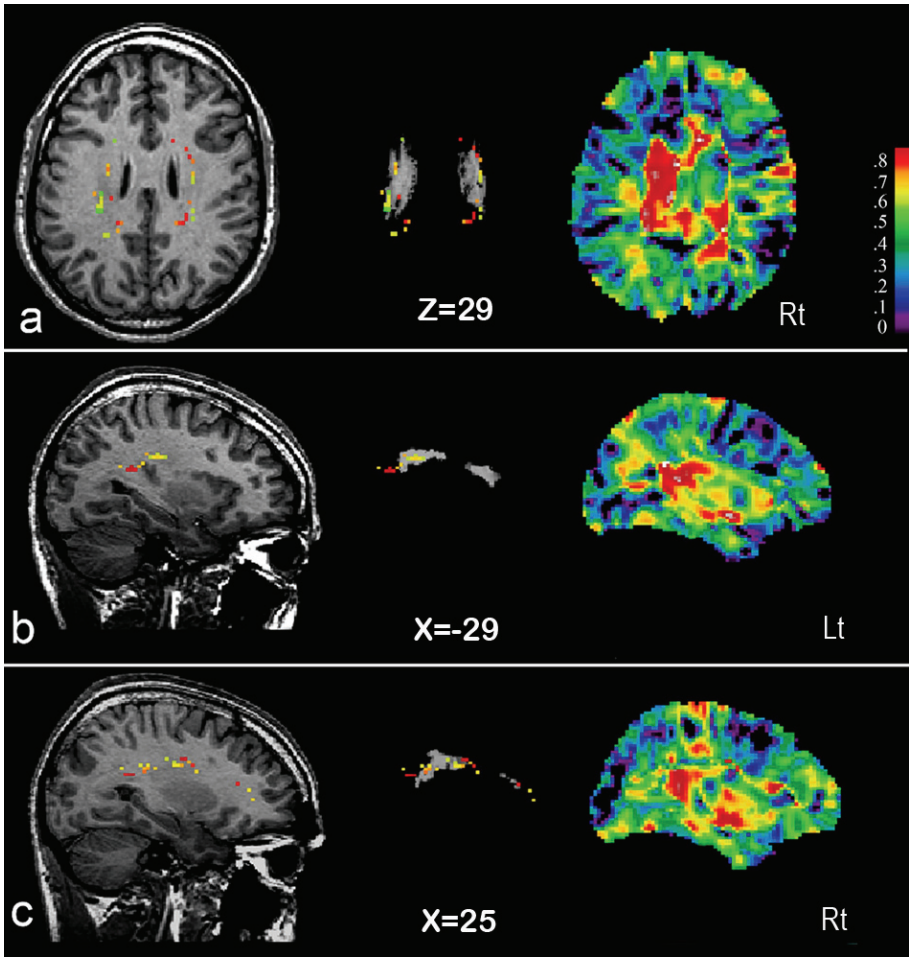

Figure 2.2 Genetically influenced focal white matter density brain areas in adult twins. Significant heritability maps are superimposed on axial and sagittal sections in the left (Lt) and right (Rt) hemisphere through the magnetic resonance image of the standardized reference brain (left) and superimposed on the histologically defined map of the occipitofrontal superior fascicle (middle). The complete heritability maps are shown on the right. (for details see Hulshoff Pol et al, 2006a, reprinted with permission of the Journal of Neuroscience).

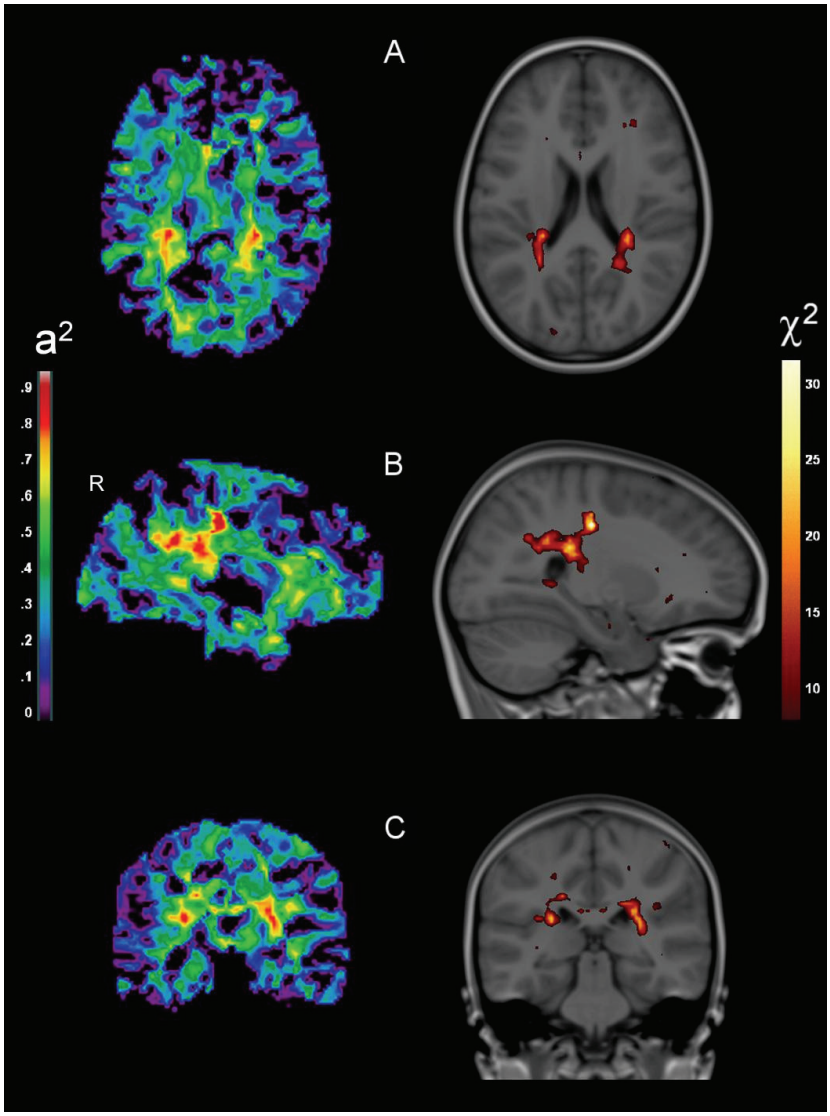

Figure 3.1 Genetically influenced regional white matter density in 9-year-old twins
A) Superior occipitofrontal fascicle (Z=36), B) Superior occipitofrontal fascicle, superior longitudinal fascicle (X=27), C) Superior longitudinal fascicle (Y=-22). Images are according to neurological convention (left=left). The left side displays heritability-estimates. The right side displays significant genetic effects in χ^2-values, overlaid on the model-brain: CE versus ACE model (critical level of significance is 17.7, corrected for multiple comparisons according to the False Discovery Rate (FDR), α=.05). For visualization purposes, this threshold is relaxed to an FDR of α=.15 (corresponding to a χ^2-value of 10.5). χ^2-maps are resampled to anatomical resolution for overlap with anatomical boundaries.

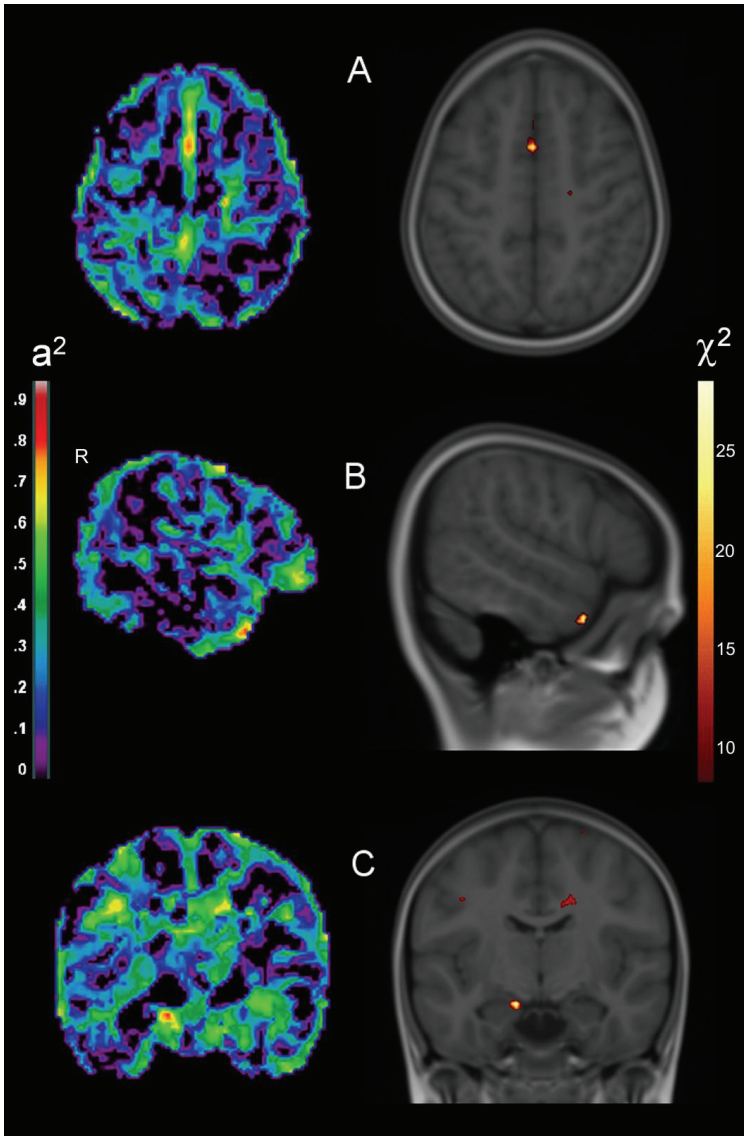

Figure 3.2 Genetically influenced regional gray matter density in 9-year-old twins
A) Superior frontal gyrus (Z=57) B) Middle temporal gyrus (X=55) C) Amygdala (Y=-9). Images are according to neurological convention (left=left). The left side displays heritability-estimates. The right side displays significant genetic effects in χ^2-values, overlaid on the model-brain: CE versus ACE model (critical level of significance is 20.5, corrected for multiple comparisons according to the False Discovery Rate (FDR), α=.05). For visualization purposes, this threshold is relaxed to an FDR of α=.15 (corresponding to a χ^2-value of 12.2). χ^2-maps are resampled to anatomical resolution for overlap with anatomical boundaries.

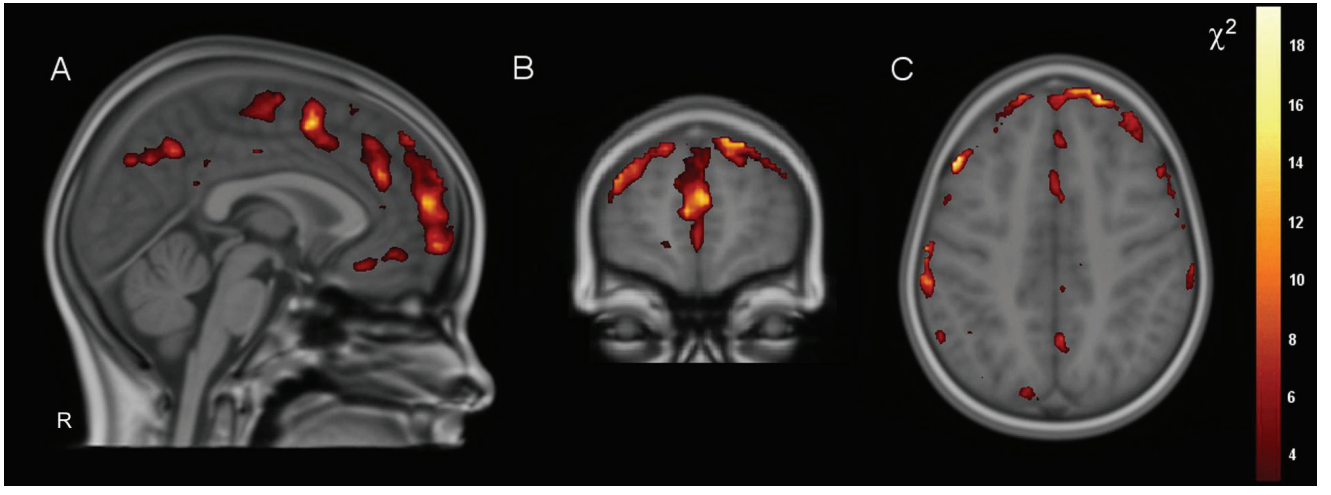

Figure 3.3 Decreases in (pre)frontal and parietal gray matter density in pubertal children, compared to non-pubertal children at 9 years.
Shown here are χ^2-values of the worsening in fit of a (means) model with Tanner-status versus without Tanner-status, corrected for sex and handedness. Level of significance: $\chi^2 > 3.84$ ($\alpha<0.05$, uncorrected for exploratory purpose). To measure decreases or increases in density, χ^2-maps were multiplied by negative and positive regression maps respectively. Significant reductions in gray matter density are mainly located in the (pre)frontal cortex (A (X=2), B (Y=60) & C (Z=41)) and parietal cortex (A & C) bilaterally with χ^2-values up to 18.5. Images are according to neurological convention (left=left) χ^2-maps are resampled to anatomical resolution for overlap with anatomical boundaries.

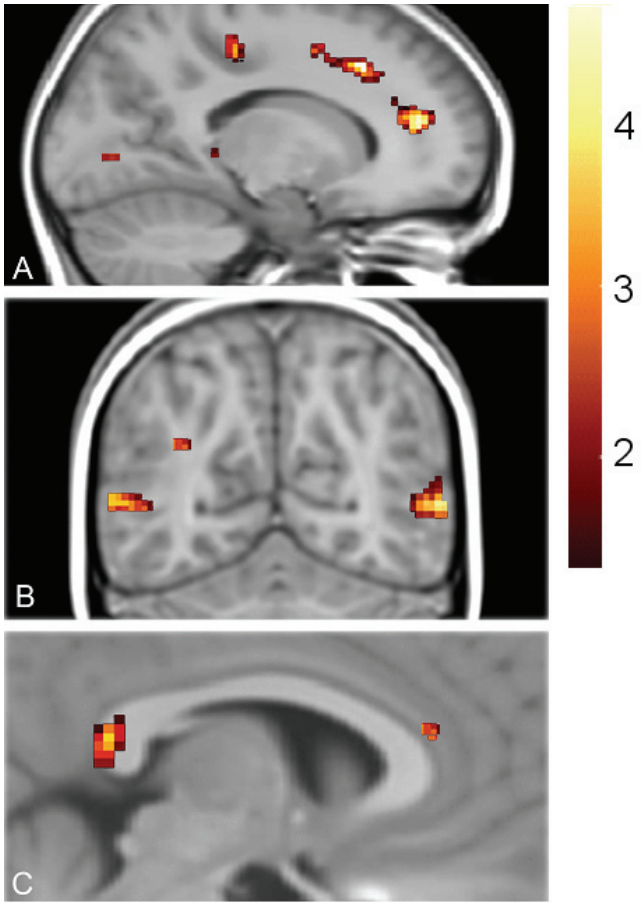

Figure 4.1 Positive phenotypic associations between LH level and regional white matter density in 104 9-year old twins.
A) Left cingulum B) Bilateral middle temporal gyrus C) Splenium of the corpus callosum. Displayed are z-values. The critical z-value was 3.39 (corrected for multiple comparisons according to the False Discovery Rate α=.05

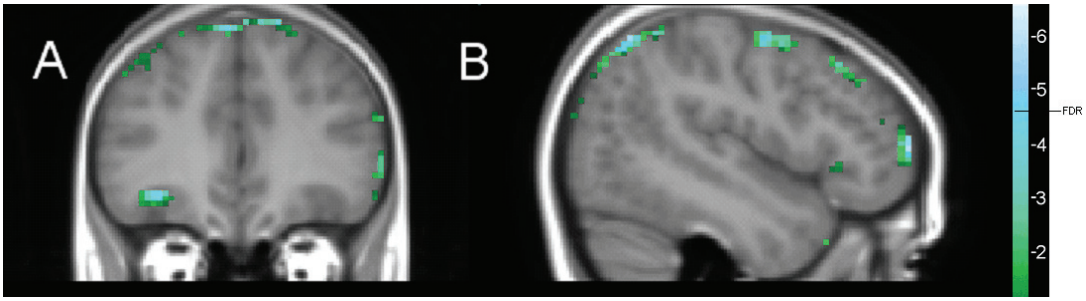

Figure 5.1a Estradiol associated with gray matter decreases (N=35 girls (10-15 years old)), corrected for age.
Bilateral superior- and left orbitofrontal gyri (A), Y=42, and right inferior frontal and angular gyri (B), X=-48. Images are according to neurological convention (left=left). Critical level of significance is -4.60, corrected for multiple comparisons according to the False Discovery Rate, α=.05, two-tailed). Significant voxels are overlaid on our created model brain.

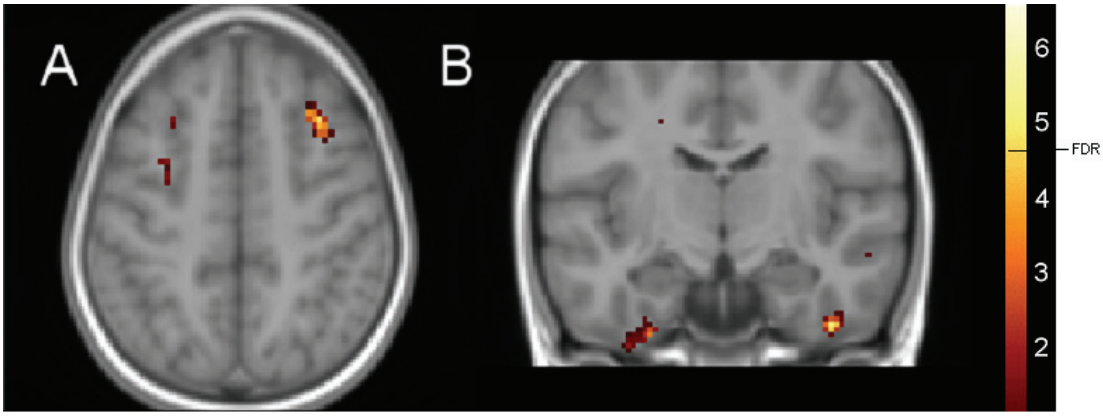

Figure 5.1b Estradiol associated with gray matter increases (N=35 girls (10-15 years old)), corrected for age.
Right middle frontal gyrus (A), Z=47 and right inferior temporal gyrus (B), Y=-7. Images are according to neurological convention (left=left). Critical level of significance is 4.60, corrected for multiple comparisons according to the False Discovery Rate, α=.05, two-tailed. Significant voxels are overlaid on our created model brain.

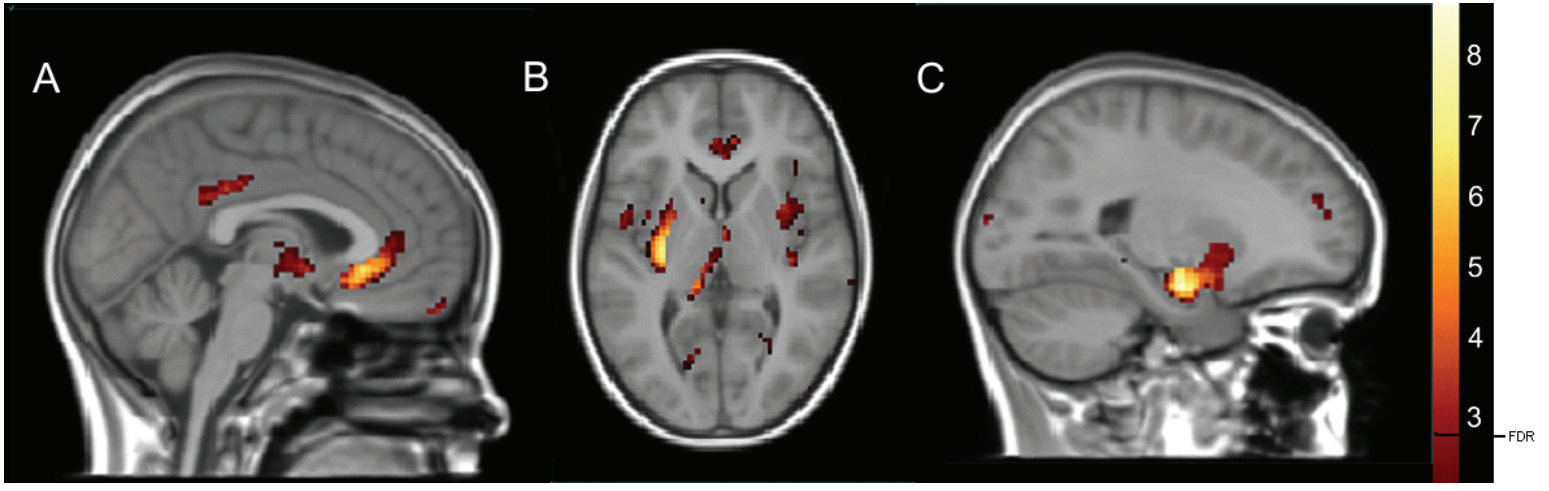

Figure 5.3a. Larger gray matter density in boys compared to girls between 10 and 15 years (corrected for age). A) left rostral anterior cingulate gyrus (X=-1), B) left putamen (Z=4) C) left amygdala (X=-21). Critical level of significance is 2.88, corrected for multiple comparisons according to the False Discovery Rate, α=.05, two-tailed. Significant voxels are overlaid on our created model brain.

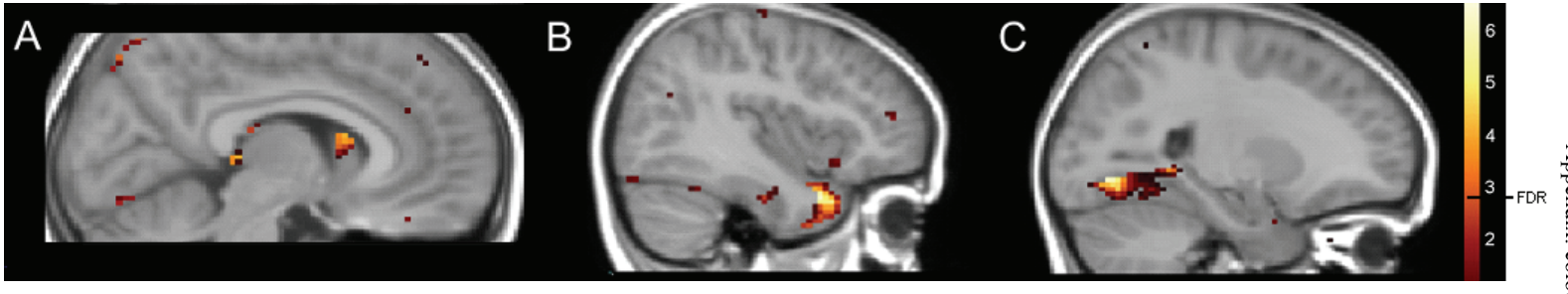

Figure 5.3b. Larger gray matter density in girls compared to boys between 10 and 15 years (corrected for age). A) Right caudate nucleus (X=10), B) right superior and middle temporal gyri (X=45) C) left inferior occipital gyrus (X=-47) Critical level of significance is 2.88, corrected for multiple comparisons according to the False Discovery Rate, α=.05 (two-tailed). Significant voxels are overlaid on our created model brain.