Cerebral Cortical Gray Matter Overgrowth and Functional Variation of the Serotonin Transporter Gene in Autism

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Context: Autism is a heritable neurodevelopmental disorder characterized biologically by enlargement of the head and brain and abnormalities of serotonin neurotransmission.

Objective: To evaluate whether 5-HTTLPR, a functional promoter polymorphism of the serotonin transporter gene SLC6A4, influences cerebral cortical structure volumes in young male children with autism.

Design: Association study of a genetic variant with quantitative traits.

Setting: Autism research centers at the University of North Carolina (UNC), Chapel Hill, and the University of Washington (UW), Seattle.

Participants: Forty-four male children, 2 to 4 years old, with autism participating in longitudinal brain magnetic resonance imaging studies.

Main Outcome Measures: Cerebral cortical and cerebellar gray and white matter volumes.

Results: We found that 5-HTTLPR genotype influenced gray matter volumes of the cerebral cortex (F_{2,23}=7.29, P = .004) and of 3 lobe-based subregions in the UNC sample of 29 children (frontal lobe gray matter: F_{2,23}=6.36, P = .01). The 5-HTTLPR short allele appeared to be additively associated with increasing gray matter volumes, an observation affirmed by tests of linear genotype effects (cortical gray matter: F_{1,24}=14.11, P = .001; frontal lobe gray matter: F_{1,24}=13.20, P = .001). Genotype did not influence cerebellar volumes. Confirmation was pursued by means of the UW sample of 15 children. While effects were not significant in the UW sample alone, the patterns of adjusted means resembled those found in the UNC sample. Positive Cochran-Mantel-Haenszel test results supported the concordance of relationships across the 2 sites, and analyses of covariance of the combined sample that included a site covariate showed significant linear genotype effects on structure volumes (cortical gray matter: F_{1,38}=5.73, P = .02; frontal lobe gray matter: F_{1,38}=11.73, P = .002). Effect sizes of 5-HTTLPR genotype on total cortical and frontal lobe gray matter volumes were 10% and 16%, respectively.

Conclusion: The SLC6A4 promoter polymorphism 5-HTTLPR influences cerebral cortical gray matter volumes in young male children with autism.

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Autism is a behaviorally defined neurodevelopmental disorder with a strong heritable component. Core symptoms, which emerge before the age of 3 years, include delayed development of language and communication, impaired social interaction, and excessively rigid and repetitive behaviors. The most consistently identified neurobiological abnormalities in autism have arisen from studies of brain size and of serotonin. Individuals with autism, when compared with normal control subjects, have been found to have an increased frequency of macrocephaly and heavier postmortem brain weight (reviewed by Cody et al) and, from an early age, increased volumes of a number of brain structures on magnetic resonance (MR) imaging. Our autism research group at the University of North Carolina (UNC), Chapel Hill, recently found generalized enlargement of cerebral cortical gray and white matter, but not of the cerebellum, in a sample of 2-year-old children with autism, the largest study of children this young reported to date. Serotonin’s role was first suggested by studies showing elevated platelet serotonin levels in autism, with subsequent studies demonstrating impaired serotonin synthesis in the brains of autistic individuals and worsening of repetitive behav-

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The UNC participants were drawn from a sample of 51 children with autism, 18 to 35 months old, who had participated in the initial stage of a longitudinal MR imaging study of the brain. Study approval was acquired from both the UNC and Duke University (Durham, NC) institutional review boards, and parents or guardians provided written, informed consent after the study had been fully explained to them. Diagnosis of participants was confirmed by means of the Autism Diagnostic Interview–Revised36 and the Autism Diagnostic Observation Schedule–G,37 and all cases met DSM-IV criteria38 for autism. Diagnosis will be reassessed at 42 to 59 months—the more conventional time for assessment by these instruments—at which point it is possible that some participants may no longer meet full criteria for autistic disorder. Exclusion criteria included notable dysmorphology, neurocutaneous abnormalities, significant neurologic deficits, fragile X syndrome via molecular testing, and disorders known to be associated with autism, such as neurofibromatosis and tuberous sclerosis. To diminish genetic heterogeneity, data from only white participants were analyzed. DNA was available for genotyping from 29 boys and 5 girls who met these criteria; given well-established sex differences in brain development and morphologic features,39,40 only data from boys were analyzed. Their mean ± SD age at the time they underwent imaging was 2.71 ± 0.30 years.

**UW SAMPLE**

The UW participants were drawn from a sample, described in detail elsewhere,1 of 45 children with autism who are participating in a longitudinal MR imaging study in which they were enrolled between the ages of 34 and 50 months. A diagnosis of autism was confirmed using the Autism Diagnostic Interview–Revised and the Autism Diagnostic Observation Schedule–G, and all cases met DSM-IV criteria for autistic disorder. Exclusion criteria were the same as those for the UNC sample. DNA was available for genotyping from 15 boys and 2 girls, although, as with the UNC sample, data from only boys were analyzed. These children were also white, and their mean ± SD age at the time they underwent imaging was 4.0 ± 0.3 years. Study approval was acquired from the UW institutional review board, and parents or guardians provided written, informed consent after the study had been fully explained to them.

**UNC MR IMAGE ACQUISITION AND PROCESSING**

All UNC participants underwent imaging at the Duke-UNC Brain Imaging and Analysis Center, on a 1.5-T MR imager (Signa; GE Healthcare, Chalfont St Giles, England). Image acquisition was described in detail in our previous publication.3 Briefly, the protocol was designed to maximize gray-white tissue contrast and included (1) a coronal T1 inversion recovery prepared image with T1 of 300 milliseconds, repetition time of 12 milliseconds, echo time of 5 milliseconds, and 20° flip angle, at 1.5-mm thickness with 1 excitation, 20-cm field of view, and 256 × 192 matrix and (2) a coronal proton density–T2 two-dimensional dual full spin echo, with a repetition time of 7200 milliseconds and echo time of 17/75 milliseconds, at 3.0-mm thickness with 1 excitation, 20-cm field of view, and 256 × 160 matrix. Children underwent imaging while under moderate sedation (a combination of pentobarbital sodium and fentanyl citrate as per hospital sedation protocol) administered by a sedation nurse under the supervision of a pediatric anesthesiologist. Physi-
logic monitoring was conducted throughout the imaging and recovery. All images were reviewed by a pediatric neuroradiologist and screened for significant abnormalities.

The T1 and proton density–T2 scans were registered and aligned along the anterior commissure–posterior commissure axis by means of BRAINS2 image processing software. The images were then segmented with the Expectation Maximization Segmentation (EMS) software originally developed at the Catholic University of Louvain and adapted by our laboratory. We developed a pediatric EMS template derived from averaging of MR images from 14 randomly selected children that were first tissue classified by means of semiautomated procedures in BRAINS2. This probabilistic brain atlas was then aligned to each participant brain by a fully automated linear affine transformation. Then, after bias estimation, correction of inhomogeneities, and stripping of nonbrain tissues, individual images were processed with EMS to produce segmented gray, white, and cerebrospinal fluid (CSF) images.

To obtain regional lobe measurements, we developed a template from a manually parcellated MR image of a 2-year-old brain that was selected because of its high image quality. Anatomic landmarks were chosen on the basis of consultation with pediatric neuroradiology experts, standard neuroanatomy references, and published protocols. Delineated regions included the frontal and temporal lobes, a combined parietal and occipital region, and the cerebellum (see Hazlett et al for a complete description of structure delineation protocols). The individual T1 images were adjusted for intensity differences, affine registered with the Rview program, and then mapped onto the template brain with its regional labeling by an automated deformation algorithm. Label maps were then combined with the EMS tissue classified images to produce gray, white, and CSF volumes for each region.

Finally, head circumference was measured on the MR image by means of Head Circumference, a locally developed program (http://www.ta.unc.edu/dev/download/headcircumference/index.htm). Measurement was taken in the axial plane by tracing the skull on the mid sagittal section through the supraorbital prominence and occipital protuberance. The intraclass correlation coefficient for both intrarater and interrater reliability was 0.90.

**UW MR IMAGE ACQUISITION AND PROCESSING**

Measures available from the UW sample for comparison with the UNC sample included total cerebral cortex tissue volume, total cortical gray and white matter volumes, total frontal lobe tissue volume, frontal lobe gray and white matter volumes, and total cerebellar tissue volumes. These were obtained according to the following protocols.

The protocols for MR imaging data acquisition and processing are described in detail by Sparks and colleagues. Scans were performed at the UW Diagnostic Imaging Sciences Research Center on a 1.5-T imager (Signa), using a 3-dimensional spoiled gradient acquisition in the steady state (SPGR) pulse sequence for image acquisition. Repetition time was 33 milliseconds, echo time was minimum, flip angle was 30°, field of view was 22 cm, and a 256 × 256 matrix was used. To improve resolution, section thickness was reduced from 3 mm to 1.5 mm by zero filling in the third-phase encoding direction. Children underwent imaging during continuous intravenous infusion of prophol at 180 to 220 mg/kg per minute.

The high-resolution SPGR images were corrected for field inhomogeneity by the N3 technique and segmented with a Bayesian classifier. Segmentation was performed only for the cerebrum, not the cerebellum. To accomplish segmentation, histogram analyses were performed to obtain pixel intensity intervals for starting parameters for each tissue type (gray, white, and CSF). On a section-by-section basis, these statistical populations were used to create probability distributions for input into a sequential maximum and posterior estimator, a Bayesian classifier that uses a multiscale approach to automatically generate classified images of gray matter, white matter, and CSF.

For the UW sample, the only cortical subdivision that had been measured was the frontal lobe. To accomplish this, scans had been stripped of extra cerebral tissue and CSF by means of a semiautomated program (MEASURE), yielding images that left all cerebral tissue outlined. From this image, a 3-dimensional reconstruction was performed. The central sulcus was identified according to boundaries previously described in the most superior axial sections and traced to its inferior end. Images were then resected in the axial plane, parallel to the anterior commissure–posterior commissure line. Starting in the most superior section, the central sulcus was identified, with the precentral and postcentral gyri used as guides. All brain area posterior to the central sulcus was erased. When the central sulcus no longer completely separated the frontal from the parietal lobes, a line was traced from the deepest point of the central sulcus to the innerhemispheric fissure; all pixels posterior to this line were erased. By the corpus callosum, lines were drawn connecting the most medial point of the central sulcus to the corpus. By the insular cortex, lines were drawn from where the precentral and postcentral gyri met across to the sylvian fissure, up to the fissure’s most anterior point, and then across to the corpus callosum, with tissue posterior to these lines being removed. Inferior to the corpus, tissue lateral to the sylvian fissure or anterior to lines drawn from the deepest point of the sylvian fissure to the innerhemispheric fissure was removed. Inferior to the optic chiasm, tissue posterior to a line drawn from the suprasellar cistern to the sylvian fissure was removed. All remaining brain tissue inferior to the most inferior section of frontal lobe was then erased. The computer software automatically summed the areas in each section and multiplied by section thickness to yield total frontal lobe volume.

Head circumference was measured on the UW scans by the same method as that described for the UNC sample.

**GENOTYPING**

Polymerase chain reaction amplification of 5-HTTLPR was performed at both UW and UNC according to a previously described protocol.

**STATISTICAL ANALYSIS**

The UNC sample was the subject of initial analyses, with the UW sample incorporated into follow-up, confirmatory analyses. Analyses of covariance (ANCOVAs) were used to test the UNC sample for relationships between genotypes and brain volumes. Structure volumes were the dependent measures, genotype was the independent predictor, and covariates included age at the time of image acquisition and head circumference as a general measure of head size. In addition, the proper genotype-based grouping for 5-HTTLPR is currently not clear. Early studies examining the effect of 5-HTTLPR on SLC6A4 expression reported higher rates of expression in L/L homozygotes than S allele carriers, while more recent studies have shown either no relationship or an additive genotype effect. We therefore used what we believed to be the most conservative model, testing a 3-genotype 5-HTTLPR effect that compared structure volumes in L/L vs S/S vs S/L genotypes. We also tested all interaction terms, which were kept in the model when significant.

For any significant genotype effect, we calculated effect size measures. For 5-HTTLPR as a categorical variable, we calcu-
lated $\omega^2$, which estimates the proportion of variance in a dependent measure accounted for by an independent categorical variable in the population from which the sample was drawn.\(^6\) The $\omega^2$ value is given by the following equation:

$$\omega^2 = \frac{SS_{\text{effect}} - (dfe_{\text{effect}})(MS_{\text{error}})}{MS_{\text{error}} + SS_{\text{total}}},$$

where, for our models, $SS_{\text{effect}}$ is the type III sums of squares for genotype, $dfe_{\text{effect}}$ is the number of degrees of freedom for genotype (2 in this case), $MS_{\text{error}}$ is the mean square error for the entire model, and $SS_{\text{total}}$ is the corrected total sums of squares for the entire model. For $5-HTTLPR$ genotype as an ordinal variable, we calculated $\tau$ after partialing out the covariates.

Follow-up of positive UNC findings in the UW sample included ANCOVAs and Cochran-Mantel-Haenszel correlation tests, focusing on brain regions that were significantly influenced by genotype in the UNC sample and that were measured in the UW sample. The ANCOVAs of the UW sample alone included the head circumference and age covariates, while ANCOVAs of the combined UW-UNC data set also included site as a covariate. Rank-based Cochran-Mantel-Haenszel tests were used to test for ordinal associations between genotype and structure volumes of interest while stratifying for site, Kendall $\tau$-$b$ was calculated as a measure of association, and $\omega^2$ and partial $r^2$ were calculated for significant ANCOVA genotype effects.

## RESULTS

Genotype frequencies and age and head circumference data for the UNC and UW samples are provided in Table 1. There was no evidence of Hardy-Weinberg disequilibrium, and genotype frequencies were not different between the 2 samples ($\chi^2 = 0.68, P = .71$). Within the UW sample, age was different across genotype ($F_{1,42} = 3.87, P = .05$), with $S/S$ individuals being slightly older than the others. Between sites, age and head circumference were greater in the UW sample (age, $F_{1,42} = 146, P < .001$; head circumference, $F_{1,42} = 4.81, P = .03$). These differences were accounted for in the ANCOVAs by the use of appropriate covariates.

For the UNC sample, an ANCOVA of total cerebral cortical volume showed a significant $5-HTTLPR$ main effect (Table 2). This led us to perform separate genotype tests for cortical gray matter, which was significant, and for cortical white matter, which was not. On this basis, we examined genotype effects on the 3 cortical lobe gray matter volumes derived from our automated segmentation protocol and found significant genotype effects for all of them, with genotype $\omega^2$ values ranging from 6% to 11% (Table 2). In contrast, no genotype effects were observed for the cerebellum, whether examining gray, white, or total tissue volumes (Table 2).

Examination of the adjusted means showed a pattern suggestive of a linear, additive allelic effect, with the $S$ allele associated with larger structure volumes (or, conversely, the $L$ allele associated with smaller structures).

### Table 1. Demographic Data for UNC and UW Samples

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>UNC Sample</th>
<th></th>
<th>UW Sample</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (N = 29)</td>
<td>L/L (n = 11)</td>
<td>S/L (n = 12)</td>
<td>S/S (n = 6)</td>
</tr>
<tr>
<td>No.</td>
<td>29</td>
<td>11</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Age, y*</td>
<td>2.7 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Head circumference, cm†</td>
<td>51.2 ± 1.3</td>
<td>50.9 ± 1.2</td>
<td>51.4 ± 1.4</td>
<td>51.3 ± 1.3</td>
</tr>
</tbody>
</table>

**Abbreviations:** $L$, long allele; $S$, short allele; UNC, University of North Carolina, Chapel Hill; UW, University of Washington, Seattle.

*Mean ± SD. Within the UW group, age was different across genotype ($F_{1,12} = 3.87, P = .05$). Age was also different between the 2 sites ($F_{1,42} = 146, P < .001$).

†Mean ± SD. Head circumference was different between sites ($F_{1,42} = 4.81, P = .03$).

### Table 2. Analysis of Covariance Results for Brain Structure Volumes: UNC

<table>
<thead>
<tr>
<th>ROI</th>
<th>Adjusted Mean ± SD Volume, cm³</th>
<th>F Tests for S/S vs S/L vs L/L</th>
<th>F Test for Linear Genotype Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (N = 29)</td>
<td>L/L (n = 11)</td>
<td>S/L (n = 12)</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>933 ± 33.7</td>
<td>913 ± 41.2</td>
<td>930 ± 29.4</td>
</tr>
<tr>
<td>Cortical gray matter</td>
<td>674 ± 22.9</td>
<td>656 ± 28.0</td>
<td>674 ± 20.0</td>
</tr>
<tr>
<td>Cortical white matter</td>
<td>259 ± 13.6</td>
<td>257 ± 16.3</td>
<td>256 ± 12.9</td>
</tr>
<tr>
<td>Frontal lobe gray matter</td>
<td>256 ± 10.2</td>
<td>247 ± 12.1</td>
<td>257 ± 10.4</td>
</tr>
<tr>
<td>Temporal gray matter</td>
<td>161 ± 8.9</td>
<td>156 ± 8.3</td>
<td>161 ± 8.9</td>
</tr>
<tr>
<td>P-O gray matter</td>
<td>259 ± 9.5</td>
<td>253 ± 11.3</td>
<td>256 ± 9.7</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>117 ± 9.6</td>
<td>116 ± 11.6</td>
<td>119 ± 10.0</td>
</tr>
<tr>
<td>Cerebellar gray matter</td>
<td>99 ± 8.6</td>
<td>98 ± 10.4</td>
<td>100 ± 8.6</td>
</tr>
<tr>
<td>Cerebellar white matter</td>
<td>18.0 ± 1.4</td>
<td>18.0 ± 1.4</td>
<td>18.3 ± 1.6</td>
</tr>
</tbody>
</table>

**Abbreviations:** $L$, long allele; P-O, parieto-occipital; ROI, region of interest; $S$, short allele; UNC, University of North Carolina, Chapel Hill; ellipses, not applicable.
The ANCOVAs with genotype coded as an ordinal variable (L/L = 1, S/L = 2, and S/S = 3) supported this, showing strongly significant linear effects of genotype on cerebral cortical gray and subregion gray matter volumes (Table 2). Partial $r^2$ values indicated that 5-HTTLPR, considered in this additive fashion, accounted for 16% of total cortical gray variance, an effect that was strongest in the frontal lobe (partial $r^2=0.23$).

Finally, we performed all the ANCOVAs with nonparametric analogues that used the rank transformation. The results of these tests were nearly identical to those from the raw values, providing assurance that the observed effects were not due to nonnormal distributions of the data (data not shown).

On the basis of these findings, we performed similar tests using the UW sample. In the UW sample alone, which was smaller, genotype did not exert statistically significant effects on brain structure volumes as either a categorical or a continuous variable (Table 3). The ANCOVAs of the combined samples that included site as a covariate, however, showed significant genotype effects on cortical gray matter volumes that were strongest for linear effects, particularly for frontal lobe gray matter volume (Table 4). Furthermore, the patterns of predicted values within the UW sample were similar to those from the UNC sample (Figure), and partial $r^2$ values relating total cortical gray and frontal lobe gray matter volumes to genotype for the combined sample were 0.10 and 0.18, respectively (Table 4), again indicating a substantial effect of 5-HTTLPR genotype on variability in the volumes of these structures. Cochran-Mantel-Haenszel tests that were stratified by site and used ranked

![Figure. Predicted frontal lobe gray matter volumes. The individual values are derived from an analysis of covariance that includes covariates of head circumference, age at the time of imaging, site, and a second-order head circumference interaction term. The horizontal bars show the adjusted means, and the vertical bars, the standard deviations for each genotype group. The test result for a linear genotype effect was $F_{3,89}=11.73$, $P=.002$, with a partial $r^2=0.16$. 5-HTTLPR indicates the functional promoter polymorphism of the serotonin transporter gene SLC6A4. L, long allele; S, short allele; UNC, University of North Carolina, Chapel Hill; UW, University of Washington, Seattle.](https://www.archgenpsychiatry.com/content/64/6/713/F2.large.jpg)
values of the data provided further support for concordance of relationships across the 2 sites, indicating the presence of significant linear correspondences between genotype and structure volumes (Table 5). The Cochran-Mantel-Haenszel tests were significant for both the raw structure volume data and, more strongly, for predicted structure volumes from the ANCOVAs.

**COMMENT**

Autism is characterized by serotonergic dysfunction and enlargement of the head and brain. Serotonergic neurons are generated early in brain development and establish extensive cortical and subcortical connections. Serotonin regulates growth cone motility, synaptogenesis, synaptic plasticity, and the development and activity of multiple neuronal subtypes. Given these effects, a plausible hypothesis can be made that the serotonin dysfunction seen in autism contributes to the brain enlargement. Such a hypothesis has indirect support from animal model studies and now more direct support from the demonstration that the SLC6A4 promoter polymorphism 5-HTTLPR influences gray matter volumes of cerebral cortical structures in young males with autism. Specifically, we found the S 5-HTTLPR allele to be associated in an additive fashion with different patterns of gene-trait relationships.

This assertion is supported by previous association studies with autism and, to some degree, with brain morphologic features. Numerous studies have reported associations of SLC6A4 polymorphisms with autism. Of these polymorphisms, 5-HTTLPR has been examined most extensively. Although overtransmissions of both the L and S alleles have been described, as has no association, the reports supporting S are in general larger and more recent. Devlin et al, in the largest study reported thus far, found significant overtransmission of the S allele in a sample of 390 families with multiplex autism; multiple SLC6A4 variants were examined, but only 5-HTTLPR showed association. The S allele has also been associated with numerous childhood psychiatric disorders and traits such as childhood-onset depression, aggression, and attention-deficit/hyperactivity disorder behaviors in males, and with phenotypic traits directly relevant to autism such as neuroticism, childhood shyness, and symptom severity and amygdala excitability in social phobia.

Although our study is the first to examine 5-HTTLPR and brain morphologic features in autism, similar studies have been performed in other psychiatric disorders. Frodl et al examined the influence of the polymorphism on hippocampal volumes in individuals with depression. Within the depressed group, they found that L/L homozygous patients had smaller hippocampal gray and white matter volumes than did patients with at least 1 S allele. Taylor et al, in a study of elderly patients with depression, found that, for patients in whom depression occurred early in life, S/S homozygotes had smaller hippocampal volumes than did L allele carriers, while, for patients with a later age at onset, L/L was associated with smaller volumes. In comparisons with controls, both Frodl et al and Taylor et al found significant differences only between affected and normal L/L homozygotes. In studies of normal adults, Pezawas et al found that adult S carriers had reduced gray matter volumes in the perigenual cingulate and amygdala compared with L/L homozygotes, whereas Canli et al found that S allele carriers had greater left cerebellar volumes and smaller volumes of the left superior, medial frontal, left anterior cingulate, and right inferior frontal gyri than did L/L homozygotes. Thus, although evidence supporting pathogenicity of the S allele is not consistent, previous studies of both healthy and psychiatrically ill individuals have found associations of 5-HTTLPR with brain morphologic features that are in the same direction as our report. The comparability of these studies with ours is, however, ultimately limited by significant differences in samples and designs. All of these previous reports compared L/L homozygotes with S allele carriers, whereas we examined a 3-genotype model. These studies examined either individuals with depression or normal controls, whereas we examined individuals with autism; and these studies all examined adults, whereas we examined very young children. Any of these distinctions, particularly those relating to diagnosis and age, could give rise to different patterns of gene-trait relationships.

### Table 5. CMH Tests of 5-HTTLPR and Structure Volumes

<table>
<thead>
<tr>
<th>ROI</th>
<th>Kendall $\tau-b$</th>
<th>Raw Values</th>
<th>CMH Tests</th>
<th>Predicted Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNC</td>
<td>UW</td>
<td>CMH</td>
<td>$P$ Value</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>0.30</td>
<td>0.28</td>
<td>4.60</td>
<td>.03</td>
</tr>
<tr>
<td>Cortical gray matter</td>
<td>0.31</td>
<td>0.19</td>
<td>4.30</td>
<td>.04</td>
</tr>
<tr>
<td>Cortical white matter</td>
<td>0.15</td>
<td>0.21</td>
<td>1.23</td>
<td>.27</td>
</tr>
<tr>
<td>Frontal lobe gray matter</td>
<td>0.31</td>
<td>0.33</td>
<td>5.40</td>
<td>.02</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>-0.03</td>
<td>-0.09</td>
<td>0.08</td>
<td>.78</td>
</tr>
</tbody>
</table>

Abbreviations: CMH, Cochran-Mantel-Haenszel; ROI, region of interest; UNC, University of North Carolina, Chapel Hill; UW, University of Washington, Seattle.

*CMH tests use ranked values of structure volumes and are stratified by site. Predicted values are derived from analyses of covariance.
Elaborating on our results, we found that, when coded as a categorical 3-genotype variable, 5-HTTLPR explained approximately 8% of total cerebral cortex volume variability. The effect was specific to the cortex because it was not observed in the cerebellum. This specificity resonated with our recent report that, when compared with controls, these same autistic children had increased cerebral cortical, but not cerebellar, volumes.\textsuperscript{77,78} Within the cerebrum, the genotype effect was found primarily in gray matter, explaining approximately 10% of volume variance, as opposed to white matter, where it was not significant. When cortical gray matter is parsed into subregions, the 5-HTTLPR effect is found in all 3: frontal, temporal, and parieto-occipital. The specificity to gray matter stood in contrast to our case-control comparison, which found significant differences in both white and gray matter volumes, suggesting that the effect of 5-HTTLPR on brain morphologic features is specific to gray matter. This is not necessarily surprising, however, given that distinct genetic factors are known to influence white and gray matter development.\textsuperscript{77,78}

Further analyses showed that a categorical treatment of 5-HTTLPR genotype did not best represent the observed results. The adjusted means for the gray matter volumes followed a pattern of S/S>S/L>L/L (Table 2). Total and regional gray matter volume adjusted means for the S/S group were 5% to 6% larger than those for S/L, which were in turn 3% to 4% larger than those for L/L. Similarly, S/S gray matter volume means were 1.1 to 1.5 SDs greater than the grand mean, S/L gray matter means were near the grand mean, and L/L gray matter means were 0.6 to 1.0 SD less than the grand mean. When this pattern was tested by treating genotype as an ordinal (additive in this case) variable, the genotype main effects increased in strength and came to account for approximately 16% and approximately 23% of total cortical gray and frontal lobe gray matter volume variability, respectively. Although these data were not perfectly linear—there is some suggestion of a homozygous, recessive S/S effect (or, conversely, a dominant L carrier effect)—the additive model best fit the data. Tests performed comparing S/S homozygotes with L allele carriers accounted for proportions of variance similar to or smaller than those for the 3-genotype comparisons and substantially less than those derived from additive models (data not shown).

To validate these findings, we examined a second, independent sample of young males with autism who had undergone brain MR imaging. Although they were fewer, were older by an average of nearly 1.5 years, and had undergone imaging with a different protocol, analyses of the UW sample were consistent with our UNC findings. While tests of 5-HTTLPR effects in the UW sample alone were not significant, the adjusted means for the 3 genotype groups followed the UNC pattern: S/S>S/L>L/L. As with the UNC sample, this pattern was observed for total cortex, cortical gray matter, and cortical white matter but not for cerebellar volumes. The same pattern was also found for frontal lobe gray matter, the only cortical subregion for which UW data were available. We note, however, that the strength of the genotype effects was similar for frontal and total cerebral gray matter. Thus, the frontal lobe gray matter enlargement cannot account for the total cerebral gray matter enlargement, which is more likely to be diffusely spread across the lobes as in the UNC sample.

Congruences of associations across the 2 samples were confirmed by Cochran-Mantel-Haenszel tests of the combined samples that were stratified by site, and by ANCOVAs of the combined samples that included site as a covariate. As with the UNC sample alone, ANCOVAs of an additive genotype model in the combined sample produced the strongest effects and accounted for the greatest amount of variance: 10% and 16% for cortical and frontal lobe gray matter volumes, respectively. Adjusted mean volumes of frontal and total gray matter were 3% to 4% (0.8-1.0 SD) greater in S/S than in S/L, which were in turn 2% to 3% (0.4-0.6 SD) greater than those in L/L. While these differences were somewhat less than those in the UNC sample alone, given that the UW sample was older, this may be due in part to the finding that brain enlargement in autism appears to be most pronounced very early and to normalize with age.\textsuperscript{2}

We therefore find that relationships between 5-HTTLPR and brain morphologic features in young children with autism are substantial and concordant across 2 independent samples. Our data must be interpreted, however, in the context of a number of limitations. First, our samples, although large for this type of study, are nonetheless, by objective standards, small. While we have addressed this limitation with replication and careful statistical analysis, we cannot rule out the possibility of false-positive findings. Second, recent data have emerged suggesting that additional serotonin transporter genetic variants interact with 5-HTTLPR to influence serotonin transporter expression.\textsuperscript{56,62,79} We have not yet genotyped these variants, but as we do we may be able to delineate genotype-phenotype relationships with more precision than our current data permit. Furthermore, because we did not analyze normal controls, we cannot determine whether the observed relationships are specific to autism, nor can we determine what proportion, if any, of the brain enlargement that characterizes autism is attributable to 5-HTTLPR. Finally, we cannot state with certainty the directional causality of the association. It may be, for example, that the L allele causes diminished gray matter volume, or that 5-HTTLPR exerts a more complex influence on gray matter volumes, rather than the S allele causing increased gray matter volume. In other words, our data do not tell us whether 5-HTTLPR has a pathological effect on brain morphologic features in autism. We can nonetheless conclude that 5-HTTLPR is strongly associated with cerebral cortical gray matter volumes in young males with autism. The testing of further related hypotheses awaits similar studies in autistic cohorts with different age and sex compositions, in individuals with other psychiatric disorders, and in healthy children.

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