

A preliminary report of the dopamine receptor D₄ and the dopamine transporter 1 gene polymorphism and its association with attention deficit hyperactivity disorder

Helmut Niederhofer¹
 Frauke Menzel²
 Karl Göbel³
 Brigitte Hackenberg^{4,5}
 Rainer Richter^{2,6}
 Maria Hildegard Walter²
 Christian Gross⁷
 Markus Huber⁸
 Roger Pycha⁸
 Hans-Jürgen Menzel^{9,10}

¹Ospedale Regionale di Bolzano, Via Guncina, Bolzano, Italia; ²Institute for Psychology, University of Innsbruck, Innsbruck, Austria; ³Freiburger Allee 67, Böblingen, Germany; ⁴University Clinic for Psychiatry, ⁵Institute for Medical Biology and Human Genetics, Medical University of Innsbruck, Innsbruck, Austria; ⁶University Clinic for Pediatrics, Medical University Vienna, Wien, Austria; ⁷Department of Psychosomatic Medicine and Psychotherapy, University Hospital, Eppendorf, Hamburg, Germany; ⁸Institut für Heilpädagogik, Klessheimer Allee 81, Salzburg, Austria; ⁹Krankenhaus Bruneck, Bruneck, Italia

Correspondence: Helmut Niederhofer
 Ospedale Regionale di Bolzano, Via
 Guncina, 54/A, I - 39100 Bolzano, Italia
 Tel +39 348 936 0281
 Fax +39 0471 466 609
 Email helmutniederhofer@yahoo.de

Abstract: Attention deficit hyperactivity disorder (ADHD) is one of the most prevalent childhood-onset psychiatric syndromes affecting 5%–10% of school-age children worldwide. Distortions in the catecholaminergic system seem to be responsible for this condition. Within this system there are several candidate genes, the dopamine receptor D₄ (*DRD4*) and the dopamine transporter 1 (*DAT1*), with common polymorphism which might be associated with ADHD. We performed a family based association study with 36 trios and 19 parent proband pairs. All diagnoses were confirmed by the “Hypescheme” diagnostic computer program. In this study we did not observe an association of ADHD with *DRD4* and *DAT1* polymorphism neither by the haplotype relative risk (HRR) method nor by the transmission disequilibrium test (TdT) method. The odds ratio for the *DRD4* 7-allele was 1.01 and 0.94 for both statistical tests, respectively, and the respective odds ratio for the *DAT1* 6-allele were 0.91 and 0.88.

Keywords: ADHD, dopamine receptor D₄, dopamine transporter, haplotype relative risk, transmission disequilibrium test

Introduction

Attention deficit hyperactivity disorder (ADHD) is a rather common (5%–10%) (Burd et al 2003) psychiatric disorder in schoolchildren between the age of six to ten years. The details of the etiology and pathophysiology of this disorder have still to be worked out, but available data implicate a dysregulation of the catecholaminergic system and this malfunction might be effected by polymorphisms of several genes involved in this system (Kronenberg et al 1999; Faraone et al 2001). Twin studies have demonstrated a high degree of heredity (0.6–0.9) in this disorder (Todd et al 2005). Molecular genetic studies of ADHD have focused on genes in catecholaminergic pathways because animal models, theoretical considerations, and the effectiveness of stimulant treatment implicate catecholaminergic dysfunction in the pathophysiology of the disorder (Faraone et al 2001).

Candidate genes for association and linkage studies are those involved in the dopamine pathway like the dopamine receptor D₄ (*DRD4*) and the dopamine transporter 1 (*DAT1*) genes. Numerous molecular genetic studies have been conducted with ambiguous findings. These studies were lately used for two meta-analyses to increase the statistical power of the findings (Kronenberg et al 1999; Maher et al 2002; Menzel 2003; Todd et al 2005).

Most of these studies have been almost exclusively conducted in North America and none were conducted in central Europe during the last eight years. We decided to recruit patients with ADHD in southern Germany and the western part of Austria. To increase the comparability of the diagnosis we used a computer-based diagnostic scheme for evaluation of all diagnosis. This computer program was developed by Sarah Curran and colleagues at the Institute of Psychiatry, London, United Kingdom under the name "Hypescheme" and can be downloaded from the following webpage <http://iop.kcl.ac.uk/IoP/Departments/SGDPsy/Hypescheme.shtml> (Curran et al 2000) and is closely related to the Conner's rating system (Conners et al 1998).

The hyperactive patients were recruited in three different locations. To circumvent problems of stratification due to differences in ethnic background, we choose to perform a family based study (Ewens and Spielman 1995; Todd 2000). Children with diagnosed ADHD were recruited together with their parents.

In the present paper the diagnosis of attention deficit hyperactivity disorder was evaluated with the "Hypescheme" program. The patients with ADHD and their parents were analyzed for the repeat polymorphism in the *DRD4* (48bp-repeat) and the *DAT1* (3'-40bp repeat) genes. Statistical analysis was performed by the haplotype relative risk (HRR) and the transmission disequilibrium test (TDT) methods (Ewens and Spielman 1995; Todd 2000). To also include pairs (1 affected child and one parent), the possible genotype of the missing parent was reconstructed and added to the analysis after weighting the genotypes according to occurrences in the normal population (Doucette-Stamm et al 1995; Kustanovich et al 2003).

Methods

Patient recruitment and diagnosis evaluation

Patients and their parents were recruited at institutions specialized in diagnosis and treatment of children with ADHD. These institutions were located in Innsbruck and Salzburg, Austria and Böblingen, Germany. In order to standardize the diagnosis, all patients were evaluated with the "Hypescheme" program after its release in 2000. Also children with other or no psychiatric disorders were diagnosed with the "Hypescheme" program to evaluate this program with respect to false – positive results. All parents gave informed consent for this study.

Table 1 Allele count for the haplotype relative risk calculation of the *DRD4* polymorphism of the 36 Trios

	Alleles					
	2	3	4	6	7	8
Cases	10	7	43	1	11	–
"controls"	5	3	50	–	12	2

Statistics: Allele 7 against all others: OD 0.93 (95% CI 0.35–2.47) $p = 0.88$.

DNA isolation

DNA was isolated from 5–10 ml of EDTA-blood. First, erythrocyte-lysis puffer (155 mM NH_4Cl , 10 mM KHCO_3) was added to the blood (10 to 1 v/v) and left for 30 min on ice. After centrifugation at 3000 g for 15 min the pellet was washed with erythrocyte-lysis puffer and the centrifugation was repeated. Only the tightly precipitated pellet was used. The pellet was solubilized with 10x Taq-polymerase buffer (0.5 M KCl, 0.1M Tris-HCl pH 9.0 and 15 mM MgCl_2), 2% Triton X-100 and 0.2 mg/ml Pronase A (Roche, Switzerland). This solution was about 1/10 of the whole blood volume and incubated for 3 h at 55 °C. After an inactivation at 95 °C for 15 min, the solution was vortexed, placed immediately on ice, and Pefabloc (Roche, Switzerland) was added at a final concentration of 0.1% and the solution was kept at 4 °C and used for polymerase chain reaction (PCR).

PCR of *DRD4* and the *DAT1* polymorphism

PCR for the *DRD4* polymorphism was done according to Nanko and colleagues (1993) with some modification. Dimethylsulfoxide was omitted and we used "Hot start"-Taq polymerase with Q-solution from Quiagen (Quiagen, Valencia, USA). The 200 $\mu\text{mol/L}$ of deazaguanosine was replaced by 100 $\mu\text{mol/L}$ deazaguanosine and 100 $\mu\text{mol/L}$ guanosine. Electrophoresis of the PCR-fragments was run on a 4% Metaphor[®] gel (BMA Bioproducts, Rockland USA). PCR for the *DAT1* polymorphism was performed according to Doucette-Stamm and colleagues (1995).

Table 1a Allele count for the haplotype relative risk calculation of the *DRD4* polymorphism of the 36 Trios and 13 Duos

	Alleles	
	7	others
Cases	19	79
"controls"	19,4	79,6

Statistics: OD = 1.01 (95% CI 0.47–2.18) $p = 1.0$.

Table 2 Allele count for the transmission disequilibrium test of the *DRD4* polymorphism of the 36 Trios

		Not transmitted					
		2	3	4	6	7	8
transmitted	2			7		2	
	3			7		1	
	4	6	1			8	2
	6					1	
	7	2		9			
	8						

Statistics: Allele 7 against all others: $\chi^2 = 0.04$ OD = 0.92 (95% CI 0.24–3.47).

Statistical analysis

For the statistical evaluation of our data we used two different approaches. One was the HRR estimation (Terwilliger and Ott 1992) and the other was the TDT (Ewens et al 1995).

For HRR, we counted all alleles that were passed to the patient in each trio and assigned them to the “cases” group whereas the untransmitted alleles were assigned to the “control” group. All alleles were counted also for homozygous parents. These numbers can be analyzed by 2×2 contingency tables for odds ratio and significant differences.

For the TDT, only the alleles of the informative trios were counted for transmission (T) or no-transmission (NT). Noninformative cases were omitted. To test if one allele is significantly more transmitted, the following formula is used: $(T-NT)^2/T+NT = \chi^2$ (McNemar-test). To calculate the odds ratio in a 2×2 contingency table the T and NT values were placed in the first row and the value for $(T+NT)/2$ twice in the second row.

Results

Evaluation of the “Hypescheme” program

We have recruited 43 male and 6 female Caucasian patients (6–13 years, mean 8.4, SD 2.2) in three centres (Innsbruck, Salzburg, and Böblingen) encompassing 36 trios (parents and child) and 13 pairs (one parent and child). All children had ADHD according to the DSM-IV criteria. 10 suffered from

Table 2a Allele count for the transmission disequilibrium test of the *DRD4* polymorphism of the 36 Trios and the 13 Duos

		Not transmitted	
		7	others
transmitted	7		18,15
	others	17,125	

Statistics: $\chi^2 = 0.04$ OD = 0.94 (95% CI 0.33–2.70).

Table 3 Allele count for the haplotype relative risk calculation of the *DAT1* polymorphism of the 36 Trios

		Alleles		
		5	6	7
Cases		16	56	
“controls”		14	56	2

Statistics: Allele 6 against all others: $\chi^2 = 0.0$ OD = 1.0 (95% CI 0.42–2.35).

additional conduct disorders and 5 from learning disorders. Other psychiatric/psychological disorders such as depression or anxiety disorders were defined as exclusion criteria. Diagnosis was confirmed by the “Hypescheme” program (16 attention deficits, 4 hyperactive, and 29 combined). The sensitivity and specificity of the “Hypescheme” program are both 1.0 according to DSM-IV criteria. The diagnostic algorithm of the program identifies hyperactive children with a specificity of 1.0 (sensitivity 0.33) according to ICD-10 criteria (Menzel 2003).

Dopamine receptor *D₄* polymorphism and ADHD

The analysis of our sample of parents and children with ADHD demonstrated the existence of six different alleles in the *DRD4* gene. We observed 2, 3, 4, 6, 7, and 8 alleles according to the nomenclature of van Tol and colleagues (1992). None of these was significantly associated with ADHD in the 36 trios, neither by the haplotype relative risk nor by the transmission disequilibrium test (Table 1 and 2). When the “risk” allele (number 7 allele) was compared with the other alleles, a nonsignificant ($p = 0.88$) odds ratio of 0.93 (95% CI 0.35–2.47) by the HRR method and a $\chi^2 = 0.04$ (odds ratio = 0.92; 95% CI 0.24–3.47) by the TDT method was obtained. If the 13 pairs were added to the calculation, similar results were observed (Table 1a and 2a).

Dopamine transporter 1 polymorphism and ADHD

The examination of our sample of parents and children with ADHD exhibited three different alleles of the *DAT1*

Table 3a Allele count for the haplotype relative risk calculation of the *DAT1* polymorphism of the 36 Trios and 13 Duos

		Alleles		
		5	6	7
Cases		24	73	
“controls”		20,6	75,4	2

Statistics: Allele 6 against all others: $\chi^2 = 0.04$ OD = 0.91 (95% CI 0.46–1.89).

Table 4 Allele count for the transmission disequilibrium test of the DAT1 polymorphism of the 36 Trios

		Non transmitted		
		5	6	7
transmitted	5		14	1
	6	13		1
	7			

Statistics: Allele 6 against all others: $\chi^2 = 0.0$ OD = 1.0 (95% CI 0.31–3.25).

gene which were designated 5, 6, and 7 according to the nomenclature of Doucette-Stamm and colleagues (1995). We did not observe any significant association with ADHD in the 36 trios, neither by the haplotype relative risk nor by the transmission disequilibrium test (Table 3 and 4). Comparison of the “risk” allele (number 6 allele) with the other alleles gave a nonsignificant result. An odds ratio of 1.0 (95% CI 0.42–2.35) ($p = 0.0$) was obtained by the HRR method and a $\chi^2 = 0.0$ (odds ratio = 1.0; 95% CI 0.31–3.25) was obtained by the TDT method. If the 13 pairs were added to the calculation, similar results were observed (Table 3a and 4a).

Discussion

We present a study that is the first to use the computer-based program, “Hypescheme”, which is based on DSM-IV for diagnosis of ADHD. It is also the first that is conducted with a sample from central Europe.

Using the “Hypescheme” computer program to diagnose ADHD introduces a new diagnostic tool that shows high sensitivity and specificity. Since it can be used by other investigators in the same way, it might be a good tool to standardize the diagnosis of ADHD.

This study does not support the previous findings of an association between the *DRD4* 7-allele and the *DAT1* 6-allele and ADHD.

Two meta-analyses (Kronenberg et al 1999; Menzel 2003) have only come up with a significant association when several studies were combined, although the odds ratios were not very different from 1.00. Meta-analyses are

Table 4a Allele count for the transmission disequilibrium test of the DAT1 polymorphism of the 36 Trios and the 13 Duos

		Non transmitted		
		5	6	7
transmitted	5		19,95	1
	6	17,5		1
	7			

Statistics: Allele 6 against all others: $\chi^2 = 0.17$ OD = 0.88 (95% CI 0.33–2.44).

always prone to publication bias. Although one publication (Kronenberg et al 1999) claims to have checked for this bias with the method published by Egger and colleagues (1997) the later method has not been mathematically correctly applied.

Just recently a new publication (Li et al 2006) has been issued from a group that has formerly observed a positive association with the *DRD4* polymorphism and was included in the meta-analyses (Kronenberg et al 1999; Menzel 2003). Now in a larger sample they no longer detect this association and also found no association with the *DAT1* polymorphism.

It is very important to publish all results from association studies regardless as to whether they reveal positive, negative, or no association at all.

Limitations

Although we have to admit that our study is quite small (36 trios and 13 pairs), it fits into the general picture that only a very few publications have been able to demonstrate: a significant association of these alleles with ADHD (Kronenberg et al 1999; Menzel 2003).

Acknowledgments

We thank the families who participated in this study. The authors report no conflicts of interest.

References

- Burd L, Klug MG, Coumbe MJ, et al. 2003. Children and adolescents with attention deficit-hyperactivity disorder: 1. Prevalence and cost of care. *J Child Neurol*, 18:555–61.
- Conners CK, Sitarenios G, Parker JD, et al. 1998. The revised Conner's Parent Rating Scale (CPRS-R): factor structure, reliability, and criterion validity. *J Abnorm Child Psychol*, 26:257–68.
- Curran S, Newman S, Taylor E, et al. 2000. Hypescheme: An operational criteria checklist and minimum data set for molecular genetic studies of attention deficit and hyperactivity disorders. *Am J Med Genet*, 96:244–50.
- Doucette-Stamm LA, Blakely DJ, Tian J, et al. 1995. Population genetic study of the human dopamine transporter gene (*DAT1*). *Genet Epidemiol*, 12:303–8.
- Egger M, Smith GD, Schneider M, et al. 1997. Bias in meta-analysis detected by a simple, graphical test. *Br Med J*, 315:629–34.
- Ewens WJ, Spielman RS. 1995. The transmission/disequilibrium test: History, subdivision and admixture. *Am J Hum Genet*, 57:455–64.
- Faraone SV, Doyle AE, Mick E, et al. 2001. Meta-analysis of the association between the 7-repeat allele of the dopamine D₄ receptor gene and attention deficit hyperactivity disorder. *Am J Psychiatry*, 158:1052–7.
- Kronenberg MF, Menzel HJ, Ebersbach G, et al. 1999. Dopamine D₄ receptor polymorphism and idiopathic Parkinson's disease. *Eur J Hum Genet*, 7:397–400.
- Kustanovich V, Ishij J, Crawford L, et al. 2003. Molecular Psychiatry, Dec 16 (Epub ahead of print).
- Li D, Sham PC, Owen MJ, et al. 2006. Meta-analysis shows significant association between dopamine system genes and attention deficit hyperactivity disorder (ADHD). *Hum Mol Genet*, 15:2276–84.

- Maher BS, Marazita ML, Ferrell RE, et al. 2002. Dopamine system genes and attention deficit hyperactivity. *Psychiatr Genet*, 12:207–15.
- Menzel F. 2003. “Hypescheme international entry system” (HSIES) – Evaluation eines Computerprogramms zur Diagnose von Hyperaktivität, sowie Durchführung einer Untersuchung mit dessen Hilfe an Tiroler Volksschulen. unpublished thesis, University Innsbruck, Austria.
- Nanko S, Hattori M, Ikeda K, et al. 1993. Dopamine D4 receptor polymorphism and schizophrenia. *Lancet*, 341:689–90.
- Terwilliger JD, Ott J. 1992. A haplotype-based ‘Haplotype Relative Risk’ approach to detecting allelic association. *Hum Hered*, 42:337–46.
- Todd R, Huang H, Smalley SL, et al. 2005. Collaborative analysis of *DRD4* and *DAT* genotypes in population-defined ADHD subtypes. *J Child Psychol Psychiatry*, 46:1067–73.
- Todd RD. 2000. Genetics of attention deficit/hyperactivity disorder: Are we ready for molecular genetic studies? *Am J Med Genet*, 96:241–3.
- van Tol HHM, Wu CM, Guan HC, et al. 1992. Multiple dopamine D4 receptor in the human population. *Nature*, 358:149–52.

