Association of the Dopamine Transporter (*SLC6A3/DAT1*) Gene 9–6 Haplotype With Adult ADHD

B. Franke,[1,2]* M. Hoogman,[1] A. Arias Vasquez,[1,2,3] J.G.A.M. Heister,[2] P.J. Savelkoul,[1] M. Naber,[2] H. Scheffer,[2] L.A. Kiemeney,[3] C.C. Kan,[1] J.J.S. Kooij,[4] and J.K. Buitelaar[1]

[1]*Department of Psychiatry, Radboud University Nijmegen Medical Centre, Donders Institute for Brain, Cognition and Behavior, Nijmegen, The Netherlands*
[2]*Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands*
[3]*Department of Epidemiology & Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands*
[4]*PsyQ, Psycho-Medical Programs, The Hague, The Netherlands*

ADHD is a neuropsychiatric disorder characterized by chronic hyperactivity, inattention and impulsivity, which affects about 5% of school-age children. ADHD persists into adulthood in at least 15% of cases. It is highly heritable and familial influences seem strongest for ADHD persisting into adulthood. However, most of the genetic research in ADHD has been carried out in children with the disorder. The gene that has received most attention in ADHD genetics is *SLC6A3/DAT1* encoding the dopamine transporter. In the current study we attempted to replicate in adults with ADHD the reported association of a 10–6 *SLC6A3*-haplotype, formed by the 10-repeat allele of the variable number of tandem repeat (VNTR) polymorphism in the 3′ untranslated region of the gene and the 6-repeat allele of the VNTR in intron 8 of the gene, with childhood ADHD. In addition, we wished to explore the role of a recently described VNTR in intron 3 of the gene. Two hundred sixteen patients and 528 controls were included in the study. We found a 9–6 *SLC6A3*-haplotype, rather than the 10–6 haplotype, to be associated with ADHD in adults. The intron 3 VNTR showed no association with adult ADHD. Our findings converge with earlier reports and suggest that age is an important factor to be taken into account when assessing the association of *SLC6A3* with ADHD. If confirmed in other studies, the differential association of the gene with ADHD in children and in adults might imply that *SLC6A3* plays a role in modulating the ADHD phenotype, rather than causing it.   © 2008 Wiley-Liss, Inc.

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INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is among the most heritable and common behavioral disorders in childhood. It affects ∼5% of school-age children. At least 65% of patients retain ADHD symptoms into adulthood, with at least 15% of them showing syndromal persistence [Faraone et al., 2006]. The influence of familial (possibly genetic) factors on ADHD etiology appears to be stronger in adults than in children [Faraone et al., 2000].

One of the most extensively studied genes in childhood ADHD is *SLC6A3/DAT1* encoding the dopamine transporter. A 40 base pair (bp) variable number of tandem repeats (VNTR) polymorphism in the 3′ untranslated region (UTR) of the gene has received most attention. Meta-analysis of both family-based and case–control association studies in children with ADHD has indicated overrepresentation of the 10-repeat allele of the VNTR in ADHD [Faraone et al., 2005]. More recent meta-analyses have challenged this view, and have suggested heterogeneity in the findings [Todd et al., 2005; Li et al., 2006; Yang et al., 2007].

Recent work by us and others indicates that the 10-repeat allele of the 3′ UTR VNTR might only increase ADHD risk in children in a haplotype with allele 6 of a VNTR in intron 8 of the gene [Brookes et al., 2006a; Asherson et al., 2007]. Both, the 3′ UTR VNTR and the intron 8 VNTR have been suggested to influence the expression of *SLC6A3*. However, there is inconsistency in the findings of in vitro and in vivo studies investigating allele-specific expression [Spencer et al., 2005; Brookes et al., 2007]. This may suggest that neither VNTR is functional by itself, but (incompletely) tags an unknown functional site. It is likely that the haplotype of both VNTRs improves tagging of this functional variant.

A first study of the two *SLC6A3* VNTRs in adults with ADHD, though of limited samples size, could not confirm the association of the 10–6 haplotype with the persistent form of the disorder [Bruggemann et al., 2007]. In the current study we, again, attempted to replicate the haplotype association findings from childhood ADHD in a second sample of adult ADHD patients and also explored the role of a recently described additional VNTR in intron 3 of *SLC6A3* [Mijyajima et al., 2006].

MATERIALS AND METHODS

Participants

Patients (n = 216) had been referred for assessment of ADHD to the outpatient clinic of GGZ Delfland in Delft, to

Parnassia, psycho-medical centre in The Hague, or to the department of Psychiatry at the Radboud University Nijmegen Medical Centre in Nijmegen, The Netherlands. Subjects were included if a clinical diagnosis of adult ADHD with childhood onset was established. Part of the patients has been described before [Bekker et al., 2005; Kooij et al., 2005].

Controls (n = 528) were obtained from the Nijmegen Biomedical Study (NBS, www.nijmegenbiomedischestudie.nl), a population-based survey conducted by the Departments of Epidemiology & Biostatistics and of Clinical Chemistry of the Radboud University Nijmegen Medical Centre [Hoogendoorn et al., 2006]. The control group was frequency-matched for gender with the patient group.

Patients and controls were of Caucasian ethnic background. The study was approved by regional medical ethics committees. All participants completed written informed consent.

Diagnostic Assessments

Prior to inclusion, all patients underwent a standard clinical assessment consisting of a psychiatric evaluation by experienced psychiatrists using a semi-structured diagnostic interview for ADHD and comorbid disorders, the Dutch version of structured diagnostic interviews for retrospective diagnosis of childhood onset ADHD and current symptoms. For current ADHD symptoms during the last 6 months, also a Dutch version of the DSM-IV ADHD Rating Scale, based on the 18 DSM-IV items for ADHD, was used [DuPaul et al., 1998; Kooij et al., 2005]. The ADHD Rating Scale has been used in epidemiologic and clinical research in adults in the United States and in The Netherlands [Murphy and Barkley, 1996; Kooij et al., 2005]. To be given a full diagnosis of adult ADHD, subjects had to (A) meet at least 6 out of 9 DSM-IV criteria of inattention and/or hyperactivity/impulsivity for a diagnosis of ADHD in childhood and at least 5 out of 9 criteria in adulthood, (B) describe a chronic persisting course of ADHD symptoms from childhood to adulthood, and (C) endorse a moderate to severe level of impairment attributed to the ADHD symptoms. A cut-off point of 5 of 9 criteria was set for adult diagnosis of ADHD based on literature and epidemiological data using the same DSM-IV ADHD Rating Scale [Kooij et al., 2005]. In order to obtain information about lifetime ADHD symptoms and impairment, the patient, the partner and, if available, the parents were interviewed. Information on school reports was examined in order to sustain the diagnosis in childhood. Diagnostic criteria have been described in more detail elsewhere [Bekker et al., 2005; Kooij et al., 2005]. Table I shows the demographics of the patient sample. Controls were also screened for presence of current ADHD symptoms using the DSM-IV ADHD Rating Scale [Kooij et al., 2005] and were excluded if 4 or more symptoms were present.

Genotyping and Statistical Analysis

Genotyping of the VNTRs in the 3′ UTR and intron 8 has been described earlier [Brookes et al., 2006b; Boonstra et al., 2008]. The 63 base pair (bp) VNTR in intron 3 of the gene was genotyped using a PCR-based method on 62.5 ng genomic DNA using 0.4 μM of forward (5′-GAAGTTGGCTGGTTGGTGAG-3′) and reverse primer (5′-ACCAGAGTCCCCCTTACCAA-3′), respectively, 0.25 mM dNTPs and 0.5 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands) in a buffer containing 60 mM Tris–HCl pH 9.5, 15 mM NH₂SO₄ and 2 mM MgCl₂. The cycling conditions for amplification involved 5 min at 92°C, followed by 35 cycles of 1 min 92°C, 1 min at 61°C and 1 min 72°C and an extra 10 min at 72°C. Analysis on a 2% agarose gel yielded distinct bands at 596 and 659 bp for the most common

Characteristics	n	Percentage
Number of subjects	216	
Age (mean and range)	36.0	18–62
Males	105	48.6
Marital status		
Married/relationship	129	59.7
Single/divorced/living with parents	84	38.9
Unknown	3	1.4
Educational level		
Lower educational level	56	25.9
High school	141	65.3
University	15	6.9
Unknown	4	1.9
DSM IV axis I disorder		
ADHD combined type	187	86.6
ADHD hyperactive/impulsive type	8	3.7
ADHD inattentive type	21	9.7
Any comorbid axis I disorder[a]	177	81.9
Multiple (≥ 2) comorbid axis I disorders[a]	91	42.1
Any mood disorder[a]	129	59.7
Any anxiety disorder[a]	74	34.3
Any substance use disorder[a]	49	22.7
Bulimia Nervosa[a]	9	4.2
Co-morbid Borderline Personality Disorder[b]	35	17.9

TABLE I. Patient Characteristics

[a]Past or present.
[b]Data available for 206 subjects.

alleles 7 and 8. Generally, all three genotyping assays have been validated earlier and 5% duplicates and blanks were taken along as quality controls during genotyping. Hardy–Weinberg equilibrium (HWE) was assessed for all genotypes in all available samples using a χ^2 statistic. Only the most common alleles/genotypes were taken into account in the haplotype analysis and the tests of association with ADHD. Genotype frequencies of the *SLC6A3* polymorphisms were compared between cases and controls using a χ^2 test. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using SPSS (version 14.0). *SLC6A3* haplotypes of the 3′ UTR and intron 8 (and intron 3) VNTRs with ADHD were estimated using the haplo.em function implemented in the haplo.stats package [Schaid et al., 2002], which computes maximum likelihood estimates of haplotype probabilities, together with posterior probabilities of haplotype pairs for each subject. Haplotype association analyses were done using the haplo.score function implemented in haplo.stats. Briefly, this package computes score statistics to test associations between haplotypes and a trait, and allows adjustment for other determinants. This analysis was corrected for multiple testing by applying the simulate = TRUE parameter in haplo.score which gives simulated p values. These simulated haplotype score statistics are calculated from a permuted re-ordering of the trait (ADHD status) and *SLC6A3* polymorphisms. We used 1,000 permutations for all the analyses.

RESULTS

Genotype distributions of all three polymorphisms were in Hardy–Weinberg equilibrium ($P > 0.05$). The overall haplotype distributions were significantly different in cases and controls. We found global evidence for association of *SLC6A3* with adult ADHD (haplo.score global $P = 0.01$). This difference was not explained by the 10–6 haplotype, but rather by the 9–6 haplotype which was significantly more frequent in cases than in controls (haplotype-specific $P = 0.001$) (Table II). Inclusion

TABLE II. Genotype Analysis of the 3′ UTR and Intron 8 and 3 VNTRs of the *SLC6A3* Gene and Haplotype Analysis for the 3′ UTR and Intron 8 VNTRs

Genotypes	Frequency (%)		Pearson χ^2 *P*-value	OR	95% CI
	Controls	Cases			
3′ UTR					
10/10	294 (58.0)	108 (50.7)	0.085	1	
10/9	191 (37.7)	89 (41.8)		1.27	0.91–1.8
9/9	22 (4.3)	16 (7.5)		1.98	1.00–3.91
Intron 8					
6/6	306 (60.4)	128 (62.7)	0.731	1	
6/5	178 (35.1)	69 (33.8)		0.93	0.66–1.31
5/5	23 (4.5)	7 (3.4)		0.73	0.30–1.74
Intron 3					
7/7	336 (63.9)	139 (65.0)	0.809	1	
7/8	176 (33.5)	71 (33.2)		0.975	0.695–1.369
8/8	14 (2.7)	4 (1.9)		0.691	0.224–2.135
Haplotypes*	Frequency (%)		Score test *P*-value	OR	95% CI
	Controls	Cases			
10–6	70.0	66.2		1	
9–5	16.5	16.3	0.99138	1.06	0.77–1.46
9–6	6.4	11.7	0.00106	2.05	1.35–3.1
10–5	4.6	3.2	0.18033	0.74	0.39–1.42

Four haplotypes were found at frequencies above 1%, representing 97.4% of all haplotypes in the sample.
*Haplo.score global *P* = 0.01.

of the intron 3 VNTR did not change the results (data not shown).

DISCUSSION

Although the *SLC6A3/DAT1* gene is the best-studied gene in childhood ADHD, only few studies have looked at this gene in adult ADHD genetics research. Only one earlier study has tried to replicate the 10–6 haplotype association with childhood ADHD in adult patients. This study, in 122 adult patients and 174 controls, did not find association with *SLC6A3*, neither with the 10–6 haplotype, nor with any other haplotype formed by the 40 bp VNTR polymorphism in the 3′ untranslated region of the gene and the 30 bp VNTR in intron 8 [Bruggemann et al., 2007]. Using a larger sample, our data also suggest a lack of association with 10–6, but we find the adult disorder to be associated with the 9–6 haplotype in *SLC6A3*. Our findings converge with recent data of the 3′ UTR VNTR from a prospective 13-year follow-up study in 147 young adults with ADHD and 73 controls, indicating that more ADHD symptoms and externalizing behaviors were present in the 9/10 than in the 10/10 genotype for the group as a whole [Barkley et al., 2006]. Interestingly, the effects of the genotype became more pronounced with increasing age of the participants. More individuals with a DSM diagnosis of ADHD in adulthood were also found among those having the 9/10 genotype (53%) than among the 10/10 homozygous group (35%) [Barkley et al., 2006]. Our own neuropsychological studies in a subset of 45 adults with ADHD from the current study also support the current findings in that the heterozygous 10-allele carriers (mostly 9/10) were shown to have slower inhibition during the Change task [Boonstra et al., 2008]. Similar results were recently reported in 75 young healthy adults [Caldu et al., 2007].

Rather than age being the factor modifying the association of *SLC6A3* with ADHD, geographical origin might form an alternative explanation for the results of the current study. However, we do not think that this is the cause of our findings, given our recent findings within the IMAGE study, in which we were able to confirm the association of the 10–6 haplotype with childhood ADHD [Asherson et al., 2007]. This sample contains more than 300 families from The Netherlands, who also show the expected overtransmission of the 10–6 haplotype to affected offspring, when analyzed separately from all other samples (unpublished results).

Of course, our findings of the *SLC6A3* 9–6 haplotype association with adult ADHD are in need of replication, before we can judge if the association is really true. Actually, although *SLC6A3* has been the subject of numerous studies in children, the evidence for the involvement of the gene in ADHD is still rather slim. Meta-analyses of the 3′ UTR VNTR have suggested association of either of the two common alleles (10-repeat and 9-repeat) or neither allele with the disorder and related phenotypes [Faraone et al., 2005; Todd et al., 2005; Li et al., 2006; Yang et al., 2007]. Even in those meta-analyses that did show association with ADHD, the significance of the findings was limited and far from reaching genome-wide levels of significance, though large numbers of patients were included. Clinical heterogeneity and environmental factors—but also age—might be important factors to take into account when assessing the association of *SLC6A3* with ADHD.

In conclusion, if confirmed in other studies, our data bear the intriguing suggestion that the *SLC6A3* haplotype associated with ADHD in adults is different from the one associated with the childhood disorder. Since dopamine transporter density decreases during life [Spencer et al., 2005] and ADHD symptoms are known to change during adolescence [Biederman et al., 2000], the differential association of *SLC6A3* with ADHD might reflect changing requirements on the dopaminergic system during life. Furthermore, regulation of the dopamine transporter is influenced by environmental factors like smoking, ethanol and several drugs [Madras et al., 2005], that are used more often by adults than by children. A differential association of the *SLC6A3* gene with ADHD in children and in adults might imply that the gene plays a role in modulating the ADHD phenotype, rather than causing it.

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