Effect of Dopamine Transporter Gene (SLC6A3) Variation on Dorsal Anterior Cingulate Function in Attention-Deficit/Hyperactivity Disorder

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Although attention-deficit/hyperactivity disorder (ADHD) is associated both with brain alterations in attention and executive function (EF) circuitry and with genetic variations within the dopamine system (including the dopamine transporter gene [SLC6A3]), few studies have directly investigated how genetic variations are linked to brain alterations. We sought to examine how a polymorphism in the 3’ untranslated region (UTR) of SLC6A3, associated with ADHD in meta-analysis, might contribute to variation in dorsal anterior cingulate cortex (dACC) function in subjects with ADHD. We collected fMRI scans of 42 individuals with ADHD, all of European descent and over the age of 17, while they performed the multi-source interference task (MSIT), a cognitive task shown to activate dACC. SLC6A3 3’ UTR

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variable number tandem repeat (VNTR) polymorphisms were genotyped and brain activity was compared for groups based on allele status. ADHD individuals homozygous for the 10R allele showed significant hypoactivation in the left dACC compared to 9R-carriers. Exploratory analysis also showed trends toward hypoactivation in the 10R homozygotes in left cerebellar vermis and right lateral prefrontal cortex. Further breakdown of genotype groups showed similar activation in individuals heterozygous and homozygous for the 9R allele. Alterations in activation of attention and EF networks found previously to be involved in ADHD are likely influenced by SLC6A3 genotype. This genotype may contribute to heterogeneity of brain alterations found within ADHD samples. © 2009 Wiley-Liss, Inc.

**Key words:** dopamine transporter gene; functional magnetic resonance imaging; genetics; adhd; anterior cingulate

**INTRODUCTION**

Attention deficit/hyperactivity disorder (ADHD) affects up to 10% of children [Faraone et al., 2003], and 5% of adults worldwide [Faraone and Biederman, 2005]. ADHD is increasingly recognized as a brain disorder, with alterations found across the lifespan in studies of both brain structure [Seidman et al., 2005] and function [Dickstein et al., 2006]. The regions most frequently implicated in ADHD are anterior cingulate cortex (ACC), lateral prefrontal cortex (PFC), striatum, and cerebellum [Dickstein et al., 2006; Valera et al., 2007], congruent with the executive and attentional processes that these networks support and that are often dysfunctional in the disorder.

ADHD has a strong genetic component, with results from twin studies estimating that the additive effect of multiple genes explains approximately 80% of its variance, making it one of the most heritable psychiatric disorders [Faraone et al., 2005]. Although the genetic underpinnings of ADHD are complex, molecular genetic studies have identified several candidate genes associated with the disorder, most within catecholamine systems [Faraone et al., 2005]. These associations are consistent with the longstanding “dopamine hypothesis” of ADHD, supported by multiple lines of evidence from neuroanatomic, pharmacologic and molecular imaging studies all implicating dopamine in the disorder [see Swanson et al., 2007 for review].

One of the candidate genes most extensively studied in ADHD is SLC6A3, which codes for the dopamine transporter (DAT), the primary protein responsible for clearing dopamine from the synaptic space in the striatum. The SLC6A3 gene is of particular interest to ADHD because DAT is the principal target of the two most effective pharmacological treatments for the disorder (both methylphenidate [MPH] and amphetamine [AMP]; [Madras et al., 2005]), because altered levels of DAT availability have been found across studies of unmedicated patients with ADHD [Krause, 2008], and because the SLC6A3 knockout mouse is an animal model for ADHD with very high external validity [van der Kooij and Glennon, 2007]. SLC6A3 contains a variable number tandem repeat (VNTR) polymorphism in the 3′ untranslated region (UTR). The 9-repeat (9R) and 10-repeat (10R) alleles are the most common forms of the gene, and it is the 10R allele that shows a weak but significant association with ADHD [Faraone et al., 2005; Yang et al., 2007]. Although contrary reports have been published [van Dyck et al., 2005], the 9R allele has been found in vivo [Heinz et al., 2000], ex vivo [Mill et al., 2002], and in vitro [VanNess et al., 2005] to result in lower expression of DAT, presumably reducing synaptic DA clearance rates.

While genetic studies showing associations between genotype and diagnosis have made a significant impact on our understanding of the genes involved in ADHD, findings on any one particular gene, including SLC6A3, lack statistical strength, and studies are relatively equivocal [Faraone et al., 2005]. Imaging genetics may be a more powerful technique for finding associations as it allows the observation of biologically based endophenotypes, presumably under control of fewer genes than the complex sets of behaviors used to diagnose a disorder [Meyer-Lindenberg and Weinberger, 2006]. Further, this technique can help us to understand the specific neurobiological consequences of genes previously identified in association studies. Specifically, imaging genetics can help us to understand how one variant of a gene might predispose towards one type of neural outcome, while another variant may have a different or additive effect. In a disorder with such substantial clinical and neurocognitive heterogeneity as ADHD [Sonuga-Barke, 2005], these types of studies can help elucidate the different causal paths leading from gene to brain to expression of the disorder. Identifying these heterogeneous pathophysiologic trends may lead eventually to refined diagnoses and more individualized treatments.

To date, only a handful of imaging genetics studies of ADHD have been published, most using structural imaging in children. These studies have focused on dopamine system genes previously associated with ADHD, several finding genetic effects on ADHD regions of interest (ROIs) such as frontal cortex, striatum, and cerebellum [Durston et al., 2005, 2008; Shaw et al., 2007; Monuteaux et al., 2008]. In the current study we investigated the role of the SLC6A3 VNTR in adults with ADHD, targeting its effect on the dorsal anterior cingulate cortex (dACC).

The ACC has been found to be affected in multi-modal neuroimaging studies of ADHD in both children and adults. It has shown reduced volume [Seidman et al., 2006], decreased cortical thickness [Shaw et al., 2006; Makris et al., 2007], and functional hypoactivation [Bush et al., 1999, 2005; Dickstein et al., 2006] in ADHD. Although the causative factors behind ACC pathology in ADHD are unknown, it does seem to be particularly affected by changes in brain dopamine levels, and specifically to changes in DAT function. For example, in studies of brain effects of MPH, which principally targets the DAT [Volkow et al., 1998], acute treatment increases fMRI BOLD signal [Bush et al., 2008], and rCBF [Udo de Haes et al., 2007] in ACC. An MR spectroscopic study found that chronic MPH treatment affects NAA and choline levels in anterior cingulate but not in lateral frontal cortex [Kronenberg et al., 2008]. In addition, at least two previous imaging genetics studies have found significant differences between SLC6A3 genotype groups on ACC functioning during cognitive tasks [Bertolino et al., 2006; Schott et al., 2006]. Taken together, these findings suggest that changes in DAT function are linked to ACC function, and for this reason, we sought to investigate if variation in ACC activity within a group of subjects
with ADHD might be partially explained by SLC6A3 genotype. We used the multi-source interference task (MSIT) to probe SLC6A3 effects on dACC as it was designed specifically for interrogating the dACC and related executive attention network [Bush and Shin, 2006], and appears to be sensitive to changes in DA system functioning as probed with MPH [Bush et al., 2008].

In this study we investigate if subjects with ADHD differ neurofunctionally based on SLC6A3 genotype. Based on results from previous fMRI studies showing regional hypoactivity in 10R homozygotes in both control and ADHD samples [Bertolino et al., 2006; Schott et al., 2006; Durston et al., 2008], the association of the 10R allele with ADHD, and the likely low activity nature of the 9R allele, we hypothesized that ADHD subjects homozygous for the SLC6A3 10R allele would show hypoactivity in the dACC.

MATERIALS AND METHODS

This study is a secondary data analysis, integrating neuroimaging data collected in an NIH funded study (MH 062152), and genetic data previously collected in MH 062152, or four other NIH funded studies (MH 57934, HD 37694, MH 064019, HD 36317). All studies were conducted in accordance with the Declaration of Helsinki and the standards established by the Partners Healthcare Human Research IRB. Written informed consent was obtained from all adult subjects, and for subjects under 18 (17-year olds), consent was obtained from a parent and assent was obtained from the subject. Subjects described below include all individuals diagnosed with ADHD for whom both the fMRI task (MSIT) and DNA were obtained from a parent and assent was obtained from the subject. Written informed consent was obtained from all adult subjects, and for subjects under 18 (17-year olds), consent was obtained from a parent and assent was obtained from the subject. Subjects described below include all individuals diagnosed with ADHD for whom both the fMRI task (MSIT) and DNA were collected, and who met the inclusion criteria for this analysis. There was insufficient overlap of control subjects between the neuroimaging and genetic studies to include a control sample in the study.

Subjects

Subjects were 42 participants (52% female), aged 17–59, who were diagnosed with ADHD, recruited from Massachusetts General Hospital clinics and advertisements posted in the Boston area. Previous imaging reports have been published on subsets of this sample [Valera et al., 2005; Seidman et al., 2006; Makris et al., 2007; Monuteaux et al., 2008], but none using the MSIT, and the current report extends this work by examining the subset of ADHD subjects who had both genetic and MSIT functional imaging data available. Exclusion criteria for all subjects were as follows: an estimated full scale IQ <80; lifetime history of psychosis; current alcohol or substance abuse; inadequate command of the English language; sensoriomotor handicaps. In order to reduce the risk of stratification bias, for the current report we only included subjects who identified themselves as Caucasian. Eight subjects had pharmacological intervention for ADHD in the past, and 16 were prescribed psychostimulants near the time of scan (the remaining 18 participants were naïve to pharmacological treatment for ADHD). All subjects prescribed stimulants at the time of scan underwent a 24 hr washout period before the MRI visit. Five subjects had taken medications for other psychiatric conditions within 24 hr of the MRI scan: three were on SSRIs, two on benzodiazepines.

Clinical and Behavioral Assessment

All subjects were assessed with the Structured Clinical Interview for DSM-IV [SCID-IV; First et al., 1997]. To assess childhood ADHD, an additional module was administered, derived from the Schedule of Affection Disorders and Schizophrenia for School Age Children [Kiddie SADS-E; Orvaschel and Puig-Antich, 1987]. Previous work in our lab has shown that retrospective childhood diagnoses of ADHD can be made in a reliable and valid manner using this method [Faraone et al., 2000]. We considered a subject positive for ADHD if DSM-IV diagnostic criteria were met in childhood. A committee of board-certified child and adult psychiatrists and psychologists blind to referral source resolved diagnostic uncertainties. To obtain a multi-dimensional assessment of mood near the time of brain imaging, we administered the Profile of Mood States [POMS; McNair et al., 1992]. Block Design and Vocabulary subtests from the Wechsler Adult Intelligence Scale-III [WAIS-3; Wechsler, 1997] were used to estimate IQ. Academic achievement was assessed with the Reading and Arithmetic modules of the Wide-Range Achievement Test [WRAT-3; Jastak and Jastak, 1993].

fMRI Paradigm

The MSIT combines multiple dimensions of cognitive interference including "Stroop" [Stroop, 1935], "Simon" [Simon and Berbaum, 1990] effects with response competition and selection in order to maximally recruit dACC neurons. The task is described in detail elsewhere [Bush and Shin, 2006]. To summarize, in each session we presented eight blocks of stimuli alternating between interference and control conditions beginning and ending with 30 sec of fixation. In both conditions, subjects responded to the identity of the number that differed from two other numbers. In the control blocks, distractors were zeros, and target numbers were congruent with their positions on the button press. In the interference blocks, distractors were drawn from the set of potential target numbers (i.e., 1, 2, or 3), and targets were always placed incongruently with their button press positions (see Fig. 1).

Each subject completed a 5-min practice session just prior to the fMRI scan and then completed two test runs during scanning, each 6 min, 42 sec. MSIT stimuli were generated using MacStim software (WhiteAnt Occasional Publishing, http://www.brainmapping.org/WhiteAnt) running on a Macbook G4, and projected onto a screen situated in the rear of the magnet bore. MSIT performance outcome measures were percent correct and reaction time during the control and interference conditions.

fMRI Data Acquisition and Analysis

Imaging was performed on a Siemens Sonata 1.5T full-body MR scanner equipped with high-speed imaging gradients and quadrature head coil. Head movement was minimized with padded stabilizers surrounding the head of each individual. A sagittal localizer scan was performed for correct placement of slices, followed by a coronal T2-weighted sequence to rule out unexpected
neuropathology. A T1-weighted MPRAGE image was collected for anatomical coregistration of functional images. Functional imaging was performed using a gradient-echo EPI pulse sequence (22 sagittal slices, TR = 1,500 msec, 5 mm thick, 1 mm interslice interval, TE = 21 msec, flip angle = 90°; 264 volumes + 4 dummy scans per session). Imaging parameters (optimized for coverage of cingulate cortex) precluded full coverage of the anterior and posterior poles.

fMRI data were analyzed using Statistical Parametric Mapping (SPM2; Welcome Department of Cognitive Neurology, London). Preprocessing included: (1) correction for bulk head motion, (2) coregistration of functional volumes to individual T1-weighted anatomy, (3) spatial normalization of T1 images to a template and application of normalization parameters to coregistered functional data for template space transformation, and (4) spatial smoothing with a Gaussian filter (8 mm full-width half maximum). Individual runs exhibiting a spike of more than 3 mm of scan-to-scan head motion and/or stimulus correlated motion of r ≥ 0.5 were dropped. As a result, one run was dropped from a single subject.

Following preprocessing, statistical analyses were performed at the single-subject level. Each epoch of trials was modeled using a boxcar function convolved with a canonical hemodynamic response function. Low-frequency components of the BOLD signal were modeled as confounding covariates using a set of discrete cosine basis functions with a cutoff of 168 sec in order to increase sensitivity. Six motion correction parameters were also included in the model as confounding covariates in order to increase sensitivity and reduce the possibility of motion artifacts. Voxel-wise t-tests were conducted at the individual-subject level using our contrast of interest (interference > control), which isolates the signal associated with the MSIT interference effect [Bush and Shin, 2006]. Second level two-sample t-tests by genotype group were conducted for the whole volume, as well as restricted to the ROI that was hand drawn using MARINA [MAsks for Regions of Interests Analyses; Bender Institute of Neuroimaging, University of Giessen, Germany; Walter et al., 2003]. The ROI was limited to dorsal anterior midcingulate cortex (anterior to y = 0, posterior to y = 30, and within 15 mm of midline), defined based on a meta-analysis of imaging studies reporting ACC activation during cognitively demanding tasks [Bush et al., 2000]. The height threshold for genotype comparisons was set to P < 0.005-uncorrected, and only clusters with spatial extent of k ≥ 5 were displayed. Cluster-level P values for the dACC data were corrected for number of voxel-wise comparisons within the total bilateral ROI, while in other regions cluster level P values were corrected for number of voxels in the whole volume. In our whole volume analysis, for exploratory purposes, we report any clusters emerging at significant or trend cluster-level uncorrected P values (P < 0.1). Because of the wide age range in our sample, we also conducted an ANCOVA to assess for the potential confounding effect of age on genotype effects, testing effects both within the ROI and the entire volume, using the above thresholds.

In order to investigate whether resulting differences were due to an allele dosing effect, in SPSS® we ran Bonferroni corrected post hoc tests on raw beta values averaged across the voxels of resulting clusters, with number of 9R alleles as the independent variable. In order to assess if an allele dosing effect could explain activity anywhere in the entire volume we additionally conducted a linear contrast within SPM2 with number of 9R alleles as the independent variable.

Genotyping Methods

Genotyping of SLC6A3 was conducted at the Massachusetts General Hospital Psychiatric and Neurodevelopmental Genetics Unit using the following protocol: Genomic DNA (5 ng) was amplified in a 7 μl reaction using HotStarTaq DNA Polymerase (0.2 U), the proprietary HotStarTaq Buffer (1×), dNTPs (200 μM), and the marker specific primers (0.2 μM). Primers were ordered from Applied BioSystems and are as follows: SLC6A3-F 6FAM-TGTGGTGTAAGGAAGCCTGAG, SLC6A3-R CCTCCTGGA-GGTCAGGCTCAAGG. The SLC6A3-R primer also contains the proprietary tail. For amplification, samples were heated at 92°C for 9 min to activate the HotStarTaq Polymerase. This is followed by 12 cycles of denaturation for 30 sec at 93°C, annealing for 30 sec beginning at 64.5°C and dropped 0.5°C every cycle, and primer extension at 72°C for 30 sec; 37 cycles of denaturation for 30 sec at 93°C, annealing for 30 sec at 58°C, and primer extension at 72°C for 30 sec; 72°C for 1 hr. Amplified products were pooled and combined with size standard (LIZ-250) before being analyzed on an ABI 3730. GeneMapper v3.5 was used to analyze the raw results from the ABI3730, however, a genotype was not considered final until two laboratory personnel had independently checked (and corrected) the GeneMapper results and both individuals were in agreement.

FIG. 1. The MSIT paradigm. Example stimuli from the control and interference conditions of the multi-source interference task.
RESULTS

Genotype, Demographic, and MSIT Performance Data

Genotype frequencies of the SLC6A3 gene were as follows: 19 subjects were homozygous for the 10R allele and 23 were 9R carriers. Of the 9R carriers, 6 were homozygous for the 9R allele, 16 were 9R/10R, and 1 was 9R/11R. There was no evidence that these data were not in Hardy–Weinberg Equilibrium ($P = 0.89$).

As Table I shows, there were no significant differences between SLC6A3 genotype groups on any of the demographic, mood, or ADHD variables including age of onset, number of childhood symptoms, and number of symptoms at time of interview. No differences were found between groups on frequencies of lifetime alcohol or substance abuse, anxiety disorders, or major depression. MSIT performance data did not differ significantly between the two genotype groups, although there was a trend for higher accuracy on the interference task in the 10R/10R group ($P = 0.077$). We found no differences between our genotype groups in frequencies of subjects who were naïve to psychostimulant treatment, those who had been prescribed psychostimulants in the past, or those who were taking psychostimulants near the time of scan but that were washed out for at least 24 hr (Table II).

Main Effect of Task on BOLD Signal Change

Across the entire group of 42 subjects in our contrast of interest (interference > control), we found significant activation in BILATERAL inferior parietal lobules and cerebellar hemispheres, LEFT lateral PFC, precentral gyrus, caudate and insula, and RIGHT fusiform gyrus.

fMRI Results by Genotype

Table III and Figure 2 show these results. Within the dACC ROI, a significant cluster (left dACC) indicated hypoactivity in the 10R/10R group compared to the 9R carriers. Whole volume exploratory analyses additionally suggested hypoactivity in right lateral PFC and

<table>
<thead>
<tr>
<th>TABLE I. Demographic and MSIT Performance Data for Genotype Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10R/10R</strong> (N = 19)</td>
</tr>
<tr>
<td>Left-handers</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Psychiatric comorbidities (lifetime history)</td>
</tr>
<tr>
<td>Multiple (≥2) anxiety disorders</td>
</tr>
<tr>
<td>Major depressive disorder</td>
</tr>
<tr>
<td>Alcohol abuse</td>
</tr>
<tr>
<td>Substance abuse</td>
</tr>
<tr>
<td><strong>10R/10R</strong> (N = 19)</td>
</tr>
<tr>
<td>Mean SD</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>IQ</td>
</tr>
<tr>
<td>WRAT—arithmetic</td>
</tr>
<tr>
<td>WRAT—reading</td>
</tr>
<tr>
<td>POMS—tension/anxiety</td>
</tr>
<tr>
<td>POMS—depression</td>
</tr>
<tr>
<td>Age ADHD onset</td>
</tr>
<tr>
<td>Inattentive symptoms, childhood</td>
</tr>
<tr>
<td>Hyperactive symptoms, childhood</td>
</tr>
<tr>
<td>Inattentive symptoms, recent</td>
</tr>
<tr>
<td>Hyperactive symptoms, recent</td>
</tr>
<tr>
<td>MSIT performance data</td>
</tr>
<tr>
<td>Neutral %</td>
</tr>
<tr>
<td>Neutral RT</td>
</tr>
<tr>
<td>Interference %</td>
</tr>
<tr>
<td>Interference RT</td>
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<tr>
<td>Interference RT—Neutral RT</td>
</tr>
</tbody>
</table>

IQ, estimated full scale IQ; WRAT, Wide Range Achievement Test; POMS, Profile of Mood States; RT, reaction time. All RTs in milliseconds. Due to computer error, MSIT performance data was lost for two 9R carriers.
left cerebellar vermis (though neither of these clusters survived correction for multiple comparisons when considering the entire search volume). The ANCOVA treating age as a covariate of no interest yielded only minimal differences from the two-sample t-test, most notably that the R PFC and L dACC findings dropped to trend level ($P = 0.052$ and 0.068, respectively).

No areas, either within the dACC ROI or the whole volume showed greater activation in 10R/10R subjects than 9R carriers.

Regarding the effect of allele load, one-way ANOVAs revealed significant differences in activation in left dACC, left cerebellar vermis, and right lateral PFC ($P < 0.01$) as expected. Post hoc Bonferroni corrected pair-wise comparisons of mean beta values in dACC and right lateral PFC showed significantly more activation in the 9R-heterozygous group versus 10R/10R ($P < 0.01$), but not for 9R/9R group versus 10R/10R ($P > 0.1$). In the left cerebellar vermis, the 10R/10R group showed significant hypoactivity compared to both the 9R groups ($P < 0.01$). In none of the regions did the 9R groups differ from each other. Although differences between the 9R/9R and 10R/10R groups did not reach statistical significance in dACC and lateral PFC, effect sizes were large (Cohen’s $d = 0.96$ and 0.94 for dACC and lateral prefrontal, respectively, and power was restricted due to the small n of the 9R/9R group), and subjects with one 9R allele had very similar levels of activation to 9R homozygous subjects (see Fig. 3). We were unable to find any areas in the entire volume which were significantly predicted by a linear dosing effect. This pattern of results is most consistent with the hypothesis that differences were due to homozygosity of the 10R allele and not to a dosing effect.

### TABLE II. Stimulant Medication History in Genotype Groups

<table>
<thead>
<tr>
<th></th>
<th>10R/10R (N = 19)</th>
<th>9R carriers (N = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Currently prescribed stimulants$^a$</td>
<td>8 (42%)</td>
<td>8 (35%)</td>
</tr>
<tr>
<td>Stimulants prescribed only in past</td>
<td>4 (21%)</td>
<td>4 (17%)</td>
</tr>
<tr>
<td>Stimulant naïve</td>
<td>7 (37%)</td>
<td>11 (48%)</td>
</tr>
</tbody>
</table>

$\chi^2(2) = 0.513, P = 0.77$.

$^a$Subjects prescribed stimulant medications near time of scan were washed out for at least 24 hr.

### TABLE III. Areas of Greater Activation in 9R-Carriers Than in Subjects With 10R/10R (Interference > Control Contrast)

<table>
<thead>
<tr>
<th>Cluster</th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>k (cluster extent)</th>
<th>T</th>
<th>z</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region of interest</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L dACC  (BA 24/32)$^a$</td>
<td>$-3$</td>
<td>$0$</td>
<td>$30$</td>
<td>26</td>
<td>3.54</td>
<td>3.28</td>
<td>0.036</td>
</tr>
<tr>
<td>Whole volume analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R lateral PFC  (BA 46)$^b$</td>
<td>$42$</td>
<td>$18$</td>
<td>$27$</td>
<td>28</td>
<td>3.50</td>
<td>3.25</td>
<td>0.049</td>
</tr>
<tr>
<td>L cerebellar vermis$^b$</td>
<td>$-6$</td>
<td>$-48$</td>
<td>$-6$</td>
<td>25</td>
<td>4.73</td>
<td>4.19</td>
<td>0.061</td>
</tr>
</tbody>
</table>

Coordinates are given in Montreal Neurological Institute (MNI) standard space.

$^a$Cluster defined by ROI analysis, P-value corrected for number of voxels across the entire bilateral ROI.

$^b$Clusters defined by whole-volume analysis, P-values are uncorrected.

**DISCUSSION**

In this study of adult subjects with childhood-onset ADHD, we found a significant effect of the SLC6A3 gene on activation in the dACC, as well as trend effects in the lateral PFC and cerebellar vermis. Specifically, homozygosity for the ADHD risk allele (10R), but not mere carriage of the allele, predicted hypoactivation in these areas on a cognitive interference task. These brain areas are all relevant to ADHD, implicated by structural [Seidman et al., 2006; Valera et al., 2007] and functional [Dickstein et al., 2006] neuro-imaging studies, as well as neurobiological theories of the disorder [Krain and Castellanos, 2006].

The findings are of interest not only because they potentially elucidate the neurofunctional consequences of an ADHD candidate gene variant in the ADHD brain, but also because they help us to understand how SLC6A3 may produce endophenotypic heterogeneity within an ADHD sample even in the context of tight comparability of the two genotype groups on a series of demographic and phenotypic variables, such as psychiatric comorbidities, neuro-cognition, and medication history (see Tables I and II). In addition, our findings extend to ADHD those of Bertolino et al. [2006], who found ACC hypoactivity in 10R/10R control subjects compared to their 9R counterparts. Finally, our results suggest that the effect of the 10R allele on dACC function acts in a recessive manner, and that two copies of the 10R allele are necessary to produce an unexpected pattern of function (deactivation during an interference task).

While it did not reach significance, we did find a trend for better performance in our 10R/10R group during the inhibition task. This is consistent with several behavioral studies which found better performance in 10R/10R versus 9R ADHD groups on measures of attention and executive function (EF) [Oh et al., 2003; Kim et al., 2006; Boonstra et al., 2008]. These findings are also consistent with the theory that hypoactivation represents a more “focused” engagement resulting from increased SNR in the 10R group [see Bertolino et al., 2006]. However, alternative explanations must also be considered as there is a literature suggesting no significant relationship between SLC6A3 and cognitive performance [see review in Rommelse et al., 2008], and a robust imaging literature linking the ADHD diagnosis itself with hypoactivity in fronto-striatal regions [see Dickstein et al., 2006]. It is possible that non-DAT1 effects across ADHD samples tip the scale towards less optimal functioning which compared to controls appears similar to the hypoactivity...
seen in 10R versus 9R controls, but that indeed is associated with less efficient cognitive functioning. Future studies should investigate the significance of hypoactivity in ADHD versus control groups (associated with less optimal functioning), and how its quality differs from the hypoactivity in 10R versus 9R genotype groups (associated with more optimal functioning).

Our imaging findings are particularly interesting when viewed next to those of Bush et al. [2008], who found that after a 6 week trial of MPH, dACC activation was increased during the MSIT in an adult ADHD sample independent of those reported in this article. Given that one of the primary mechanisms of action of MPH is to block DAT [Volkow et al., 1998], the two studies can be seen as congruent, with our 9R-carriers analogous to the post-MPH group. In other words, increased dACC activation may be related to decreased DAT functioning, regardless of whether it is a function of genotype or of pharmaceutical intervention. Although we found no difference between our genotype groups in medication history, other groups have indeed found that 9R-carriers have an increased likelihood of response to MPH [Kooij et al., 2008], findings which have been recently supported by meta-analysis [Purper-Ouakil et al., 2008]. Future pharmacogenetic studies should be conducted that directly investigate the interaction between genotype, dACC activation, and response to psychostimulant medication.

We have found one other study investigating the impact of gene variants on fMRI measures in ADHD [Durston et al., 2008]. Using a go no-go paradigm, Durston et al. found that SLC6A3 predicted activation differences, as our study did, in cerebellar vermis (as well as striatum) in ADHD subjects. However, their vermis results were in the opposite direction to ours. Such varying results might be
explained by differences in task demands and/or their use of a pediatric sample. Our findings are also novel in that to our knowledge it is the first imaging genetics study of ADHD in adults using fMRI, and that we probed and found differences in dACC, an area of much interest in ADHD.

The effect of the SLC6A3 genotype on cortical activation may be direct through synaptic effects on the relatively low amounts of DAT in these areas, indirect through striatal DAT effects on the cortex via thalamocortical pathways, through gene–gene interactions, or any combination of these factors. In contrast to Durston et al. [2008], we did not find any effects of SLC6A3 on striatum. Although the reasons for the discrepant findings are not clear, it could be due to differences between the adult and pediatric samples studied, considering that caudate volumes tend to normalize in ADHD in adolescence [Castellanos et al., 2002], and/or the fact that our task is not specifically designed to probe the striatum.

Given our previous findings of anatomical abnormalities in the ADHD ACC as compared to a control group [Seidman et al., 2006; Makris et al., 2007], it is possible that our findings were influenced by differences in ACC morphology, such as cortical thickness or volume. Since, however, no studies that we know of have examined the effect of SLC6A3 on ACC structure, the potential impact of structure on our findings is unknown. Future genetic imaging studies involving dopamine genotypes should investigate the effect of brain structure on functional outcomes.

The current results not only link specific brain areas previously found to be altered in ADHD with a known ADHD risk genotype, but also support the complex heterogeneity of the disorder, providing insight into the likely multiple pathways to ADHD. Hypo-activations in frontocerebellar pathways as a result of SLC6A3 10R homozygosity may characterize one of the several paths predisposing individuals towards ADHD. It may be that SLC6A3 is more closely linked to anterior cingulate network dysfunction (found in many psychiatric disorders) than to the ADHD phenotypic expression, and that previous association studies finding a link between SLC6A3 and ADHD may be more driven by associations with this network’s dysfunction in a subset of subjects than to the clinical phenotype of ADHD itself. Further investigation of the effects of SLC6A3 on anterior cingulate function in controls and other psychiatric populations will be important to delineate whether this link is specific to ADHD or not.

Notably, in our analysis of main effects of task we did not find that the L dACC or R PFC were significantly activated in the interference versus control condition. Even though the MSIT has been found to robustly activate dACC in individual control subjects [Bush et al., 2003], these findings were not very surprising given that dACC (as well as PFC and cerebellum) are frequently hypoactive during multiple tasks in subjects with ADHD [Bush et al., 1999; Rubia et al., 1999; Booth et al., 2005; Valera et al., 2005; Konrad et al., 2006]. As can be seen in Figure 3, however, it is evident that activation is in fact in the opposite direction in the two genotype groups. These results are of great interest as they suggest that averaging across SLC6A3 genotypes groups in ADHD samples may wash away effects seen in these areas.

It is also important to note that in the dACC, cerebellar vermis, and lateral PFC, the 9R carriers had more activation in the interference condition as compared to the control condition, whereas 10R/10R subjects had less activation in the interference condition as compared to the control condition (see Fig. 3). The lack of effect of SLC6A3 on activation in the control condition alone (results not shown) suggests that the variant likely impacts brain function associated with MSIT interference processes and/or baseline. Alternative methods of analysis should be employed to test the effect of SLC6A3 on “default-mode networks,” which have indeed found to be altered in samples with ADHD [Castellanos et al., 2008; Fassbender et al., 2009].

Limitations of our study include modest power, which likely accounts for the cerebellar and frontal findings not surviving correction for multiple comparisons despite large Cohen’s d effect size estimates (1.34 and 1.02, respectively). Given that classical association studies (including studies of this SLC6A3 polymorphism with ADHD) sometimes require hundreds of subjects for adequate power, these effect sizes are promising. Nonetheless, interpretations of statistically non-significant findings should be made with caution until demonstrated to be significant in larger samples. Future studies should test specific hypotheses in multiple ADHD ROIs including lateral PFC and cerebellum, as they are essential nodes of networks found to be altered in ADHD [Krain and Castellanos, 2006]. Further, even though genotypes did not differ in terms of ADHD medication history, we would advise future replication using a medication-naïve sample given the effect of psychostimulants on both DAT and ACC, and the unknown effect of previous psychopharmacological treatment on brain function. Finally, as mentioned in the methods, since our primary hypothesis was regarding an effect in the ACC, we did not acquire data from the anterior or posterior poles. Therefore, we were unable to determine if any effect of SLC6A3 is present in these areas.

As mentioned above, our sample had a wide age range, potentially confounding our data with multiple stages of brain development. When we ran the ANCOVA removing variance associated with age, our significant findings did drop to trend level. Given that our results were just above trend level before removing the effects of age, we suggest the P-value reduction was likely due to a reduction of power by adding the additional covariate into the model, rather than to an actual effect of age. Future studies with better power should examine the effect of ageing on genotype effects.

Because this study is limited to only subjects with ADHD, we were unable to determine how the genotype effect we found is comparable to that seen previously in a healthy control sample. It is therefore possible that the DAT1 effect we found on interference processing is limited only to samples with ADHD, or that the magnitude of the effect would be greater or lesser than in a control sample. Because Schott et al. [2006] found effects in the same direction (less signal change in 10R/10R) with a control group during an episodic memory task, as did Bertolino et al. with a working memory task [2006], we expect that at least the directionality during the MSIT task would be similar in a control sample to what we found in the ADHD sample. Future studies should include healthy controls and comparison psychiatric groups to test how the magnitude of this effect might differ between diagnoses.

Despite these considerations, our findings suggest that activation of dACC, lateral PFC, and cerebellar vermis, all component parts of attentional and EF networks affected in ADHD, are influenced by SLC6A3 genotype. These data help to elucidate the neurofunctional
consequences of a risk gene in ADHD, and how this variation may produce endophenotypic heterogeneity within the disorder.

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