# 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.<br>2) Regionally: high heritability of GM in frontal cortex, WM of SOF & SLF.<br>3) Somewhat lower heritability of (para-) hippocampus. |
| **3** | 1) Study heritability of brain volume and structure in 9-year old twins.<br>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%.<br>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.<br>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.<br>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.<br>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.<br>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).<br>2) In boys: no association between T or E and brain structure, nor between T and brain structure in girls.<br>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.<br>2) Current T and E level could not explain the enlarged brain volumes.<br>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.