Multicenter Analysis of the *SLC6A3/DAT1* VNTR Haplotype in Persistent ADHD Suggests Differential Involvement of the Gene in Childhood and Persistent ADHD

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Attention deficit/hyperactivity disorder (ADHD) is one of the most common neuropsychiatric disorders with a worldwide prevalence around 4–5% in children and 1–4% in adults. Although ADHD is highly heritable and familial risk may contribute most strongly to the persistent form of the disorder, there are few studies on the genetics of ADHD in adults. In this paper, we present the first results of the International Multicentre Persistent ADHD Genetics Collaboration (IMpACT) that has been set up with the goal of performing research into the genetics of persistent ADHD. In this study, we carried out a combined analysis as well as a meta-analysis of the association of the *SLC6A3/DAT1* gene with persistent ADHD in 1440 patients and 1769 controls from IMpACT and an earlier report. *DAT1*, encoding the dopamine transporter, is one of the most frequently studied genes in ADHD, though results have been inconsistent. A variable number tandem repeat polymorphism (VNTR) in the 3'-untranslated region (UTR) of the gene and, more recently, a haplotype of this VNTR with another VNTR in intron 8 have been the target of most studies. Although the 10/10 genotype of the 3'-UTR VNTR and the 10-6 haplotype of the two VNTRs are thought to be risk factors for ADHD in children, we found the 9/9 genotype and the 9-6 haplotype associated with persistent ADHD. In conclusion, a differential association of *DAT1* with ADHD in children and in adults might help explain the inconsistencies observed in earlier association studies. However, the data might also imply that *DAT1* has a modulatory rather than causative role in ADHD. *Neuropsychopharmacology* (2010) **35**, 656–664; doi:10.1038/npp.2009.170; published online 4 November 2009

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INTRODUCTION

Attention deficit/hyperactivity disorder (ADHD) is one of the most common neuropsychiatric disorders in children worldwide. The prevalence of the disorder in children is estimated to be 4–5% (Polanczyk *et al*, 2007). Although ADHD is classically considered a disorder of children and adolescents, only a subset of affected individuals remit (Faraone *et al*, 2000; Faraone *et al*, 2006), and the prevalence in adults lies between 1 and 4% (Kessler *et al*, 2006; Polanczyk *et al*, 2007; Kooij *et al*, 2005). ADHD in adults causes a considerable burden to patients, their families, and society as a whole (Kessler *et al*, 2005a; Goodman, 2007; Bernfort *et al*, 2008). Adult patients have difficulties in social, educational, and professional fields, such as developing and maintaining stable social relationships, completing educational programs, and holding jobs.

Many twin and adoption studies have shown that ADHD symptoms in children are highly heritable, with about 76% contribution of heritable factors to phenotypic variance (Faraone *et al*, 2005). Although the heritability of the adult form of ADHD has not been formally established, the contribution of familial factors to this form of the disorder may be even larger than to childhood ADHD (Faraone, 2004). Numerous molecular genetic studies have been carried out to identify the genetic risk factors for ADHD. This has resulted in a limited number of significant and replicated findings, but all with modest effect sizes (for review see Faraone *et al*, 2005; Li *et al*, 2006) and explaining only a very small part of the genetic contribution to the disorder. Surprisingly, most molecular genetic studies report on children with ADHD, persistent ADHD has been largely neglected in genetics research so far.

In 2007, the International Multicentre Persistent ADHD CollaboraTion (IMpACT) was formed by researchers participating in the ADHD Molecular Genetics Network (Faraone, 2003; 2002). Currently, research groups from Germany, Norway, Spain, The Netherlands, the United Kingdom, and the United States participate in IMpACT. The collaboration was set up with the goal of performing and promoting research into the genetics of persistent ADHD. This publication presents its first results.

SLC6A3/DAT1, encoding the dopamine transporter, is probably the most studied gene in ADHD. The transporter is the direct target of stimulant-based medication effective in treating ADHD symptoms (Medori *et al*, 2008; Faraone *et al*, 2004). The polymorphism identified as a risk factor for ADHD is a variable number of tandem repeat (VNTR) in the 3'-untranslated region (UTR) of the gene. Since the original publication in 1995 (Cook *et al*, 1995), many studies have investigated the association of this VNTR with ADHD, with variable results. Meta-analyses of the data have also not been consistent, with the most comprehensive ones showing little or no significant effect (Maher *et al*, 2002; Faraone *et al*, 2005; Todd *et al*, 2005; Yang *et al*, 2007; Li *et al*, 2006). Because of the number of positive reports and because of significant evidence of heterogeneity between data sets, systematic differences between data sets might explain the apparent discrepancies (Li *et al*, 2006). Recently, two studies have suggested that a haplotype of two VNTRs in *DAT1*, including the 3'-UTR VNTR and a VNTR in intron 8, is more strongly associated with ADHD than the 3'-UTR

VNTR alone (Asherson *et al*, 2007; Brookes *et al*, 2006). Recently, two studies also tested the involvement of the VNTR haplotype in persistent ADHD: whereas one of them did not find an association with the disorder (Bruggemann *et al*, 2007), the other concluded that the 9–6 haplotype, which differs from the 10–6 haplotype associated with childhood ADHD, has a role in the persistent disorder (Franke *et al*, 2008).

To resolve the apparent discrepancies in the literature, we performed a meta-analysis of published data and unpublished data from the IMpACT samples to further investigate the association between the *DAT1* VNTR haplotype and persistent ADHD.

MATERIALS AND METHODS

The study reported here has been carried out in accordance with the Declaration of Helsinki.

Patients and Controls, Assessment of Psychopathology at the IMpACT Nodes

All patients were evaluated by experienced psychiatrists and diagnosed with persistent ADHD according to DSM-IV (Diagnostic and Statistical Manual for Mental Disorders) criteria. Consensus eligibility criteria for this study across all study sites were a diagnosis of ADHD according to the diagnostic criteria of DSM-IV, onset before the age of 7 years by retrospective diagnosis (which was confirmed by a family member, wherever possible), life-long persistence and current diagnosis. Most controls were screened for the presence of ADHD too (see Supplementary File S1 for more detailed information). All subjects were of Caucasoid origin. Diagnosis was blind to genotype. A detailed description of the samples, instruments, and procedures used by the different sites is provided in Supplementary File S1 and Supplementary Table S1. In total, 1525 patients and 1711 controls are part of IMpACT. Studies were approved by the ethics committees of the participating institutions, and written informed consent was obtained from all patients and controls. Genotyping data for both *DAT1* VNTRs were available for 421 patients and 405 controls from IMpACT Germany, for 450 patients and 548 controls from IMpACT Norway, IMpACT Spain contributed 264 patients and 195 controls, and from IMpACT The Netherlands 269 patients and 532 controls with complete genotyping data were available. The total numbers of genotyped cases and controls were 1404 and 1680, respectively (see Table 1).

Genotyping of the Two *DAT1* VNTRs

Genotyping of the 40 bp VNTR located in the 3'-UTR of *DAT1* had been carried out earlier at the different IMpACT sites. Procedures and/or references can be found in Supplementary File S1. As part of quality control, all four sites each sent 24 DNA samples to Norway for genotyping of the *DAT1* 3'-UTR repeat, according to the high-resolution method used in Norway (see Supplementary File S1). Samples were dispensed to one common 96-well plate, and the operator was blinded to the sample ID and country of origin of the samples. Genotyping concordance between tests was 100% for all 96 samples. The intron 8 VNTR was

Table 1 Numbers of Cases and Controls Successfully Genotyped for both VNTRs in *DAT1*

	Cases	Cases included in analysis	Controls	Controls included in analysis
<i>Samples from the International Multicentre Persistent ADHD CollaboraTion (IMpACT)</i>				
Germany	421	406	405	393
Norway	450	432	548	530
Spain	264	249	195	184
Netherlands	269	238	532	491
Combined subtype	928 (70.0%)			
Inattentive subtype	256 (19.3%)			
Hyperactive/impulsive subtype	66 (5.0%)			
Unknown	75 (5.7%)			
<i>Other samples</i>				
Germany (26)	116	115	174	171
Total number of samples in this study	1520	1440	1854	1769

Only samples with common alleles were included in the analysis. The subtyping data refer to the samples from IMpACT included in the analysis only.

genotyped in The Netherlands (Norwegian, Spanish, and Dutch samples) or Germany according to the protocol used by the Dutch IMpACT partner (see Supplementary File S1).

Statistical Analysis

Hardy–Weinberg equilibrium (HWE) was assessed for all available samples using the Markov Chain Monte-Carlo approximation of the exact test implemented in the GENEPOP package V 3.3 (Raymond and Rousset, 1995), and genotype distributions were consistent with HWE for both polymorphisms in all four samples ($p > 0.01$). Before haplotype estimation, the VNTRs were recoded, lumping all rare alleles into one group, so that three alleles for the 3'-UTR VNTR (ie, the 9-repeat and 10-repeat alleles plus a rare alleles pool), and three alleles for the intron 8 VNTR (ie, the 5-repeat and 6-repeat alleles plus a rare alleles pool) were considered. Haplotypes 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 frequencies are shown in Table 2. In the further analysis, we only considered the four most common haplotypes, ie, 10-6, 9-6, 9-5, and 10-5. All haplotypes included in the analysis had a posterior probability of 97% or higher.

A combined analysis was carried out including the samples of IMpACT only. A trend test was used to evaluate the ADHD risk conferred by carrying the 9-6 haplotype using basic χ^2 and logistic regression tests. We focused on the 9-6 haplotype because this had been implicated by one of our earlier studies (Franke *et al*, 2008). The effect of the 9/9 genotype vs all other genotypes was also tested using a χ^2 -test. These tests used SPSS (version 16.0).

The meta-analysis examined the haplotype association of the 9-6 allelic combination with the risk of ADHD relative to all other alleles in the entire sample, and in a subsample of

patients with combined or hyperactive/impulsive ADHD subtype. In addition, allelic and genotypic ORs were calculated for the two VNTRs, separately, with the most common allele or genotype as the reference.

To combine the individual study results, we conducted meta-analyses using RevMan (version 5.0.2) (The Cochrane Collaboration, 2008). The heterogeneity between studies was tested using the Q-statistic (Lau *et al*, 1997; Fleiss, 1981). Inconsistency across studies was quantified with the I^2 metric ($I^2 = Q - df/Q$) (Zintzaras and Hadjigeorgiou, 2004). When no heterogeneity was present, the pooled OR was estimated using fixed effects model (Mantel and Haenszel, 1959). Otherwise, random effects model (DerSimonian and Laird, 1986) were applied to obtain the pooled OR (Whitehead, 2002). The results of the association tests are indicated as pooled ORs with the corresponding 95% confidence intervals (CIs) of the haplotype-/allele- or genotype-induced risk of persistent ADHD. $P < 0.05$ was considered statistically significant.

RESULTS

Within the IMpACT group, 1404 persistent ADHD patients and 1680 controls, in which both VNTRs had been genotyped, were considered for inclusion in the study (Table 1). Supplementary Table S2 shows the characteristics of the IMpACT patient samples.

The haplotype frequencies in the different samples are shown in Table 2. Of the total number of patients and controls, we included only those individuals that carried the four most common haplotypes for both VNTRs (ie, those containing the 9-repeat or 10-repeat allele of the 3'-UTR VNTR and the 5-repeat or 6-repeat allele of the intron 8 VNTR). This led to the exclusion of 80 patients and 85 controls (Table 1). Haplotype frequencies varied among countries, with the 9-6 allele being more frequent in the

Table 2 Frequencies of Haplotypes per Country (Haplotypes Coded as 'Other' Consist of a Mixture of Rare Haplotypes)

	IMpACT Germany	IMpACT Norway	IMpACT Spain	IMpACT The Netherlands	Germany (from the study by Bruggemann et al, 2007)
10-6 (%)					
Cases	68.7	65.5	59.6	63.9	68.1
Controls	68.7	67.9	60.5	69.2	71.1
Total	68.7	66.8	60.0	67.5	69.9
9-5 (%)					
Cases	17.5	19.0	17.7	15.5	17.2
Controls	17.1	21.0	20.9	16.1	15.4
Total	17.3	20.1	19.1	15.9	16.1
9-6 (%)					
Cases	9.2	9.0	15.5	11.1	10.5
Controls	9.8	4.9	13.2	6.4	9.1
Total	9.5	6.8	14.5	7.9	9.6
10-5 (%)					
Cases	2.8	4.0	4.4	3.8	3.7
Controls	3.0	4.6	2.6	4.5	3.6
Total	2.9	4.3	3.6	4.3	3.6
Other (%)					
Cases	1.8	2.4	2.8	5.8	0.5
Controls	1.5	1.7	2.8	3.9	0.9
Total	1.6	2.0	2.8	4.5	0.7

Table 3 Analysis of the Association of the 9-6 Haplotype Formed by the 3'-UTR and Intron 8 VNTRs of the *SLC6A3* Gene vs all Other (frequent) Haplotypes

Number of 9-6 alleles	Frequency (%)		Pearson's χ^2 p-value ^a	OR ^b	95% CI
	Controls	Cases			
0	1370 (85.7)	1062 (80.2)	20.363	1.5	1.25–1.79
1	221 (13.8)	244 (18.4)			
2	7 (0.4)	19 (1.4)			
Total	1598 (100)	1325 (100)			

^adf = 2.^bLogistic regression analysis.

Spanish sample compared with the other Northern European samples (Table 2).

We first performed a combined analysis in the IMpACT sample only. In a sample of 1325 patients and 1598 controls, we evaluated if there was a difference in the distribution of the 9-6 haplotype between cases and controls. As shown in Table 3, this haplotype indeed was significantly more frequent in the cases ($\chi^2 = 20.36$; df = 2; $p < 0.001$). The allelic trend test showed a risk increase of 1.5 (95% CI 1.25–1.79)

for carrying a 9-6 haplotype. Interestingly, an analysis of the 9/9 genotype vs all other genotypes showed essentially the same result (9/9 homozygotes = 99 (6.2%) in controls and 119 (9.0%) in cases; $\chi^2 = 8.15$; df = 1; $p = 0.005$; OR = 1.5, 95% CI 1.13–1.97).

Given the differences in haplotype frequencies between samples (Table 2), we considered a meta-analysis design more appropriate than a combined analysis for further analysis. In addition to the IMpACT samples, we also

included data from another published report on the *DAT1* VNTR haplotype in adults (Bruggemann *et al*, 2007) into this meta-analysis, which increased the total number of genotypes included further to 1440 patients with adult ADHD and 1769 controls. Meta-analysis of the data using a random effects model showed that the 9-6 haplotype was significantly associated with ADHD in adults, with an OR of 1.39 (95% CI 1.03–1.88), $p = 0.03$ (Figure 1). As *DAT1* has been suggested to be more relevant for those ADHD subtypes including hyperactivity (Diamond, 2007), we repeated the analysis excluding patients with inattentive subtype ADHD from the four IMPACT samples (see Table 1 for the numbers of samples included). As shown in Figure 2, the point estimate for the OR increased somewhat numerically, but nonsignificantly (OR 1.47, 95% CI 1.02–2.12).

We also analyzed the two VNTRs, separately, in the samples included in the haplotype analysis. For the VNTR in the *DAT1* 3'-UTR the homozygous 10/10 genotype, which is thought to be the risk factor for ADHD in children, did not show association with ADHD in adults (OR = 0.93, 95% CI 0.93–1.07) (Figure 3a). However, as in the combined analysis, we did observe an association of the homozygous 9/9 genotype with persistent ADHD (Figure 3b), with an effect size similar to the one observed for the 9-6 haplotype (OR 1.34, 95% CI 1.03–1.76), $p = 0.03$. The intron 8 VNTR by itself did not have any effect on ADHD risk in the adults (Supplementary Figure S1A–C).

DISCUSSION

In this study we investigated two VNTRs within the *SLC6A3/DAT1* gene and the haplotypes formed by them for association with persistent ADHD. The study included genotype information on 1440 patients, of whom 1100 were formerly unpublished ones from the IMPACT study group. Both the 9-6 haplotype (3'-UTR VNTR/Intron 8 VNTR) and the 9/9 genotype of the 3'-UTR VNTR showed association with the disorder in adults using two different analysis methods, ie, a combined analysis and a meta-analysis design. The intron 8 VNTR by itself did not seem to increase persistent ADHD risk.

The two VNTRs within *SLC6A3/DAT1* have both been suggested to influence the regulation of the gene (Brookes *et al*, 2007; Spencer *et al*, 2005; Guinda-Lini *et al*, 2006). However, *in vivo* and *in vitro* studies for the 3'-UTR VNTR have not been consistent, and the intron 8 VNTR has so far only been studied once. It might therefore also be possible that—instead of being directly involved in regulating gene expression—both VNTRs (incompletely) tag an unknown functional site, with the haplotype increasing the efficiency of the tagging (Asherson *et al*, 2007).

The finding of association with persistent ADHD for the 9-6 haplotype supports an earlier report in the Dutch IMPACT subsample (Franke *et al*, 2008). This finding, as well as the finding that the 9/9 3'-UTR VNTR genotype is associated with persistent ADHD, is contrary to findings in

Figure 1 Forest plot showing the analysis of the 9-6 VNTR haplotype vs all other haplotypes.

Figure 2 Forest plot showing the analysis of the 9-6 haplotype vs all others for patients with ADHD subtypes containing hyperactivity (combined subtype and hyperactive/impulsive subtype) only.

Figure 3 (a) Forest plot showing the analysis of the *DAT1* 3'-UTR VNTR 10/10 genotype vs all other genotypes. (b) Forest plot showing the analysis of the *DAT1* 3'-UTR VNTR 9/9 genotype vs all other genotypes.

children with ADHD, where the 10-6 haplotype and the 10/10 genotype have been suggested to be risk factors for the disorder (Brookes *et al*, 2006; Asherson *et al*, 2007; Faraone *et al*, 2005). This is not likely to reflect a mere population-specific effect, as the populations represented in this adult study are also represented (apart from the Norwegian sample) in the studies showing the effects of the 10-6 haplotype (eg, in the IMAGE study; Asherson *et al*, 2007). A major difference between most samples of childhood and adult ADHD is the gender distribution. Although in children the male to female ratio lies between 3:1 and 9:1 (Staller and Faraone, 2006), the gender distribution is much more equal in adults with ADHD (Kessler *et al*, 2006). However, it is unlikely that gender causes the differences in the findings for children and adults, as a male-specific analysis in this study did not change the results presented here (not shown). Another difference between samples of children and adults may be the comorbidity profile. Although comorbidity is the rule rather than the exception in both groups of patients, the prevalence of specific comorbid disorders may differ. This issue is further discussed below.

Despite an impressive number of studies already performed, the role of *DAT1* in ADHD, even in children, is still far from clear. The 3'-UTR VNTR has been investigated in multiple studies, but meta-analyses of the genotyping data are controversial (Maher *et al*, 2002; Faraone *et al*, 2005; Todd *et al*, 2005; Yang *et al*, 2007; Li *et al*, 2006). The same holds true for the current findings: although our meta-analysis included 1440 patients and 1854 controls, the

p-values we report are only nominally significant (we did not carry out correction for multiple testing for the correlated tests). Hence, for both children and adults, the evidence for an association of the *DAT1* gene with ADHD is still far from reaching genome-wide significance. Apart from the possibility that the gene is simply not associated with ADHD after all, additional explanations for the limited significance of the findings are as follows: first, only a subgroup of patients might show the association with the gene. For example, data from studies by Diamond (2007) suggest that *DAT1* has a more important role in ADHD subtypes featuring hyperactivity symptoms than in the inattentive subtype. In this study, we did not find evidence to support this hypothesis. Also, *DAT1* might be linked to ADHD only in the absence or presence of a given comorbidity. This hypothesis recently found some support from the work within the IMAGE study, in which Zhou *et al* (2008) showed that *DAT1* was only associated with ADHD in children without comorbid conduct problems. This possibility needs to be tested in future studies. A second possible explanation, as mentioned above, is that the VNTRs and their haplotype incompletely tag the real ADHD risk variant in *DAT1*, or that additionally, other variants in or near the gene also exert an effect on ADHD risk. The latter view is again supported by the IMAGE study, which suggests that two different loci within *DAT1*, one 5' and one 3' site, influence ADHD risk in children (Brookes *et al*, 2008). As most studies up to now have studied children with the disorder, a third possible explanation is that age is an important factor to take into account when studying the role of *DAT1* in ADHD.

The putative differential association of *DAT1* with ADHD in children and adults might arise from different causes. Possibly, the 9/9 3'-UTR VNTR genotype and/or the 9-6 haplotype predispose(s) to a more severe ADHD phenotype characterized by persistence into adulthood. As only a subgroup of children with ADHD remit (Faraone *et al*, 2006; Barkley *et al*, 2006a; Kessler *et al*, 2005b), this subgroup might not be equally represented in all association studies of childhood ADHD. Some support for this hypothesis is provided by a prospective 13-year follow-up study 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, and that the effects of the genotype became more pronounced with increasing age of the participants. Importantly, more individuals with a DSM diagnosis of ADHD in adulthood were found among those having the 9/10 genotype (53%) than among the 10/10 homozygous group (35%) (Barkley *et al*, 2006b). On the other hand, as 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 *DAT1* with ADHD might reflect changing requirements on the dopaminergic system during life. Furthermore, adults more often than children consume cigarettes, alcohol or drugs, environmental factors that are known to influence the regulation of the dopamine transporter (Madras *et al*, 2005). An additional potential explanation might be that *DAT1* genotype effects on ADHD depend on the effect of another gene, which shows development-specific association with ADHD. A good candidate for this is *COMT*, encoding the catechol-O-methyltransferase, a major contributor to (prefrontal cortex) dopaminergic metabolism. Several recent studies suggest a double dissociation of dopamine effects, depending on *COMT* and *DAT1* genotype in dopamine-related brain activity (Bertolino *et al*, 2008; Yacubian *et al*, 2007). However, an involvement of *COMT* in ADHD has been suggested for both children and adults (eg, Lasky-Su *et al*, 2008; Hallett *et al*, 2008). Future studies of the *DAT1* VNTR haplotype might want to use brain imaging to investigate the neural substrates of the differences between children and adults. One would predict that these substrates are different for the different developmental stages. However, as this study is cross-sectional we cannot exclude the possibility that individuals seeking treatment as children are different from those seeking treatment as adults. This might be suggested by findings of other phenotypes associated with *DAT1*, such as decreased delinquency and promiscuous behavior in teenagers with the 9/9 genotype of the 3'-UTR VNTR (Guo *et al*, 2007).

In conclusion, our data bear the intriguing suggestion that the *DAT1* haplotype and genotype associated with ADHD in adults might be different from the one associated with the childhood disorder. A differential association of the *DAT1* gene with ADHD in children and in adults might help to explain the inconsistencies observed in association studies, where age is not commonly taken into account. However, the data might also imply that the gene has a role in modulating the ADHD phenotype, rather than causing it.

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DISCLOSURE

The authors declare no conflict of interest.

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