Sex Differences in the Human Brain and the Impact of Sex Chromosomes and Sex Hormones

E. Lentini1, M. Kasahara2, S. Arver3 and I. Savic1

1Department of Women and Child Health, 2Department of Clinical Neuroscience and 3Department of Medicine, Karolinska Institute, Stockholm, Sweden

Address correspondence to Ivanka Savic, Department of Clinical Neuroscience, Stockholm Brain Institute, Karolinska Institutet, Retziusväg 8, 171 77 Solna, Sweden. Email: ivanka.savic-berglund@ki.se

Ivanka Savic designed and wrote the paper; Emilia Lentini analyzed the data and performed research; Stefan Arver performed research; and Maki Kasahara ran analyses.

While there has been increasing support for the existence of cerebral sex differences, the mechanisms underlying these differences are unclear. Based on animal data, it has long been believed that sexual differentiation of the brain is primarily linked to organizational effects of fetal testosterone. This view is, however, in question as more recent data show the presence of sex differences before the onset of testosterone production. The present study focuses on the impact that sex chromosomes might have on these differences. Utilizing the inherent differences in sex and X-chromosome dosage among XXY males, XY males, and XX females, comparative voxel-based morphometry was conducted using sex hormones and sex chromosomes as covariates. Sex differences in the cerebellar and precentral gray matter volumes (GMV) were found to be related to X-chromosome dosage, whereas sex differences in the amygdala, the parahippocampus, and the occipital cortex were linked to testosterone levels. An increased number of sex chromosomes was associated with reduced GMV in the amygdala, caudate, and the temporal and insular cortices, with increased parietal GMV and reduced frontotemporal white matter volume. No selective, testosterone-independent effect of the Y-chromosome was detected. Based on these observations, it was hypothesized that programming of the motor cortex and parts of cerebellum is mediated by processes linked to X-escapee genes, which do not have Y-chromosome homologs, and that programming of certain limbic structures involves testosterone and X-chromosome escapee genes with Y-homologs.

Keywords: brain, MRI, XXY, sex chromosome, sex dimorphism, sex hormone

Introduction

Many neuropsychiatric disorders show an uneven sex distribution in regard to their prevalence, and, in some types, their age at onset and treatment response (Bao and Swaab 2010). The origins of this are largely unknown. One possible explanation is that sex differences in cerebral anatomy, connectivity and function render men and women differently susceptible to certain aspects of psychopathology.

Cerebral sex differences have been shown at the group level in regard to structural volumes as well as regional volumes of gray matter (GM) and white matter (WM), even when correcting for individual differences in total brain volume. In general, women have been found to have larger structural volumes in the hippocampus, caudate nucleus (Filipek et al. 1994; Murphy et al. 1996; Giedd et al. 1997, 2006), and the anterior cingulate gyrus (Paus et al. 1996), whereas the volume of the amygdala (Giedd et al. 1997, 2006; Neufang et al. 2009) has been found to be larger in men. With some exceptions (Goldstein et al. 2001; Carne et al. 2006), men have been found to have larger GM volumes in the mesial temporal lobe, the cerebellum, and the lingual gyrus (Good et al. 2001; Carne et al. 2006). Women, on the other hand, seem to have larger GM volumes in the precentral gyrus, the anterior cingulate, the right inferior parietal, and the orbitofrontal cortex (Nopoulos et al. 2000; Good et al. 2001; Luders et al. 2005; Luders, Gaser et al. 2009; Luders, Sanchez et al. 2009; Savic and Arvis 2011). In addition, there are reports of larger GM volumes among women in the dorsolateral prefrontal cortex (Schlaepfer et al. 1995) and planum temporale (Witelson et al. 1995).

To date, the mechanisms underlying the observed sex differences remain uncertain. Unraveling these mechanisms is of considerable interest not only for improving our understanding of some of the basic aspects of human biology, but also for investigating the pathophysiology of those disorders which have a skewed sex distribution, including attention deficit hyperactivity disorder (ADHD), autism spectrum disorders, (male/female prevalence ratio of 6:1–2:1 in various reports), major depression, and anxiety (male/female ratio of ca. 1:3). These conditions are associated with changes in the basal ganglia, the amygdala, the anterior cingulate gyrus, which, according to some studies, are brain regions whose structural volumes differ between healthy men and women (American Psychiatric Association 2000; Baron-Cohen et al. 2000; Andreasen 2005; McAlonan et al. 2008; McAlonan et al. 2008; Balint et al. 2009; Lai et al. 2012; Tomasi and Volkow 2012).

For many years, the dominant hypothesis among biologists was that sex differences were linked to intrauterine exposure to testosterone (Phoenix et al. 1959; Ehrhardt and Meyer-Bahlburg 1979), which after metabolization to estrogen would induce organizational effects on an initially undifferentiated “female” brain, leading to its masculinization during a limited period of development. This hypothesis was based on animal experiments showing that the absence of androgen in male rats and exposure to androgen in female rats during sexual differentiation of the brain leads to a complete inversion of sexual behavior (Phoenix et al. 1959; Ehrhardt and Meyer-Bahlburg 1979). The possible effects of excess intrauterine testosterone may be studied in humans by investigating females with adrenal cortical hyperplasia (CAH). Contrary to expectations, no sex atypical features in any of the cerebral volumes measured were found in CAH women, compared with female controls (Merke et al. 2003; Giedd et al. 2006; Ciumas et al. 2009). This absence of masculinization in...
women, despite their high level of fetal testosterone, suggests that factors other than fetal testosterone might be involved in the development of cerebral sex dimorphism in humans. Such a scenario is supported by elegant studies by Arnold’s group, showing that cerebral sex dimorphism exists long before the fetus starts to produce testosterone (Arnold et al. 2004; Arnold and Chen 2009). Further support is given through findings using animal data, which have demonstrated that several genes are expressed in the brain in a sexually dimorphic manner (Jazin and Cahill 2010). Altogether, these more recent scientific achievements encourage investigations of genomic effects on the human brain. In this respect, the possible impact of X-chromosome gene dosage deserves special attention.

In the somatic cells of females with 46, XX, 1 of the 2 X-chromosomes is randomly inactivated. About 15% of X-linked genes escape this process, and are denoted as escapee genes (Carrel and Willard 2005). In 46 XX females, these genes will be expressed from both X-chromosomes, whereas in 46 XY males, only from one X-chromosome. Only a few of the escapee genes have homologs on the Y chromosome. They are located in the so-called pseudoautosomal region (Xu and Disteche 2006). Consequently, the remaining X-linked escapee genes may play a major role in sexual differentiation by virtue of their higher expression in XX females.

The possible effects of sex chromosome linked gene expression on cerebral sex dimorphism in humans can be studied by examining the unique condition of sex chromosome aneuploidy (Good et al. 2003; Shen et al. 2004; Giedd et al. 2007). To the best of our knowledge, magnetic resonance imaging (MRI) data from subjects with sex chromosome aneuploidy have not been analyzed regarding sex dimorphism of cerebral GM and WM. Such studies are, however, warranted, as they may potentially generate important information about the effects of sex chromosome gene dosage. Optimally, they should be carried out so that the possible effects of sex hormones can be assessed in parallel (not withstanding that some hormonal effects may be indirect X-chromosome gene effects). This would require measuring the sex hormone levels and evaluating the possible effects of fetal testosterone. Fetal testosterone cannot, for natural reasons, be assessed in adult subjects. Several scientific reports suggest, however, that the ratio between the length of the second and fourth digit (2D:4D), may serve as its correlate (Manning et al. 1998, 2004; Williams et al. 2000; Lutchmaya et al. 2004; Coates et al. 2009; Honekopp and Watson 2010).

In the present MR study, men with Klinefelter’s syndrome (47, XXY males) were studied together with 46, XX and 46, XY controls to explore whether sex chromosomes (X-chromosomes in particular) and sex hormones have an impact on the described sex differences in the GM and WM volumes. An attempt was also made to find out whether the possible effects of sex chromosomes and sex hormones could be regionally differentiated. XXY males have a supernumerary X-chromosome, and although their phenotype may vary, the findings from brain imaging studies have been rather consistent (Giedd et al. 2007; Lenroot et al. 2009). The testosterone levels in XXY males have been found to be normal or subnormal during the prenatal period and up to puberty (Carson et al. 1982; Ratcliffe et al. 1994), before becoming drastically reduced (Akslaa et al. 2006) and calling for testosterone supplementation. The gender roles of XXY males do not differ from other males; their identity is male, and the majority of XXY men are heterosexual.

Based on the following data, we had 2 main reasons for expecting to find cerebral differences between XXY males and controls, and assumed that these differences would be primarily related to 2 types of genetic mechanisms. First, in XXY males there could be an excessive expression of genes that lie in the pseudoautosomal regions of the X-chromosome. These X escapee genes have Y-chromosome homologs, and may be expressed in a higher dose in XXY males than in both XX and XY controls, leading to cerebral differences in comparison to “both control groups.” Second, like in XX females there are also other X escapee genes, although their exact percentage has not been estimated in XXY males (Vawter et al. 2007), and these genes do not have Y-chromosome analogues. It is assumed that they are expressed in excess in XXY males “only in relation to XY males” but not to typical XX females. Thus, provided that chromosome-linked processes were involved, some differences were expected only in relation to XY males, others in relation to both control populations.

We, therefore, hypothesized: (1) that XXY men would have different GM and WM volumes from controls and that these differences are due to sex chromosome aneuploidy, and hypogonadism (in this order), rather than the social environment. (2) That differences in regional GM and WM volume between XXY and XY men, which are also found between XX women and XY men (features more closely resembling those in females), would be primarily due to the genes located on the extra X-chromosome. (2) Cerebral differences in XXY males, when compared with both control groups, should, on the other hand, be due to trisomy.

(3) Because of hypogonadism, the testosterone levels of XXY males are lower than in XY males (prior to testosterone substitution) but higher than in XX females. Consequently, regional changes in XXY males could be attributed to their lower testosterone levels, if they resulted in features “in between” those of XX women and XY men (for example, a larger regional GM volume than in XX women, but smaller than in XY men).

To test these hypotheses, 45 XX females, 41 XY males, and 33 XXY males were investigated using MRI and voxel based morphometry (VBM). The data generated were analyzed consecutively at several levels, and explored with increasing complexity utilizing the options to add nuisance variables and variables of interest in the explorative multi-regression model used [statistical parametric mapping 5 (SPM5), Wellcome Foundation].

Experimental Procedures

Subjects
Thirty-eight males with Klinefelter’s syndrome were recruited consecutively from the Center of Andrology, Department of Medicine, Karolinska University Hospital, Sweden, along with 86 controls from the general public. The exclusion criteria included not being 20–55 years, having karyotypic mosaicism; a heredity for, history of, or current psychosis; a personality disorder; a major or bipolar depression; a neurological disease; and alcohol or substance abuse problems. Because our primary purpose was to evaluate the possible effects of X-chromosome dosage on sex differences in the brain, XXY subjects with comorbid autism and ADHD were also excluded. Mild dyslexia was present in about 60% of the patients. After exclusion of 5 patients due to depression, substance abuse, or
hallucinations, the population included in the analysis consisted of 33 XXY males (age 39 ± 10 years, range 21–55; education 13.2 ± 2.7 years), 41 XY males (age 35 ± 7 years, range 27–50, education 16.2 ± 2.4), and 45 XX females (age 35 ± 7 years, range 26–51, education 15.9 ± 2.2).

All subjects underwent a medical examination at the screening visit, including an evaluation of their medical histories and routine laboratory tests. Comorbid psychiatric disorders and personality disturbances were assessed according to the Diagnostic and Statistical Manual of the American Psychiatric Association, 4th edition (DSM–IV®; 13) by a psychiatrist. This assessment included a Structured Questionnaire for DSM–IV® Axis I and II (Structured Clinical Interview for DSM–IV® (SCID-I and II) (American Psychiatric Publishing Inc., 1997), as well as scales for depression (Beck Depression Inventory scores (Beck 1961). The average Beck Depression Inventory score was 8.6 ± 7.7 for the XXY males, 2.4 ± 2.3 for the male controls, and 4.7 ± 2.9 for the female controls. Although patients scored significantly higher than controls (df = 2, $F = 9.345$, $P = 0.00025$), their values were within the normal range. Thirty patients had been diagnosed in early adolescence and 3 received the diagnosis as adults during the course of infertility investigations. All but 2 of the patients (who were deemed to not need testosterone) had received testosterone supplementation when diagnosed and were being treated at the time of the study. About half of the patient population was receiving daily gels, the other half received injections. None of the patients received medication immediately before drawing blood samples. The karyotyping test for Klinefelter’s syndrome was performed by assessing the metaphase chromosomes in cells derived from whole blood samples according to the standard procedure.

All the participants were right-handed [Edinburgh Handedness Inventory (Oldfield 1971)]. All were heterosexual (scored Kinsey 0) according to Kinsey’s Heterosexual/Homosexual Scale, (Kinsey et al. 2003), used as described previously (Berglund et al. 2006; Savic and Lindstrom 2008). The major demographic data are presented in Table 1.

All participants were Caucasian. All provided written informed consent and received reimbursement after participation. The study was approved by the Regional Ethical Review Board and the Radiation Safety Committee at the Karolinska Institute.

Finger Ratios

The 2D:4D ratio of both hands was measured using steel vernier calipers. An investigator who was blind to the diagnosis measured the fingers directly, on the ventral side of the hand, between the basal crease and the fingertip (Manning et al. 1998, 2004; Lutchmaya et al. 2004; Manno 2008; Giumas et al. 2009). For 15 subjects, the 2D:4D ratios were also measured by a second rater, and the inter-rater correlation was calculated with linear regression (Pearson’s coefficient, $P < 0.05$). Nonparametric statistics was employed for group comparisons because the Shapiro–Wilk test showed that the normality of data distribution could not be assumed among the XXY patients.

Venous Blood Samples

Venous blood samples were collected from the controls as well as from the patients, in the morning between 8 and 10 am. Plasma levels of testosterone (nmol/L), 17β-estradiol (pmol/L) (radioimmunoassay, Testosterone RIA DSL-4000, Diagnostik Systems Laboratory Inc.), and the sex hormone-binding globulin were analyzed in the Chemical Laboratory Diagnostics at the Karolinska University Hospital. The levels of bioavailable testosterone (nmol/L) were calculated using an equation developed by Sodergard et al. (1982). To avoid bias due to the menstrual cycle phase, women were measured during the mid-follicular phase, when levels of 17β-estradiol are relatively stable and low.

MRI

MRI Data Acquisition

All MRI data was acquired on a whole-body 1.5-Tesla MRI medical scanner (General Electric) equipped with an 8-channel coil. The MRI protocol included the following scans: 1) 3D-weighted $T_1$ spoiled grass (SPGR) images with 1 mm isotropic voxel size according to a previously described protocol (Giumas et al. 2010), and 2) 2D $T_2$-weighted fast spin echo images in the axial plane (effective echo time = 56 ms, repetition time = 2500 ms, field of view = 24 cm, 23 slices of 3 mm thickness). Only the 3D-weighted $T_1$ SPGR images were used for VBM.

MRI Data Pre-Processing

Voxel-based morphometry (Ashburner 2007) analysis was performed using the Gaser Toolbox (http://dbm.neuro.uni-jena.de/vbm/) with SPM5 (The Wellcome Department of Imaging Neuroscience, University College London; www.fil.ion.ucl.ac.uk/spm/) and Matlab 7.3 (Math Works). The VBM preprocessing included 5 steps: 1) visual inspection for scanner artifacts and gross anatomical abnormalities for each subject; 2) setting the image origin at the anterior commissure-posterior commissure line; 3) using the hidden Markov random field option in the segmentation of the VBM5 toolbox to minimize

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Female controls</th>
<th>Male controls</th>
<th>XXY Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N = 45$</td>
<td>$N = 41$</td>
<td>$N = 33$</td>
</tr>
<tr>
<td><strong>Age</strong> $^{a,b}$</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td></td>
<td>35.1 7.3</td>
<td>35.0 6.8</td>
<td>39.1 10.5</td>
</tr>
<tr>
<td><strong>Education</strong> $^{c,d}$</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td></td>
<td>15.9 2.2</td>
<td>16.2 2.4</td>
<td>13.2 2.7</td>
</tr>
<tr>
<td><strong>Right D2:D4</strong></td>
<td>Ratio</td>
<td>Ratio</td>
<td>Ratio</td>
</tr>
<tr>
<td></td>
<td>0.99 0.03</td>
<td>0.97 0.02</td>
<td>0.99 0.04</td>
</tr>
<tr>
<td><strong>Left D2:D4</strong></td>
<td>Ratio</td>
<td>Ratio</td>
<td>Ratio</td>
</tr>
<tr>
<td></td>
<td>0.99 0.03</td>
<td>0.97 0.03</td>
<td>0.99 0.04</td>
</tr>
<tr>
<td><strong>Oestradiol (plasma)</strong> $^{e}$</td>
<td>pmol/L</td>
<td>pmol/L</td>
<td>pmol/L</td>
</tr>
<tr>
<td></td>
<td>184.1 107.5</td>
<td>75.8 28.6</td>
<td>98.8 47.3</td>
</tr>
<tr>
<td><strong>Testosterone (bioactive)</strong> $^{f}$</td>
<td>nmol/L</td>
<td>nmol/L</td>
<td>nmol/L</td>
</tr>
<tr>
<td></td>
<td>0.46 0.28</td>
<td>6.10 1.47</td>
<td>11.16 6.48</td>
</tr>
<tr>
<td><strong>Total GM</strong> $^{g,h}$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
</tr>
<tr>
<td></td>
<td>679.1 62.3</td>
<td>768.5 68.9</td>
<td>724.6 65.6</td>
</tr>
<tr>
<td><strong>Total WM</strong> $^{i,j}$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
</tr>
<tr>
<td></td>
<td>440.4 44.2</td>
<td>517.6 51.2</td>
<td>473.6 44.1</td>
</tr>
<tr>
<td><strong>Total CSF</strong> $^{i,j}$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
</tr>
<tr>
<td></td>
<td>312.9 97.6</td>
<td>376.7 67.7</td>
<td>372.9 76.2</td>
</tr>
<tr>
<td><strong>Tissue volume</strong> $^{l,m}$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
</tr>
<tr>
<td></td>
<td>1119.5 98.9</td>
<td>1286.0 111.2</td>
<td>1183.8 112.6</td>
</tr>
<tr>
<td><strong>Total intracranial volume</strong> $^{l,m}$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
<td>cm$^3$</td>
</tr>
<tr>
<td></td>
<td>1432.3 136.9</td>
<td>1662.7 135.3</td>
<td>1556.8 139.1</td>
</tr>
</tbody>
</table>

Note: F values from group comparisons (one-way ANOVA). Possible differences in digit ratios were calculated with Kruskal–Wallis test.

$^{a}$ Difference between XXY and XX, $P < 0.05$.

$^{b}$ Difference between XXY and XX, $P < 0.01$.

$^{c}$ Difference between XX and XX, $P < 0.001$.

$^{d}$ Difference between XX and XX, $P < 0.001$.

$^{e}$ Difference between XX and XX, $P < 0.001$.

$^{f}$ Difference between XX and XX, $P < 0.001$.

$^{g}$ Difference between XX and XX, $P < 0.001$.

$^{h}$ Difference between XX and XX, $P < 0.001$.

$^{i}$ Difference between XX and XX, $P < 0.001$.

$^{j}$ Difference between XX and XX, $P < 0.001$.

$^{k}$ Difference between XX and XX, $P < 0.001$.

$^{l}$ Difference between XX and XX, $P < 0.001$.
the noise level of the segmentation; and 4) using the Diffeo-
morphic Anatomical Registration Through Lie Algebra toolbox
(DARTEL, Wellcome Department of Imaging Neuroscience,
University College London; http://www.fil.ion.ucl.ac.uk/spm)
for a high-dimensional normalization protocol. John Ashbur-
ner’s chapter in its standard version was followed, including
the Montreal Neurological Institute (MNI) space transformation
(Ashburner 2007). 5) To restore the original volume infor-
mation within each voxel, voxel values in the segmented
images were modulated (multiplied) by the Jacobian determi-
nants derived from the spatial normalization step. The analyses
of modulated data allowed direct comparisons of regional
differences in the amount of each tissue type. After pre-
processing, the segmented images were checked for homogen-
ecity across samples, smoothed with an 8-mm full-width at half-
maximum Gaussian filter, modulated, and normalized, before
being used for the statistical analyses.

Statistical Analyses of Group Differences
Overall Tissue Volumes and Hormone Levels
First, hormone levels and total volumes of GM, WM, and cere-
brosinal fluid (CSF) were tested for normal distribution.
Group differences in TV (total volume of GM + WM), total in-
tracranial volume (TIV, calculated as GM + WM + CSF), the
relative volume of GM (total GM volume/TIV), WM (total WM
volume/TIV), and CSF (total CSF volume/TIV) were calculated
using separate one-way analysis of covariances (ANOVAs)
with group as the between factor, followed by Tukey’s post
hoc tests ($P < 0.05$). The same approach was applied to test
the possible group differences in plasma testosterone and es-
tradiol, age, and education.

VBM Data
The VBM data were analyzed using the VBM toolbox of SPM
5 (SPM 5, Wellcome Foundation).

Group Comparisons. First, group comparisons of regional
GM and WM were carried out to identify the possible sex
differences among the controls, and to investigate how XXY
males differed from each control group. This was done using
a full factorial design, with age and total tissue volume (TV)
as the nuisance variables to adjust for individual differences
in these factors. Significant clusters for this first-level analysis
were assessed at $P = 0.001$ for the peak voxel value, taking
into consideration false discovery rate (FDR) correction for
multiple comparisons at $P < 0.05$ from the SPM output tables
(Genovese et al. 2002), and employing cluster extent correction
at $P < 0.05$. In addition, a correction for nonstationary
smoothness (Hayasaka et al. 2004) was applied using VBM5
toolbox.

Next, we conducted conjunctional analyses to evaluate whether, and in which regions, XXY males exhibited “female,” or “male,” features. This analysis (Friston et al. 1999) assumed that both XXY males and XX females differed in certain aspects from XY males and that these differences could be overlapping, thus showing common clusters for XX–
XY and XXY–XY contrasts, and vice versa. “Female” features in
XXY males were expected primarily because of their supernu-
merary X-chromosome and, to some extent, their hypogo-
nadism. It was further assumed that XXY males, because they
have 3 rather than 2 sex chromosomes, would differ from
both control groups at least in some regions. Possible con-
junctional clusters were calculated for (A) XX–XY and XXY–
XY, (B) XY–XX and XY–XXY, (C) XX–XXY and XY–XY, and
(D) XXY–XX and XXY–XY. The conjunctional analyses were
conducted separately for GM and WM with age and TV as the
nuisance variables, using peak voxel threshold at $P = 0.001$. A
correction for nonstationary smoothness was employed. Clus-
ters in A and B were expected primarily in regard to sexually
dimorphic areas, and the cluster level significance was, there-
fore, $P < 0.05$ uncorrected. Here we also employed small
volume correction (10-mm sphere) and FDR correction at $P <
0.05$. For C and D, $P < 0.05$ corrected was used, as there was
no a priori hypothesis about cluster location.

The peak voxel threshold was at $P = 0.001$ for all the compari-
sons.

In the following analyses, we specifically investigated possible
effects of sex hormones and sex chromosome genes, and
focused on regions, which showed group differences in the
first-level analysis.

Analyses of the Impact of Sex Hormones on Regional GM and
WM Volumes. The next set of analyses was used to evaluate
the impact of sex hormones on the observed sex differences
among the controls. Whether, and in which regions, the GM
and WM volumes in controls correlated with circulating
estrogen and testosterone levels was investigated. Only the
data from controls were included to avoid possible bias due to
the fact that the plasma levels of these hormones in XXY
males was likely to be affected by testosterone supplementation
and were therefore a less reliable indicator of any potential
long-term activational effects of sex hormones on the brain.

The possible relationship between circulating sex hor-
mones and the GM and WM volumes was tested using multi-
variate regression analyses in which each pixel value of GM
and WM volume was regressed against 17$\beta$-estradiol and tes-

tosterone, using age and TV as the nuisance variables, and the
entire brain as the search space (multiple regression analysis,
SPM5). Based on a number of previous reports (Peper et al.
2010; Mueller et al. 2011), the assumption was made that cir-
culating sex hormone levels would be significantly correlated
with the GM and WM volumes in regions that corresponded to
the sex dimorphic clusters detected in XX–XY and XY–XX
contrasts. To avoid bias due to the fact that levels of circulat-
ing estradiol and testosterone are inherently different in men
and women, and would cluster around 2 ends of the
regression line, the respective values were z-normalized for
each group. The significance level for clusters in regions
showing sex differences in the first-level analysis (the simple
XX vs. XY group comparison, Table 2) was set at $P < 0.05$ un-
corrected (peak threshold at $P = 0.001$). For all other regions,
the cluster level was $P < 0.05$ corrected.

In addition, the possible impact of fetal testosterone on regio-
nal GM and WM volumes was tested. This was done in a
separate analysis, employing right-hand 2D:4D ratios as the
linear regressor of interest, and using age and TV as the nui-
sance variables. The significance levels were the same as for
the calculations of possible effects of circulating sex hor-
mones. When significant clusters were detected, it was then
tested whether these clusters were still found when the circu-
lating hormone levels were added to the same multiple
regression analyses, in order to determine if an independent factor had been observed or not.

Analysis of the Dosage Effects of Sex Chromosomes on the Regional GM and WM Volumes. In order to test for any possible major effects of sex chromosomes on regional GM and WM volumes, all 3 groups were included. Two further explorative multiple regression analyses were used to test the possible effects of both the number of X- and Y-chromosomes, which were used as the covariates of interest (2 separate analyses, voxel threshold at $P = 0.001$, cluster extent at $P < 0.05$ corrected). First, only digit ratios, TV, and age were included as the nuisance variable. When the sex factor had been observed or not.

Finally, whether trisomy (the fact that patients had 3 sex-chromosomes) explained some of the observed differences between the XXY patients and both control groups was tested. Potentially, this analysis could reveal dosage effects of the X-escapee genes that have homologs on the Y-chromosome. Individual, preprocessed WM and GM images were, therefore, regressed in a separate analysis against the number of sex chromosomes (2 for XX females and XY males and 3 for XXY males), using right-hand 2D:4D ratios, TV, and the multiple regression analysis was re-run to control for the possible effects of sex hormones on the results. The reason for including sex hormone levels only in the adjunct analysis was that testosterone and also estrogen levels in XXY males was likely to be affected by testosterone supplementation therapy. Adult sex hormone levels could, however, theoretically, have modulating effects on regional TVs, which could be identified by adding this factor into the model.

Table 2

<table>
<thead>
<tr>
<th>Region</th>
<th>Gray matter volumes</th>
<th>White matter volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z level</td>
<td>Size, cm³</td>
</tr>
<tr>
<td>Male–female controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L uncus and amygdala</td>
<td>4.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Cerebellum (the semilunar lobe and vermis)</td>
<td>6.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Lingual gyrus</td>
<td>3.8</td>
<td>17</td>
</tr>
<tr>
<td>Superior temporal gyrus, WM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occipital WM</td>
<td>4.5</td>
<td>2</td>
</tr>
<tr>
<td>Female–male controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior cingulate cortex, subcallosum</td>
<td>4.8</td>
<td>1.6</td>
</tr>
<tr>
<td>R inferior frontal gyrus (+ portion of the orbitofrontal cortex)</td>
<td>4.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Pecuneus, (+ precentral gyrus)</td>
<td>4.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Male controls–XXY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L insular cortex and L inferior temporal gyrus</td>
<td>5</td>
<td>8.0</td>
</tr>
<tr>
<td>L hippocampus, L amygdala</td>
<td>4.2</td>
<td>6</td>
</tr>
<tr>
<td>R hippocampus, R amygdala</td>
<td>6.9</td>
<td>16.0</td>
</tr>
<tr>
<td>R caudate, R putamen</td>
<td>4.2</td>
<td>54</td>
</tr>
<tr>
<td>R insular cortex, R uncus</td>
<td>5.8</td>
<td>0.4</td>
</tr>
<tr>
<td>R superior temporal gyrus</td>
<td>4.8</td>
<td>6.4</td>
</tr>
<tr>
<td>Cerebellum (semilunar lobe)</td>
<td>5.8</td>
<td>20.0</td>
</tr>
<tr>
<td>Male–female controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L postcentral gyrus</td>
<td>4.4</td>
<td>5.4</td>
</tr>
<tr>
<td>R postcentral gyrus</td>
<td>4.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Cuneus and the lingual gyrus</td>
<td>4.3</td>
<td>5</td>
</tr>
<tr>
<td>Lingual gyrus</td>
<td>4.2</td>
<td>54</td>
</tr>
<tr>
<td>Pecuneus, and WM</td>
<td>5.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Female controls–XXY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L caudate</td>
<td>5</td>
<td>16.0</td>
</tr>
<tr>
<td>L insular cortex and superior and inferior temporal gyrus</td>
<td>4.2</td>
<td>5.4</td>
</tr>
<tr>
<td>L hippocampus, L amygdala</td>
<td>4.7</td>
<td>6.5</td>
</tr>
<tr>
<td>R caudate</td>
<td>4.7</td>
<td>6.4</td>
</tr>
<tr>
<td>R insular cortex and superior temporal gyrus</td>
<td>4.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Thalamus</td>
<td>3.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Cerebellum (semilunar lobe)</td>
<td>3.7</td>
<td>2.6</td>
</tr>
<tr>
<td>L insular WM</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>R temporal lobe WM</td>
<td>4.4</td>
<td>21.0</td>
</tr>
<tr>
<td>L temporal lobe WM</td>
<td>4.9</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Note: Clusters denoting GM and WM volumes calculated with the SPM5 VBM toolbox, using TV and age as covariates of no interest. Significant clusters calculated using voxel threshold at $P = 0.001$, FDR corrected at $P < 0.05$, with cluster significance level at $P < 0.05$ corrected, minimum cluster size 0.2 cm³. MNI coordinates indicate the peak values and the indicated regions coverage of the respective cluster. R = right, L = left.

*aConfluent cluster.
age as the nuisance variables. Again, the analysis was repeated with the z-normalized sex hormone levels as an additional nuisance variable to evaluate if this maneuver modified the results.

Results

Background Data

The demographical data are presented in Table 1. Out of the initially recruited 124 subjects 5 were excluded due to psychiatric symptoms (see procedures section). No age difference was found between the male and female controls. The patient group was significantly older than both control groups, and there was no age difference between the 2 control groups ($P = 0.019, F = 4.08, df = 2$; $P = 0.043$ for XY vs. XXY, $P = 0.035$ for the XX vs. XXY, and $P = 0.81$ for XX vs. XY). Both male and female controls had significantly higher education than the patients ($P < 0.0001, F = 14.1, df = 2$); there was no such difference between the 2 control groups (XX vs. XY $P = 0.91$; XX vs. XXY and XY vs. XXY, $P < 0.0001$). The 3 study groups did not differ in regard to handedness or sexual orientation, and no gross anatomical abnormalities were found, according to an experienced neuroradiologist.

The TIV and the total TV were normally distributed, as were the total volumes of GM, WM, and CSF. Group differences were detected in TIV ($P < 0.001, F = 30.7, df = 2$) as well as in TV ($P < 0.001, F = 27.8, df = 2$). They consisted of significantly smaller volumes in females in relation to both XY and XXY males ($P < 0.0001$ for TIV for both comparisons and $P < 0.001$ and $P < 0.008$, respectively, for the TV comparison) (Table 1), and significantly smaller TIV and TV in XXY males compared with XY males ($P = 0.017$ and $P = 0.002$, respectively). In addition, there was a significant group difference in the relative GM volume (GM/TIV) ($P = 0.005, F = 5.6, df = 2$), with a greater volume in XX females than in XXY males ($P = 0.003$), but not XY males ($P = 0.15$). No difference was detected between XXY and XY males ($P = 0.28$). The groups differed also in the relative WM volume (WM/TIV) ($P = 0.034, F = 3.47, df = 2$), with lower values among XXY males compared with XY males ($P = 0.03$), but not XX females ($P = 0.11$). No statistically significant difference was found between XX females and XY males ($P = 0.82$). Finally, there were group differences in CSF/TIV ($P = 0.0017, F = 6.71, df = 2$), with larger values among XXY males compared with both XX females ($P = 0.002$) and XY males ($P = 0.02$). No significant group difference was detected in regard to the total GM/WM ratio ($P = 0.06, F = 2.87, df = 2$).

Measures of digit ratios, carried out by 2 raters, were highly correlated ($r = 0.92; P < 0.001$). The results presented here were based on measurements from rater 1, as rater 2 performed ratings for only 15 subjects. Even though the mean values of 2D:4D ratios were higher among XX females and XXY males compared with XY males (Table 1), a Kruskal–Wallis test, with the subject group as the independent factor, showed that there was no significant group difference ($P = 0.29$). When extending the analysis to all the subjects that were initially included, a significant group difference was found for the 2D:4D ratio of the right hand ($P = 0.033, \chi^2 = 6.8, df = 2$) but not the left hand ($P = 0.070, \chi^2 = 5.2, df = 2$). A post hoc test (Wilcoxon W) showed right-hand digit ratios to be lower among XY males when compared with XX females and XXY males ($P = 0.019$, and $P = 0.032$, for the respective comparison), and no difference between XX females and XXY males ($P = 0.680$).

VBM Data

Regional Group Differences in GM and WM Volumes

Possible differences between the male and female controls were first examined to define the sex dimorphic GM and WM clusters. As previously reported (Savic and Arvis 2011), the female controls had larger GM volumes than the male controls bilaterally in the precentral gyrus, the subcallosum, and the right inferior frontal gyrus (including a portion of the orbitofrontal cortex). Male controls, on the other hand, showed a larger GM volume in the cerebellum and the left uncus and a portion of amygdala, and larger occipital GM and WM volumes (Table 2, Fig. 1). Except for the inferior frontal/orbitofrontal cluster, these clusters also appeared when conjunctural analyses were conducted to detect regional differences, which 2 groups shared in relation to the third (Table 3). Conjunctural analyses showed that both XX and XXY subjects had a greater GM volume in the precentral gyrus than XY
Table 3
Conjunctional clusters

<table>
<thead>
<tr>
<th>Region</th>
<th>Gray matter volumes</th>
<th>White matter volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z level</td>
<td>Size, cm³</td>
</tr>
<tr>
<td>XX-XX and XXY-XY</td>
<td>Precentral gyrus (bilateral)</td>
<td>3.1</td>
</tr>
<tr>
<td>XXY-XX and XY-XXY</td>
<td>Cerebellum (vermis)</td>
<td>5.4</td>
</tr>
<tr>
<td>XXY-XX and XY-XX</td>
<td>Amygdala</td>
<td>3.0</td>
</tr>
<tr>
<td>XXY-XX and XY-XX</td>
<td>Occipital cortex (covering the lingual gyrus)</td>
<td>5.0</td>
</tr>
<tr>
<td>XXY-XX and XX-XY</td>
<td>Occipital cortex (covering the lingual gyrus)</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Note: All clusters are calculated using age and TV as nuisance variables. Peak threshold at P = 0.001, cluster level at P < 0.05 corrected for XX-XXY, XY-XX, and XXY-XXY, and P < 0.05 corrected for the remaining contrasts (see Methods for clarification); italics indicate clusters at P < 0.1 corrected with (peak threshold P = 0.001). MNI coordinates. R = right; L = left.

males, whereas their GM volume in the cerebellum was smaller. The only difference from XX females that was common among both male groups was the larger occipital GM volume (primarily covering the lingual gyrus), (Tables 2 and 3).

A further finding was that XXY males differed significantly from both control groups by having a smaller GM volume bilaterally in the insular cortex, in the superior temporal gyrus, the caudate and thalamus, in the left hippocampus the amygdala, and in the cerebellum (the semilunar lobes) in comparison to XY males as well as XX females. Furthermore, their GM volume was larger in the inferior parietal lobe, whereas the WM volumes in the temporal lobe and insula were significantly smaller in comparison to both the male and female controls (Table 2). Because XXY males had significantly lower education than controls, the comparisons with controls were re-run adding education as the nuisance variable. The results did not change.

**Sex Hormone Levels, 2D:4D Ratios, and Regional GM and WM Volumes**

A positive correlation was detected between bioactive plasma testosterone and the GM volume in the parahippocampus (a cluster also covering the amygdala), the occipital (lingual gyrus and cuneus), and the insular cortex on both sides (clusters covering parts of putamen). There was also a tendency for inverse correlation with testosterone in the parietal GM (Table 4, Fig. 1). No significant correlation with estradiol was detected. Neither did we detect any significant correlation with the right-hand 2D:4D ratio among controls (a cluster showing a positive correlation with the GM volume in the precentral gyrus appeared when also including the XXY patients).

**Sex Chromosomes and the Regional GM and WM Volumes Association with the Number of X- and Y-Chromosomes**

X-chromosome dosage was associated with a greater GM volume bilaterally in the precentral gyrus (Table 5), and a smaller GM volume in the cerebellar vermis, the caudate, and the left inferior temporal gyrus. These associations remained after adding testosterone as a nuisance variable. The number of X-chromosomes was also associated with a smaller GM volume in the hypothalamus, a smaller frontal-temporal WM volume, and a greater parietal GM volume. These latter clusters, however, disappeared after adding
z-testosterone as the nuisance variable, suggesting an interaction with testosterone (Table 5, Fig. 1), which is congruent with the previously reported (Neufang et al. 2009) and presently detected correlations between testosterone and GM in these regions (Table 4). Including z-estradiol did not further change the results.

With respect to the Y-chromosome, a positive association was detected with the GM volume in the occipital lobe, and a negative association with the GM volume in the right insular cortex, and the subcallosum. These clusters did not appear when adding testosterone as a nuisance variable, suggesting that at least some of the Y-chromosome associations were due to testosterone (Table 5). Again, including z-estradiol did not further change the results.

**Association with the Total Number of Sex Chromosomes.** Broadly in accordance with conjunctual analysis, which revealed several singular features in XXY men, it was found that a higher number of sex chromosomes was associated with greater GM volume in large portions of the parietal lobe, and with smaller GM volume bilaterally in the caudate, amygdala, and the insular cortex, in the right superior temporal gyrus, and the cerebellar hemispheres (the cerebellar cluster disappeared when subsequently adding z-testosterone as nuisance covariate in the regression analysis). The number of sex chromosomes was also associated with a smaller frontotemporal WM volume in both hemispheres (a cluster covering the uncinate fascicle, external capsule, and the right internal capsule), (Table 5, Fig. 2).

**Discussion**

The present comparative study between XXY males and XY and XX controls explores the mechanisms underlying sexual dimorphism in regional volumes of cerebral GM and WM. In contrast to previous studies, which were either purely descriptive or limited to investigations of sex hormone effects, the present study included investigations of multiple etiological factors. We hypothesized that cerebral differences between XXY and XY males would mainly be a downstream effect of the fact that XXY males have 2 instead of 1 X-chromosome, and 3 instead of 2 sex chromosomes overall. Cerebral dimorphism in regional volumes of cerebral GM and WM. In contrast to previous studies, which were either purely descriptive or limited to investigations of sex hormone effects, the present study included investigations of multiple etiological factors. We hypothesized that cerebral differences between XXY and XY males would mainly be a downstream effect of the fact that XXY males have 2 instead of 1 X-chromosome, and 3 instead of 2 sex chromosomes overall. Cerebral differences between XXY males and XX females, on the other hand, could be attributed to the males’ Y-chromosome, their trisomy, and their at least slightly higher testosterone levels, as well as to the higher estrogen levels in XX females either separately or in combination. In addition, the differences between XX females and XY males could be ascribed to the presence or absence of the Y-chromosome, the difference in the number of X-chromosomes, as well as to the difference in testosterone and/or estrogen levels (Supplementary Table). This provided a multifactorial dataset for exploring several

### Table 5

Association with sex chromosomes

<table>
<thead>
<tr>
<th>Region</th>
<th>Gray matter volumes</th>
<th>White matter correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z level</td>
<td>Size, cm³</td>
</tr>
<tr>
<td>X-chromosome, positive association</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precentral gyrus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parietal cortex (superior parietal lobule, precuneus)*</td>
<td>3.9</td>
<td>2.6</td>
</tr>
<tr>
<td>X-chromosome, negative association</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothalamus*</td>
<td>4</td>
<td>2.4</td>
</tr>
<tr>
<td>Cerebellum (vermis)</td>
<td>5.6</td>
<td>6.5</td>
</tr>
<tr>
<td>R caudate</td>
<td>4.5</td>
<td>1.7</td>
</tr>
<tr>
<td>L caudate</td>
<td>4.6</td>
<td>1.7</td>
</tr>
<tr>
<td>L inferior temporal gyrus</td>
<td>4.6</td>
<td>1.8</td>
</tr>
<tr>
<td>R fronto-temporal WM*</td>
<td>4.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Y chromosome, positive association</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occipital cortex (occipital gyr, cuneus, lingual gyrus)*</td>
<td>3.8</td>
<td>6.2</td>
</tr>
<tr>
<td>Y chromosome, negative association</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R insular cortex and angular gyrus*</td>
<td>4.5</td>
<td>4.1</td>
</tr>
<tr>
<td>Subcallosum*</td>
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<td>1.6</td>
</tr>
<tr>
<td>Sex chromosome number, positive association</td>
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<td></td>
</tr>
<tr>
<td>Parietal cortex (including precuneus)</td>
<td>4.6</td>
<td>6.5</td>
</tr>
<tr>
<td>L caudate + amygdala + insular cortex</td>
<td>4.4</td>
<td>1.7</td>
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<tr>
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<tr>
<td>R caudate + amygdala + insular cortex</td>
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<td>6.8</td>
</tr>
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<td>Hypothalamus*</td>
<td>4.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Cerebellar hemispheres*</td>
<td>4.9</td>
<td>12.0</td>
</tr>
<tr>
<td>R amygdala</td>
<td>4.4</td>
<td>4.1</td>
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</tr>
<tr>
<td>L temporal WM</td>
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</tr>
<tr>
<td>R temporal WM (includes uncinate, external capsule)</td>
<td>4.5</td>
<td>7</td>
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</tbody>
</table>

Note: Calculations showing association with X-chromosome, and with the total number of sex chromosomes, taking all 3 groups in the analysis and using right hands D2:D4 ratio, age and TV as nuisance variable. Clusters calculated at peak threshold P = 0.001, cluster level P < 0.05 corrected (italics denote P < 0.1 corrected). MNI coordinates indicate the peak level, and the regions describe the coverage of the respective cluster. R = right; L = left.

*Cluster that disappears when adding testosterone as nuisance variable.
possible mechanisms that might be underlying the differences in regional GM and WM volumes among the 3 groups investigated and, ultimately, the sexual dimorphism in the measured parameters.

Broadly in line with previous studies (Filipek et al. 1994; Murphy et al. 1996; Paus et al. 1996; Giedd et al. 1997; Goldstein et al. 2001; Good et al. 2001; Luders et al. 2005; Raz et al. 2005; Carne et al. 2006; Chen et al. 2007; Hsu et al. 2008), relatively larger GM volumes were detected in XX controls bilaterally in the precentral gyrus, the subcallosum, and the right inferior frontal gyrus, whereas XY controls had larger GM volumes in the cerebellum, the left uncus and amygdala, and larger GM and WM volumes in the occipital lobe (Table 2). XXY males shared some features with XY males, some with XX females, and also displayed singular features (Tables 2 and 3).

Overall, the group differences found in regard to WM were paralleled by differences in GM volumes (Table 2). This is not surprising, considering that GM is composed predominantly of neurons, while WM is composed mainly of axons connecting these neuronal bodies.

Multiple regression analyses showed that sex differences in the precentral gyrus (overlapping with the motor cortex) and cerebellum (mainly overlapping with the vermis) were primarily linked to the number of X-chromosomes (Table 5), whereas the larger occipital GM volume in men was linked to testosterone levels (Tables 3 and 4). No specific Y-chromosome effects other than those related to testosterone were detected. Sex chromosome trisomy was found to interact with several limbic structures.

In the ensuing discussion, we will address several methodological issues, then discuss the present finding in relation to relevant literature, and finally speculate about the possible implications vis-à-vis mechanisms behind sex differences in cerebral anatomy and the skewed sex distribution in psychopathology.

**Methodological Consideration**

One may argue that our midsize sample could hamper detection of some group differences. Although this is theoretically possible, it should be stressed that our findings have a precedence in that differences between XY males and XX females were detected in regions previously reported to be sexually dimorphic, and also that the observed changes in XXY patients were consistent with previous reports (Raz et al. 1995; Murphy et al. 1996; Paus et al. 1996; Giedd et al. 1997, 2006, 2007; Nopoulos et al. 2000; Goldstein et al. 2001; Luders et al. 2005). This reproducibility of data provides reliability to the present multigroup comparisons.

The use of the 2D:4D ratio deserves comment. Since the influential article of Manning et al. (1998) on this antrophomorphic measure of fetal testosterone exposure, over 300 related works have appeared in the literature (Voracek and Lobl 2009). The exact mechanisms by which 2D:4D and prenatal androgen exposure are related are, however, not entirely clear. Knickmeyer et al. recently reported in a longitudinal study of neonates that the interaction of salivary testosterone and CAG repeats predicted right-hand digit ratio at 12 months and left hand digit ratio at 12 months and 24 months but not at 2 weeks of age. Given these observations the authors suggested that it may be more appropriate to interpret the 2D:4D ratio in adulthood as an index of early testosterone exposure rather than prenatal exposure per se (Knickmeyer et al. 2011). While these data need to be replicated, they motivate a further evaluation of how exactly this antrophomorphic measure reflects testosterone exposure. Another potential methodological concern regards differences in brain size. Because of their larger brains, the outer brain limits in men are farther away from the center of the coil in the MR scanner, and, therefore, could be located in less homogenous parts of the field. This, in turn, could result in lower signal intensity in these locations, leading to regionally decreased GM in male subjects. As a consequence, gender differences in GM volumes in the most extreme parts of the brains (frontal pole, pre- and postcentral gyrus, and occipital pole) could potentially be due to this confound. While our findings of lower GM volumes in the precentral gyrus in XY males could be due to this, such a confound is incompatible with the enlarged GM volume in the occipital GM, and the fact that other structures located far from the center of the coil, such as the frontal pole, did not show lower GM volumes in XY men. Furthermore, the XXY males had larger whole brain volumes than the XX women and yet several “female”
features were found. Also of note is that larger regional GM volumes have been found in females even when comparing their GM with that of males of matched brain size (Luders et al. 2009).

The groups differed with respect to age, TIV, TV, and their relative GM and WM volumes, but not in their GM/WM ratios. Age and TV were, therefore, used as covariates in all the calculations. As pointed out by Leonard et al. (2008), frontal lobe WM increases with the overall brain size more steeply than GM. The higher precentral GM volumes in the XX and XY groups could, therefore, be an effect of the smaller brain size in these subjects (relatively less frontal lobe WM). To test this, the group comparisons of regional TVs were re-run using total GM volume as the covariate. The differences presented in Table 2 remained, suggesting that they were independent of the type of correction variable used (TV or total GM volumes).

The GM volume cluster in the occipital lobe and cerebellum in the XY–XX contrast (Table 1) could, despite the careful normalization procedure with the DARTEL algorithm, potentially be confounded by the edge effects due to difference in head size. To test this, in a post hoc analysis, GM and WM templates were constructed separately for the male and female controls and superimposed on each other for a visual comparison. No difference was detected in size or shape between the templates, confirming that the occipital-cerebellar GM cluster was not an effect of a bias in cranial normalization.

A further issue requiring discussion is the use of sex hormone levels measured at one time point as a regressor to reveal potential sex hormone effects on cerebral dimorphism. As in previous studies, correlation analyses of GM and WM volumes were based on blood samples collected on one occasion (Peper et al. 2008; Neufang et al. 2009; Witte et al. 2010). Hormone levels vary, however, with activity, stress, and sleeping patterns. Although we tried to standardize these factors, and can claim that the measures of hormone levels and cerebral GM and WM volumes were temporally related (blood samples in the majority of subjects were taken on the same day as the MRI scans), it should be acknowledged that an approach utilizing multiple measurements of serum hormone levels over time might have been more precise for trying to link circulating hormones to brain morphology. Such a bias should, however, cause a type II rather than type I error, meaning that the detected correlations should be reliable.

The covariation analyses with z-score values of sex hormones, employed in the present study, provide inferences for how differences in testosterone (or estrogen) affect brain morphology “within-gender.” Although post hoc analysis using absolute hormone yielded similar clusters, the inherent collinearity with gender is a clear limitation when trying to test whether testosterone or estrogen may have important effects on brain regions “across genders.”

It should be mentioned that use of cluster significance in regard to VBM, employed in the present study, has been discussed by some researchers, mainly due to the problem of nonuniformity (Ashburner and Friston 2000). Nonuniformity correction was, therefore applied in all the analyses. We also carried out post hoc analysis with the alternative approach using a voxel height threshold at $P = 0.001$ with FDR correction at $P < 0.05$, and small volume correction with 10-mm sphere or $5 \times 10 \times 5$-mm box) when a specific hypothesis was tested. Interestingly, with the exception of cluster sizes, and the subsignificant clusters in Table 3 that were not detected in the post hoc analysis, the results did not differ for any of the calculations, suggesting that the reported findings are robust.

Finally, it should also be noted that since regional GM and WM volumes are composite measures of different micro units, such as neuronal bodies, dendrites, synapses, axons, myelin, glial cells, the present findings of enlargements or reductions of GM and WM volumes cannot be directly translated into function.

Possible Effects of Sex Hormones on Tissue Volumes and Sex Dimorphism

Positive correlations were found between bioactive testosterone and the GM volume in the parahippocampus (including a portion of amygdala), the putamen, and in the insular and the occipital cortices, as well as a tendency for negative correlation with the GM volume in the parietal lobe. These data accord with the notion that the density of steroid receptors is high in these regions (Simerly et al. 1990; Goldstein et al. 2001; Lepore et al. 2008), and with the observation that boys with familial male precocious puberty and an early excess of androgen secretion have higher GM volumes in the putamen and the parahippocampal gyrus (Mueller et al. 2011). They are also in accordance with Neufang et al.’s (2009) findings of testosterone having an inverse correlation with parietal lobe GM volume and a positive correlation with GM volume in the amygdala. The present study, however, was not able to reproduce findings from investigations of pubertal cohorts which showed that increasing levels of testosterone in boys contributed to the emerging sex differences in the hippocampus during adolescence (Neufang et al. 2009), and that increasing levels of 17β-estradiol in pubertal girls correlated with GM increases in the middle frontal-, inferior temporal-, and middle occipital gyri (Peper et al. 2009). We also did not find positive estrogen and negative testosterone correlations in the inferior frontal gyrus, as previously reported in adult persons (Witte et al. 2010). This inconsistency might be explained by the age of the participants investigated. Although there are some reports that activation effects of sex hormones on brain tissue may continue into adulthood (Pol et al. 2006), their major impact is expected earlier. Longitudinal MRI studies show that hormonal changes during puberty are associated with dynamic patterns of sex dimorphism in the brain, with enhancement of this dimorphism in some regions and deflation and even reversal in others (Giedd et al. 1997). Thus, investigations focused on the developmental trajectory may differ from those carried out in adults in terms of the results they reach on the hormonal relationships. Another potential explanation relates to the use of correlation analysis. Linear correlation in selected cerebral regions of interest (Witte et al. 2010) may differ from explorative analyses used in the present study. Witte et al.’s study also showed a slightly different pattern regarding sex differences than some of the other studies (for example, greater GM volume in men in the cingulate cortex, the precentral gyrus, and the right precuneus) (Good et al. 2001; Chen et al. 2007; Paus et al. 2010). The presently observed sex differences broadly agree with findings in the current literature, and the GM volume correlations with testosterone were mainly detected in the regions showing sex differences (Tables 2 and 5). Thus, assuming that the
individual trajectory of sex hormone levels over a lifespan is stable (the relation of one individual’s hormone level in relation to another’s is relatively unchanged), as suggested by Forest et al. (1976), the presently found testosterone correlations should be relevant to sex differences.

When it comes to the 2D:4D ratio, our findings showed that the right-hand ratios of the XXY males tended to be higher compared with XY males (although not statistically significant), but were similar to those of the XX females (Table 1). The right-hand 2D:4D ratio also correlated with the precentral GM volume (when including XXY males in the analysis), which could suggest that low action of fetal testosterone is linked to a greater precentral GM volume. The correlation with the precentral GM was, however, detected also when employing the X-chromosome as the covariate of interest, and persisted when adding the 2D:4D ratio as the nuisance variable. This implies that the effect of the X-chromosome dominated and that the GM correlation with the 2D:4D ratio could be a downstream effect of the 2 X-chromosomes in XXY males. Such a scenario is congruent with the fact that the androgen receptor, which mediates the effects of fetal testosterone, is coded by X-chromosome genes, and that the receptor-subtype with low affinity to androgens is more common among XXY men (Wikstrom et al. 2006; Knickmeyer et al. 2011). It also fits with the fact that degeneration of the testosterone producing seminiferous tubules in XXY males begins at the fetal stage (Aksglaede et al. 2006), although their fetal testosterone is reported to be within the lower normal range.

Possible Effects of Sex Chromosomes on Tissue Volumes and Sex Differences

The results of the regression analyses involving sex chromosomes will be discussed in terms of X-chromosome and sex chromosome dosage, not notwithstanding that this is a simplification. We prefer, however, to use the term chromosome dosage rather than gene dosage because this is what has been investigated, and to avoid speculations about gene expression, gene compensation, and gene transcription. Although the long chain of downstream mediators between the number of X and sex chromosomes and brain TVs evidently precludes any detailed elaboration of the mechanisms involved, the present observations nonetheless show a regional genetic influence on some sex differences in the human brain.

X-Chromosome Dosage

As in the majority of previous studies, XXY patients showed GM and WM reductions in a number of cortical and subcortical regions (Patwardhan et al. 2000; Rose et al. 2004; DeLisi et al. 2005; Giedd et al. 2007; van Rijn et al. 2008; Lenroot et al. 2009; Rezaie et al. 2009; Steinman et al. 2009), as well as in the TIV (Tables 1 and 2). They also exhibited an increase in parietal GM volume, as has also been found in prepubertal Klincobler boys by Bryant et al. (2011), whereas Giedd reported increased parietal WM volume, instead (Giedd et al. 2007). This discrepancy may be related to the type of normalization (as in the present study, Bryant et al. used the total TV as the covariate).

The present study is, to the best of our knowledge, the first VBM study of XXY males to include female controls as well as measurements of sex hormones and digit ratios. The observed similarities between XXY males and XX females, and the differences that these groups have in common vis-à-vis XY males, which persisted after regressing out the hormonal interaction, suggest that the X-chromosome dosage has an influence on the GM volumes in the precentral gyrus and the cerebellum (primarily the vermis). This is of interest because of the previously reported sex differences in these regions (Rhyu et al. 1999; Raz et al. 2001; Luders et al. 2005, 2009; Savic and Arvis 2011), and the reports that women with X0 monosomy, have smaller precentral GM and larger cerebellar GM volume than XX women (Brown et al. 2002; Molko et al. 2003; Cutter et al. 2006; Marzelli et al. 2011). This similarity with XY men and reciprocity to our findings in XXX men argues for processes related to the dosage of X-chromosome genes, which do not have Y-chromosome homologs. The possible effects of X-chromosome dosage on cerebral tissue may be both direct and indirect, but this does not disqualify the argument, as such effects, nevertheless, are mediated by X-chromosome genes. As to the cerebellum, it is also worth mentioning that regional cerebellar volumes begin to show sex differences early in childhood, although the developmental trajectories differ between boys and girls (Tiemeier et al. 2010), and that gene expression in the cerebellum has been reported to be sex differentiated (Vawter et al. 2004). The cerebellum, is however, a large structure and further studies are needed to understand the programming of its specific subregions.

Sex Chromosome Dosage

Explorative regression analyses showed that the GM volumes in the amygdala, the insular cortex, temporal lobe cortex, caudate, and parietal cortex were linked to the number of sex chromosomes. The GM volume clusters of the inferior temporal gyrus and caudate were detected also when the number of X-chromosomes was used as a regressor. They were, however, primarily constituted by the GM volume reductions in XXY males, which were found in relation to both control groups, and thus seem to be related to sex chromosome trisomy rather than to the X-chromosome dosage.

The presently detected trisomy effects will be discussed in the context of previous findings in subjects with sex chromosome aneuploidy. Women with X0 monosomy have been found to exhibit hypertrophy of the structural and GM volume of the amygdala, as well as in the GM volume in the superior and, in some studies, the inferior temporal gyrus (Good et al. 2003; Molko et al. 2003; Kesler et al. 2004; Marzelli et al. 2011). Since the amygdala volume has also been found to be larger in XY males than in XX females, it has been suggested to be inversely correlated to X-chromosome gene dosage, which in turn could have a major influence on the sexually dimorphic development of this structure (Good et al. 2003). However, considering that amygdala volume has been found to be only slightly reduced in XXX females but markedly reduced in XXY males (Patwardhan et al. 2002), and that circulating testosterone has been correlated with increasing amygdala GM volume (see Table 4) (Neufang et al. 2009), it might be more plausible that amygdala volume is influenced by “sex chromosome” gene dosage (rather than “X-chromosome” gene dosage) and testosterone. This also may apply to the association between trisomy and atrophy of the superior temporal gyrus, as the posterior portion of this region mediates language functions, and it has been reported
that language dysfunction in XXY males correlated with an increased expression of a PAR gene \([GTPBP6\) (Vawter et al. 2007)]. A similar scenario may also account for the present observations regarding the parietal lobe. In addition to the finding of trisomy-related GM hypertrophy, which accords with previously reported parietal atrophy in X0 females (Brown et al. 2002), we found an inverse correlation between plasma testosterone and parietal GM volume, suggesting that impaired testosterone-mediated pruning (Chen et al. 2007; Koscik et al. 2009) could have added to the increase of parietal GM volume in XXY males, when compared with XY males.

The detected negative association between the number of sex chromosomes and the GM volume in the caudate (Molko et al. 2003) is more difficult to explain. The caudate nucleus is usually reported to be larger in females (Giedd et al. 1997) (Sowell et al. 2002; Bramen et al. 2011), occurring before they reach puberty, and is not particularly affected by hormonal perturbations, which argues for genetic effects. In the majority of MR studies, however, caudate volume has been found to be smaller in X0 females just as in XXY males (Cutter et al. 2006; Marzelli et al. 2011), although there are some reports of increases (Molko et al. 2003, 2004). It is difficult to attribute these results to the detected sex chromosome dosage effect, and it would require parallel studies of XX and XY controls together with both X0 and XXY subjects in order to gain more insight.

In summary, it is proposed that there are X-dosage effects in the precentral gyrus and parts of cerebellum. These are of interest since many forms of mental retardation are linked to the X-chromosome, as are several hereditary disorders of the motor system (Fragile X, familiar X-linked dystonias, X-linked cerebellar hypoplasia, adrenoleukodystrophy, and spinocerebellar degeneration with X-linked inheritance). The study also indicates that processes related to testosterone and sex chromosome genes have a combined effect on GM development in the superior temporal gyrus, the amygdala, the insular cortex, and the parietal cortex. The observed impact of sex chromosomes is in line with reports of sex-differentiated gene expression in the brain (Dewing et al. 2003; Vawter et al. 2007), as well as with the notion that cerebral sex differences appear prior to the production of sex hormones. We hypothesize that the presently detected effects of trisomy (expressed in differences in XXY men in relation to both male and female controls) are primarily due to X-chromosome escapee genes with Y chromosome homologs, regardless of the fact that homologous genes may be differentially regulated in males and females (Xu et al. 2002). By identifying brain areas that exhibit the effects of sex chromosomes, the present results add to the animal data about genes located on X and Y chromosomes that could contribute to sex differences in GM and WM volumes. This is of interest since many common psychiatric conditions, such as autism, ADHD, schizophrenia, and depression, are characterized by sex differences in regard to their rates, age at onset, course, and symptoms. Moreover, these conditions often involve abnormalities in the caudate and amygdala (Grossman et al. 2008; Waddell and McCarthy 2012) and tend to be more prevalent among those with various forms of sex-chromosome aneuploidy (DeLisi et al. 2005; Marco and Skuse 2006). Studies of sex chromosome aneuploidies thereby provide a unique tool to help understand some of the mechanisms behind the early development of sex differences and their clinical implications.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

**Notes**

The authors thank the Insurance Council for Working Life and Social Science (FAS), the Swedish Research Council, and VINNOVA for their financial support. The authors are very grateful to Professor Hans Forsberg for the fruitful discussions. Dr Savic had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. **Conflict of Interest:** None declared.

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