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DAT1, DRD4, and DRD5 Polymorphisms Are Not Associated With ADHD in Dutch Families

Steven C. Bakker,[1]* Emma M. van der Meulen,[1] Nicole Oteman,[2] Hedi Schelleman,[4] Peter L. Pearson,[2] Jan K. Buitelaar,[3] and Richard J. Sinke[2]

[1]*Department of Psychiatry, University Medical Center Utrecht, The Netherlands*
[2]*Department of Medical Genetics, University Medical Center Utrecht, The Netherlands*
[3]*Department of Psychiatry, University Medical Center Nijmegen, The Netherlands*
[4]*Department of Biostatistics and Epidemiology, Erasmus MC, Rotterdam, The Netherlands*

Recent meta-analyses have indicated that the dopamine transporter gene (*DAT1*) and the dopamine receptor genes D4 (*DRD4*) and D5 (*DRD5*) are associated with attention-deficit hyperactivity disorder (ADHD), although single studies frequently failed to show significant association. In a family-based sample of 236 Dutch children with ADHD, we have investigated the previously described variable number of tandem repeat (VNTR) polymorphisms and two additional microsatellites at the DAT1 and DRD4 loci. DRD5 was investigated using the microsatellite that was previously found to be associated. Transmission disequilibrium tests (TDTs) did not show preferential transmission of alleles or two-marker haplotypes to affected offspring. These data suggest that DAT1, DRD4, and DRD5 do not contribute substantially to ADHD in the Dutch population.

KEY WORDS: ADHD; association study; DAT1; DRD4; DRD5

INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD) is the most common child psychiatric disorder, affecting 4–5% of children in western countries [Buitelaar, 2002]. The estimated contribution of genetic factors is approximately 80% [Levy, 1997; Thapar et al., 1999], and it is likely that multiple genetic and environmental factors interact in causing the disease. Genetic research has focused on genes involved in neurotransmission, and in particular the dopaminergic system, since effective medication was reported to block the reuptake of dopamine by the dopamine transporter molecule (DAT1) [Krause et al., 2000]. Three dopaminergic genes have recently been reported to be associated with ADHD in meta-analyzes of data from multiple studies. The *DAT1* gene has a variable number of tandem repeat (VNTR) polymorphism in the 5′ untranslated region (UTR). A recent meta-analysis concluded that having a 10-repeat allele increased the risk to develop ADHD by a factor of approximately 1.3 [Maher et al., 2002]. The VNTR may change DAT1 function, since it has been suggested to regulate gene expression [Michelhaugh et al., 2001; Mill et al., 2002].

The dopamine receptor D4 gene (*DRD4*) has a VNTR polymorphism in the third exon, which is part of the third intracellular loop of the receptor, and may therefore have functional relevance [Asghari et al., 1995; Schoots and Van Tol, 2003]. Recent meta-analyses confirmed that the 7-repeat allele increased the risk of developing ADHD 1.4–2.0 times [Faraone et al., 2001; Maher et al., 2002]. Children with the 7-repeat allele were found to have a more inaccurate, impulsive response style on neuropsychological tasks [Langley et al., 2004].

In a meta-analysis of data from 14 different centers, the common 148 base pair (bp) allele of a compound microsatellite located 18.5 kilobases (kb) from the dopamine receptor D5 gene (*DRD5*) was shown to be significantly associated with ADHD (odds ratio 1.24) [Lowe et al., 2004].

The three polymorphisms mentioned above, as well two additional microsatellites near the VNTRs in DAT1 and DRD4, were genotyped in a sample of 236 Dutch children from 144 families.

SUBJECTS AND METHODS

Most children (n = 198) were part of a previously described sample of sib pair families [Bakker et al., 2003]. Children were only included if they had ADHD of the inattentive, hyperactive, or combined subtype, according to DSM-IV criteria. Children with autism spectrum disorders were excluded. This sample was extended with 38 families with only 1 affected child, diagnosed using the same criteria. In five families, no DNA from the father was available.

DNA was isolated as described [Bakker et al., 2003]. The VNTR polymorphisms in the *DAT1* and *DRD4* genes were amplified using previously described PCR primers [Van Tol et al., 1992; Cook et al., 1995]. Reactions were performed in 50 μl, containing 50 ng of genomic DNA, 100 ng of forward primer and 100 ng of reverse primer, 150 mM of each dNTP, 67 mM Tris HCl, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 16.6 mM $(NH_4)_2SO_4$, 10% DMSO, 7.5 μg BSA, and 0.4 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA). PCR reactions were performed on a ABI 9600 GeneAmp PCR system (Applied Biosystems) using the following conditions: 2 min at 94°C, followed by 33 cycles of 30 sec at 94°C, 30 sec at 60°C (DAT1) or 54°C (DRD4), 2 min at 72°C and a final extension of 4 min at 72°C. Subsequently, 10 μl of PCR product was analyzed on a 3% agarose gel, by applying 125 V for a duration of 2 hr. Fragments were stained with ethidium bromide and sizes were determined using a PGEM DNA size marker (Promega, Leiden, The Netherlands). In order to determine the repeat numbers of the different alleles of both genes, sequence analysis of the repeat regions were performed in several individuals. The DRD4 mononucleotide repeat,

*Correspondence to: Steven C. Bakker, Department of Psychiatry, A 00.241, University Medical Center Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands.
E-mail: s.c.bakker@med.uu.nl

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located in intron 1, was amplified with primers ACAGGCCC-TGAGGTTTCC and GTGGGGAAGGGGTGTTTC [Petronis et al., 1994]. Primers for dinucleotide repeat D5S2005, which is located at 50 kb from the DAT1 VNTR, were obtained from the Ensembl database [www.ensemble.org]. These microsatellites, as well as the DRD5 repeat [Daly et al., 1999], were amplified and analyzed as described elsewhere [Bakker et al., 2003]. Two independent raters scored all genotype data, and when they disagreed genotyping was repeated. Inheritance consistency was verified using the Pedcheck program [O'Connell and Weeks, 1998], and in case of inconsistencies the entire family was "zeroed out" for one marker. Hardy–Weinberg equilibrium (HWE) was investigated using the GENEPOP program for multi-allelic markers [Raymond and Rousset, 1995]. Likelihood ratios for transmissions of marker alleles and haplotypes from parents to affected offspring, as well as linkage disequilibrium (LD) between markers, were calculated using the TDTPHASE program [Dudbridge, 2003]. This estimates missing haplotypes using the expectation maximization (EM) algorithm, and uses unconditional logistic regression on the full likelihood of parents and offspring. Only alleles and haplotypes with frequencies higher than 0.05 were taken into account. *P* values were not corrected for multiple testing.

RESULTS

All five markers were in HWE in parents as well as in children. Results of overall likelihood tests for single markers and marker haplotypes are shown in Table I. There were no signs of a distorted transmissions of any of the single polymorphisms, or of single alleles. As genetic markers, both VNTRs are not very informative, with heterozygosity values below 0.5, and this could be one explanation for the replication problems in previous studies. In order to increase the information content, we also analyzed two-marker haplotypes with polymorphic microsatellites for DAT1 and DRD4. LD between the DRD4 markers was substantial for the DRD4 polymorphisms (D' = 0.59), but lower for the DAT1 polymorphisms (D' = 0.20), which is in agreement with a previous study [Hawi et al., 2003]. Overall, no haplotypes were significantly associated.

TABLE I. Results of Transmission Disequilibrium Tests (TDTs) for Single Markers and 2-Marker Haplotypes

Marker/haplotype	Allele	T	Freq-T	NT	Freq-NT
DRD4 VNTR	2	42	0.10	52	0.13
P = 0.50	4	277	0.68	265	0.65
	7*	71	0.17	67	0.16
	Other	19	0.05	25	0.06
DRD4 mono	2	103	0.23	105	0.24
P = 0.71	4	274	0.62	282	0.64
	5	63	0.14	55	0.12
	Other	2	0.00	0	0.00
Haplotype DRD4	2_2	15	0.04	24	0.06
P = 0.65	2_4	26	0.07	25	0.06
	4_4	206	0.52	204	0.51
	4_5	49	0.12	44	0.11
	7_2	54	0.14	58	0.15
	Other	46	0.12	41	0.10
DAT1 VNTR	9	96	0.22	92	0.21
P = 0.73	10*	341	0.77	346	0.78
	Other	5	0.01	4	0.01
D5S2005	4	221	0.52	214	0.50
P = 0.54	5	99	0.23	96	0.23
	6	60	0.14	55	0.13
	7	23	0.05	33	0.08
	Other	21	0.05	26	0.06
Haplotype DAT1	9_4	33	0.08	32	0.08
P = 0.21	10_4	178	0.44	172	0.42
	10_5	75	0.18	79	0.19
	10_6	45	0.11	38	0.09
	10_7	11	0.03	25	0.06
	Other	66	0.16	62	0.15
DRD5	4	27	0.07	26	0.06
P = 0.32	5	19	0.05	25	0.06
	8	34	0.08	25	0.06
	9*	183	0.45	186	0.46
	10	48	0.12	41	0.10
	11	29	0.07	38	0.09
	12	11	0.03	21	0.05
	Other	55	0.14	44	0.11

Overall *P* values of likelihood tests are shown in italic print below the marker names. Allele numbers of the variable number of tandem repeats (VNTRs) indicate repeat numbers; previously reported at-risk alleles are indicated with an asterisk (*). T/NT, number of transmitted/non-transmitted alleles. Freq-T/Freq-NT, frequency of transmitted/non-transmitted alleles. Two-marker haplotypes are indicated by the alleles of the respective markers, separated by an underscore (_). Other, combined numbers/frequencies of all alleles and haplotypes with frequencies <0.05.

DISCUSSION

In this Dutch family-based sample, which is one of the largest described so far, no association was found between the *DAT1*, *DRD4*, or *DRD5* genes and ADHD. The DRD5 data presented in detail here were part of the recent multi-center analysis for this gene, in which the Dutch sample was one of the two studies that did not contribute to the overall detected association [Lowe et al., 2004]. These findings seem to be in agreement with the results of our recent whole genome scan, in which there were no indications for linkage in the chromosomal regions that contain the three genes [Bakker et al., 2003]. The power of the linkage study, however, may have been too low to detect small effects. Likewise, we cannot rule out that our negative findings, as well as those by others, are due to chance, given the low relative risks attributed to the individual genes, or due to insufficient LD with an unknown disease-related variant. It is also possible, however, that these genes do not play an equally important role in all populations, or in multiplex families as compared to sporadic cases [Daly et al., 1999]. In another recent study in a large sample of multiply affected families, the DAT1 and DRD4 VNTRs also did not show a distorted transmission, although a different DRD4 polymorphism, as well as the DRD5 microsatellite, were positively associated with ADHD [Kustanovich et al., 2003]. Now that combined studies have quite convincingly suggested a small but significant role for dopaminergic genes in ADHD, further studies in different samples seem to be required to assess their role as general risk factors across populations.

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