Molecular Psychiatry (2000) 5, 396–404
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ORIGINAL RESEARCH ARTICLE

Association and linkage of DRD4 and DRD5 with attention deficit hyperactivity disorder (ADHD) in a sample of Turkish children

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The search for genetic factors predisposing to Attention Deficit Hyperactivity Disorder (ADHD) has focused on genes that regulate dopaminergic pathways such as dopamine receptors and enzymes that regulate levels of dopamine in the synapse. There have been several reports of association between ADHD and polymorphic variants within or near DRD4, DRD5, DAT1, DBH and COMT. In this study we set out to investigate specific alleles of DRD4 and DRD5, previously reported to be associated with ADHD, in a sample of Turkish children with DSM-IV ADHD children, as well as their relation to methylphenidate response and dimensional measures of symptom domains. One hundred and four independent trios and seven dyads were analysed using the transmission disequilibrium test (TDT). We found increased transmission of the DRD4 7-repeat allele (DRD4*7) (TDT $\chi^2 = 2.79$, $P = 0.047$). Given that we were testing specific *a priori* hypotheses regarding the associated alleles, we have used one-tailed *P*-values throughout. There was evidence of an interaction with methlyphenidate (MPH) response and analysis of the sample excluding non-responders revealed more significant evidence for the association (TDT $\chi^2 = 4.48$, $P = 0.017$). We also detected a trend for linkage and association in the DRD5 polymorphism (TDT $\chi^2 = 2.38$, $P = 0.06$). Similar findings were obtained in relation to MPH response as analysis of MPH responders alone gave rise to a more significant association than that of the group as a whole (TDT $\chi^2 = 4.9$, $P = 0.013$). *t*-Test and logistic regression TDT analyses of DRD4*7 transmission with respect to dimensional rating scales of hyperactivity and impulsivity showed an inverse relation suggesting that in this sample DRD4*7 is associated with a lower level of ADHD symptomatology. While this may be due to stratification along a dimension of severity such that severe cases belong to a more extreme group with other specific genetic and environmental causes, similar to the model for low cognitive ability, it is more likely the result of a chance selection bias in this sample. *Molecular Psychiatry* (2000) **5,** 396–404.

Keywords: Linkage and association; dopamine D4 receptor gene (DRD4); dopamine D5 receptor gene (DRD5); attention deficit hyperactivity disorder (ADHD)

Introduction

Attention deficit hyperactivity disorder (ADHD) is a common neuropsychiatric disorder with onset in early childhood. It affects 2–5% of school age children and boys two or three times more frequently than girls. The disorder is characterised by pervasive inattention, overactivity and impulsivity and leads to significant behavioural, social and educational impairments which frequently persist into adult life.[1,2] In recent years there has been considerable progress in our understanding of the disorder and the importance of genetic factors. ADHD is known to aggregate within families[3–6] and twin studies examining the relative importance of genetic and environmental influences have consistently shown it to be amongst the most highly heritable behaviours in childhood, with heritability (h^2) in the order of 80–90%.[4–10] The mode of transmission is unknown, although in keeping with other behavioral and complex disorders, genetic influences are likely to involve the coaction and interaction of a few or many genes, each having only a minor effect on their own.

To date, several genes involved in the regulation of dopamine and monoamine neurotransmission have been implicated in the aetiology of hyperactivity. The focus on the dopamine system stems from the well-

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Received 26 October 1999; revised and accepted 4 February 2000

characterised response of hyperactivity to stimulants such as methylphenidate.[11–14] Animal studies have found that amphetamine reversible hyperactivity can be produced by a variety of models such as knockout of the dopamine transporter gene[15] and chemical lesions including depletion of cerebral noradrenaline.[16] Alterations in delayed alternation learning, a key animal model for attention deficit, have been related to density of dopamine D1-type receptors.[17] The response to stimulants has led to the predominant hypothesis that the dopamine system may be underactive (hypodopaminergic) in children with ADHD. However, evidence that methylphenidate may in fact reduce dopamine outflow through downstream inhibition by serotonin pathways[15] is consistent with a hyperdopaminergic theory for ADHD.

In this study we focus on two dopamine receptor genes, the D4 receptor gene (DRD4) and the D5 receptor gene (DRD5) that have been reported to be associated with ADHD. First, association between the 7-repeat allele of a VNTR within the coding region of DRD4 (DRD4*7) and ADHD has been studied by several groups. La Hoste *et al*[18] were the first to report an increased frequency of DRD4*7 (22% *vs* 9%) in a clinical sample of ADHD children compared to controls. They noted that children with DRD4*7 had higher DISC scores compared to those without it, suggesting a possible relationship with clinical severity. These findings were subsequently replicated in a further sample of cases with a refined ADHD phenotype that included the combined ADHD subtype, positive drug response and no significant comorbidity,[19] similar to the ICD-10 definition of hyperkinetic disorder (HKD) and hyperkinetic disorder/conduct disorder (HKD/CD).[20] Rowe *et al*[21] reported on a clinical sample who received questionnaire-based diagnoses of ADHD derived from a parental rating scale for DSM-IV symptoms (The Emory Diagnostic Rating Scale). They found an increased frequency of DRD4*7 in both the ADHD-combined subtype ($\chi^2 = 5.9$, $P < 0.01$) and ADHD-inattentive subtype ($\chi^2 = 4.6$, $P < 0.05$) compared to controls. Further support for these findings come from the results of two other studies; a clinical sample of DSM-III-R or DSM-IV diagnosed ADHD children[22] and a sample of ADHD children whose parents were diagnosed with adult ADHD.[23] A few studies have failed to detect this association, but this may be due to a combination of small sample size, different ascertainment procedures and genetic heterogeneity.[24–26] DRD4*7 has also been reported to be associated with the trait of novelty-seeking,[27,28] a personality trait whose profile includes components of ADHD such as impulsiveness and high activity levels. However further studies on novelty seeking have so far resulted in contradictory findings (reviewed in Paterson *et al*).[29]

Second, Daly and colleagues[30] reported association between ADHD and the dopamine D5 receptor gene ($P = 0.00005$). The DRD5 association was found with the 151-bp allele (DRD5*151) of a microsatellite marker that was isolated from a cosmid clone containing DRD5.

In addition to these findings, genetic studies of the dopamine transporter (DAT1) reported an association between a variable number tandem repeat (VNTR) polymorphism in the 3′ untranslated region of the gene and DSM-III-R diagnosed ADHD and undifferentiated attention deficit disorder (UADD).[31] Two further independent studies reported similar results with DAT1,[32,33] although others have failed to find evidence for this association.[30,34,35] More recently Daly and colleagues[30] replicated their earlier findings on DAT1 and also reported association between ADHD and dopamine-β-hydroxylase ($P = 0.0072$).

Dopamine receptors belong to the group of G-protein coupled receptors that have seven transmembrane domains. The dopamine D5 and D1 receptors both belong to the group of D1-like dopamine receptors, which stimulate the production of adenylate cyclase via G-protein coupling, thereby activating cyclic AMP (c-AMP) dependent protein kinases. Dopamine has a 10-fold higher affinity for DRD5 than DRD1.[36] The function of DRD5 is not well understood but there appear to be differences of expression and pharmacological characteristics from DRD1.

The D4 receptor has structural and pharmacological characteristics, which are similar to the D2-type group of dopamine receptors that includes D2, D3 and D4. It is mostly expressed in dopamine-rich areas with a restricted distribution pattern in limbic areas, suggesting a role in human cognitive, emotional and behavioral function. DRD4, situated on chromosome 11p15.5, is highly polymorphic and contains DRD4*7, which is the 7-repeat of a 48-base pair VNTR within the third exon of the gene. This region codes for the third cytoplasmic loop, which is the region responsible for G-protein coupling. Moreover, there is some evidence that DRD4*7 mediates a blunted response to agonists due to increased inhibition of cyclic AMP. DRD4*7 has further been shown to respond differently to dopamine antagonists and agonists than the shorter repeat forms in both physiological and pharmacological experiments.[37,38]

In this study we set out to investigate the relationship between the specific alleles of DRD4 and DRD5 that had previously been reported to be associated with ADHD, in a sample of Turkish children with DSM-IV ADHD. We have performed simple within-family tests of association and linkage between the ADHD and the specific alleles implicated in these studies and explored their relationship to methylphenidate response and dimensional measures of hyperactivity.

Subjects and methods

Clinical sample

The sample of children with ADHD and their biological parents used in this study was ascertained at Marmara University Medical School, Department of Psychiatry, Development and Neuropsychiatry Research Unit, Istanbul. Probands were identified from a child behavioural/neuropsychiatric clinic and given a research assessment if they were thought to have a

diagnosis of ADHD. Biological parents of children who fulfilled criteria for inclusion in this study were also asked to participate. Written informed consent was obtained from parents and the ethical approval of the study was obtained from the Marmara University Ethical Committee.

A definite diagnosis of ADHD and comorbid conditions was established and confirmed by two experienced child psychiatrists following a semi-structured interview with the Kidi-schedule for affective disorders and schizophrenia (K-SADS)[39] with the parents and the child. Each case was assessed and an initial diagnosis given by one psychiatrist (YY) who is certified in child and adolescent psychiatry and has significant clinical and research experience in ADHD and related disorders. Two other child psychiatrists with similar experience, blind to the original diagnosis, went through the clinical data and made a best estimate diagnosis. This process was a part of a phenomenological study where major diagnostic domains were Tourette's syndrome, obsessive-compulsive disorder, pervasive developmental disorder, bipolar disorder, ADHD and other developmental neuropsychiatric disorders. The diagnostic interreliability for ADHD and comorbidities was 100% for all cases. Pervasiveness of hyperactivity symptoms was confirmed from teacher reports. Exclusion criteria were physical/neurological abnormalities (eg head trauma, seizure, Fragile X), IQ score less than 80, a personal or family history of psychiatric or developmental disorders except for tics, Tourette's syndrome, oppositional defiant disorder (ODD), conduct disorder (CD), depression and anxiety. Children with reported peri-natal difficulties such as premature birth, eclampsia/perieclampsia, neonatal seizures, intensive care unit hospitalisation in the first week following delivery, and those that scored above 2 on a perinatal distress scale of 1–5, were also excluded.

Several rating scales were used to provide dimensional measures of symptom domains and to provide comparability with data from other groups. These included a DSM-IV item checklist, Connors parent and teacher rating scales[40] and the Child Behavioral Checklist (CBCL) with the associated Teacher Report Form (TRF).[41] These measures were found to be normally distributed in this sample, apart from CBCL antisociality score. Family and medical/perinatal/developmental histories were obtained from the parents (see Table 1).

Response to methylphenidate (MPH) was defined as at least 50% improvement in the number of symptoms (clinical severity) and associated impairment following a titration procedure over 12 weeks. The titration procedure was an on-off MPH various doses design (beginning with 0.3 mg kg^{-1} per dose) titrating up to clinical response, employed as part of our routine clinical practice as suggested by the MTA study.[42] This procedure includes reports from school and home as well as observational measures at the office. MPH response has been defined similar to the response requirement in Swanson *et al*[19] and others' narrow phenotype (ie response to MPH and lack of serious comorbidities). There is a possibility that this approach may have misclassified some responders as non-responders, however, with the titration strategy used in routine clinical practice as well as the MTA study, this possibility is minimized.

One hundred and eleven unrelated children with a clinical diagnosis of ADHD were fully assessed and received a diagnosis of the combined subtype of ADHD under DSM-IV criteria (*Diagnostic and Statistical Manual of Mental Disorders*. APA, 1994). Blood samples for DNA extraction were obtained from these probands and both of their biological parents (proband parent trios), except in seven cases where only one parent was available (proband-parent dyads). Mendelian segregation has been verified by genotyping six markers, including three highly polymorphic simple sequence repeats (SSRs) to date. A single family was identified with non-paternity, which has been removed from the dataset. The mean age of probands was 9.4 (SD = 2.4) years, and 86% were male. Ninety per cent of probands were treated with methylphenidate (MPH) and 79% classified as MPH responders. Comorbid diagnoses were made for Tourette's syndrome and/or tics (TS/tics) in 34%, conduct disorder and oppositional defiant disorder (CD/ODD) in 25% and anxiety/depression in 8% of probands. Family history of ADHD (having at least one other first-degree relative with ADHD) was present in 37% of cases.

DNA analysis

High molecular weight DNA was extracted from peripheral venous blood collected in EDTA tubes. DNA was isolated using a standard phenol chloroform procedure or a Rapid DNA Extraction Kit developed in the host laboratory, prior to re-suspension and storage in TE buffer (10 mM Tris HCl, 1 mM EDTA). Genotyping followed standard polymerase chain reaction (PCR) methods and allele assignments were checked by two individuals.

The DRD4 48-base pair repeat polymorphism was genotyped following the protocol described by Lichter *et al*[43] and modified by the addition of 7-Deaza-dGTP (Boehringer Mannheim, Germany) at a final concentration of 0.2 mM and 10% dimethyl sulfoxide (DMSO). AmpliTaq Gold (Perkin-Elmer/Applied Biosystems, USA) was used instead of standard Taq Polymerase. The PCR reaction was performed on a PTC-225 Peltier Thermal Cycler (MJ Research, USA) with heated lid. Following denaturation at 95°C for 9 min, 40 cycles of amplification were performed at 93°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final 72°C for 10 min. PCR products were electrophoresed on 3% Agarose gel (Multi Agarose, Advanced Biotechnologies, UK), and visualised by ethidium bromide staining under UV illumination.

The DRD5 $(CT)_n$ dinucleotide repeat was amplified with a fluorescently tagged forward primer (5′ CGT CTA TGA TCC CTG CAG 3′) and the reverse primer (5′ GCT CAT GAG AAG AAT GGA GTG 3′) in a 2 mM Mg^{2+} reaction mix.[30] Following denaturation, a *touch down* cycle of 94°C for 30 s, Ta (annealing temperature)

for 30 s, 72°C for 30 s for 28 cycles was used to amplify the region. Ta began at 56°C and was reduced by 0.50°C every three cycles until a Ta of 51°C was reached. Reaction products were separated on an Applied Biosystems (ABI) 377 sequencer and analysed using GENESCAN™ and GENOTYPER™ software.

Statistical analysis

Analyses of linkage and association were performed using the Transmission Disequilibrium test (TDT).[44,45] The TDT tests the deviation from equal transmission of alleles from heterozygous parents to their affected offspring and is therefore a robust test of linkage in the presence of association. For both DRD4 and DRD5 we restricted our analyses to allele-specific directional hypotheses since associated alleles have been reported previously. We have therefore reported one-tailed significance levels. We also performed Extention of Transmission Disequilibrium analysis (ETDT)[46] to check whether there were other alleles that might be considered as risk alleles.

Exploratory analyses were performed using the Student's *t*-test and a logistic regression extension of the TDT described by Waldman, Robinson and Rhee.[47] For both these approaches a transmission variable was created, coded 1 or 0 for transmission and non-transmission respectively, of the risk allele from a heterozygous parent. This variable is used to define categories for the *t*-test and becomes the dependent variable in the logistic regression TDT. This enables exploration of the relationship between allelic transmission and one or more continuous or categorical explanatory variables. Here we explore the relationship with dimensional ratings of ADHD, MPH response, comorbid conditions, sex of proband, sex of the heterozygous parent who is transmitting the risk allele, and family history.

Table 1 Clinical ratings scales used in this study to complement interview data for reaching diagnoses, provide dimensional ratings of symptom domain and provide measures of clinical severity

Measurement	*Mean (SD)*	*Range*
DSM-IV total	36.6 (8.2)	13–56
DSM-IV inattention	16.5 (4.5)	4–26
DSM-IV hyperactivity	10.8 (3.4)	2–18
DSM-IV impulsivity	9.3 (2.8)	2–15
Conners family sum	45.7 (13.6)	21–77
Conners family inattention	9.1 (3.3)	1–20
Conners family hyperactivity	29 (9.7)	6–48
Conners teacher sum	3 (1.6)	1–8
Conners teacher inattention	15.1 (5)	1–27
Conners teacher hyperactivity	17.3 (6.4)	1–31
CBCL internalising	62.4 (9.8)	39–82
CBCL externalising	66.1 (8.9)	35–840
CBCL total	67.7 (7.4)	45–82
TRF	70.1 (25.4)	5–149
CGI I	4.7 (.8)	3–6

Results

Analysis of the DRD4 repeat polymorphism

The results of the TDT analyses of DRD4*7 are shown in Tables 2–4. Out of 29 parents heterozygous for DRD4*7, 19 7-repeat alleles were transmitted to their affected offspring *vs* 10 non-transmitted ($\chi^2 = 2.79$, df = 1, one-tailed $P = 0.047$). Inclusion of dyads in the TDT test may falsely inflate the transmission rate where the offspring and both parents are heterozygous.[48] In this analysis we included three dyads where we knew the transmission status from the available parent. If it is assumed that all three parents of unknown genotype are heterozygous for DRD4*7, then the strength of association decreases (19 *vs* 13, $\chi^2 = 1.125$, df = 1, one-tailed $P = 0.144$). We estimated the relative risk for the combined subtype of ADHD to be 1.29 (95% CI: 1.09–1.73) and the odds ratio to be 1.77 (95% CI: 1.01–3.35) in the total sample (Table 5).

We then tested for a relationship between DRD4*7 transmission and MPH response using logistic regression TDT. Despite the small number of MPH non-responders in the sample this revealed a significant interaction ($P = 0.049$), suggesting that excess transmission of the DRD4*7 allele was more likely among MPH responders than non-responders. We subsequently repeated the TDT analysis after removing MPH non-responders from the sample and found a stronger association with DRD4*7 transmission than in the total sample ($\chi^2 = 4.48$, df = 1, one-tailed $P = 0.017$). Under the assumption that unknown parental genotypes from the three remaining dyads are all heterozygous for DRD4*7, the strength of association is decreased ($\chi^2 = 2.13$, df = 1, one-tailed $P = 0.07$).

Having found evidence in support of the association between the combined subtype of ADHD and DRD4*7, we went on to search for differences between probands with and without the DRD4*7, by comparing mean scores on parent and teacher rating scales (Table 6). This was possible using the *t*-test since the variables we examined were all normally distributed in our sample. For these analyses we made no adjustment for multiple testing since the intention was to explore the data in the hope of deriving novel hypotheses. As a result, interpretation of these results must be treated with caution. Significant differences were found for the DSM-IV total ($P = 0.03$), DSM-IV impulsivity ($P = 0.01$), and CBCL internalising ($P = 0.01$) scores. Other rating scales showed similar but non-significant trends in the same direction. These differences are in the opposite direction to that hypothesised, so that the children possessing DRD4*7 have lower symptom counts than those without DRD4*7.

Logistic regression TDT analysis of the DSM-IV rating scale gave similar results with an inverse relationship between DRD4*7 and DSM scores. Significant inverse relationships were found with the average of the total DSM score ($P = 0.002$) and DSM impulsivity scores ($P = 0.008$) and trends with the average of the DSM hyperactivity scores ($P = 0.068$), the DSM hyperactivity scores ($P = 0.08$) and the DSM total ($P = 0.059$)

Table 2 TDT results for DRD4*7 and DRD5*151 in the total sample and in MPH responders only

	*DRD4*7*		*DRD5*151*	
	All subjects (26 trios, 3 dyads)	*MPH responders only (24 trios, 3 dyads)*	*All subjects (57 trios)*	*MPH responders only (52 trios)*
Transmitted	19	19	42	42
Non-transmitted	10	8	29	24
χ^2	2.79	4.48	2.38	4.90
P one-tailed	0.047	0.017	0.061	0.013

Table 3 Transmission tests for individual alleles in DRD4 in the whole dataset using the ETDT program

	Transmissions for DRD4 individual alleles						
	*DRD4*2 (325 bp)*	*DRD4*3 (373 bp)*	*DRD4*4 (421 bp)*	*DRD4*5 (469 bp)*	*DRD4*6 (517 bp)*	*DRD4*7 (565 bp)*	*DRD4*8 (613 bp)*
Transmitted	7	6	32	0	1	19	1
Not transmitted	6	12	27	1	6	10	2
χ^2	0.07	2	0.42	1	3.57	2.79	0.33
P values	NS	NS	NS	NS	0.05	0.047	NS

Table 4 Transmission tests for individual alleles in DRD5 in the whole dataset using the ETDT program

	Transmissions for DRD5 individual alleles					
	*DRD5*1 (136 bp)*	*DRD5*2 (138 bp)*	*DRD5*3 (140 bp)*	*DRD5*4 (143 bp)*	*DRD5*5 (145 bp)*	*DRD5*6 (147 bp)*
Transmitted	2	5	11	7	9	10
Not transmitted	3	9	17	14	5	4
χ^2	0.2	1.1	1.2	2.3	1.1	2.5
P values	NS	NS	NS	NS	NS	NS
	DRD5*8 (151 bp)	DRD5*9 (153 bp)	DRD5*10 (155 bp)	DRD5*11 (157 bp)	DRD5*12 (159 bp)	DRD5*13 (161 bp)
Transmitted	42	20	17	3	1	1
Not transmitted	29	22	15	3	2	0
χ^2	2.38	0.001	0.001	0	0.3	1
P values	0.06	NS	NS	NS	NS	NS

Table 5 Odds ratio for DRD*7 and DRD5*151 in the total sample and among MPH responders alone

	Odds ratio (95% CI)	
	*DRD4*7*	*DRD5*151*
All subjects	1.77 (1.10–3.35)	1.28 (1.07–1.97)
MPH responders only	1.81 (1.10–3.31)	1.47 (1.07–2.32)

scores. When we examined comorbidity ratings, we found that CD/ODD was negatively related to the probability of DRD4*7 transmission ($P = 0.035$). We also found a significant relationship with anxiety/depression scores on the CBCL, such that probands without DRD4*7 were found to be more anxious and depressed than those possessing a copy of DRD4*7 ($P = 0.008$). Finally, there was no relationship with the sex of heterozygous parents transmitting DRD4*7 or family history, but there was a significant sex difference ($P = 0.008$) such that DRD4*7 was preferentially transmitted for girls (6 *vs* 0) but not for boys (10 *vs* 10).

Table 6 Mean differences on behavioral rating scales between ADHD probands that have transmitted or not transmitted the DRD4*7 allele from their parents

Clinical scores	*Means DRD4*7 transmitted (DRD4*7 not transmitted)*	*t-test**	*df*	*P values*	*SE difference*
CGI 1	*5.0* (4.6)	0.967	24	0.343	0.3
CBCL total T	*69.6* (63.3)	1.988	24	0.58	3.1
CBCL internalising T	*66.2* (56.7)	2.751	24	0.011	3.4
CBCL externalising T	*68.3* (62.5)	1.431	24	0.165	4
DSM-IV total	*38.8* (31.3)	2.195	24	0.038	3.3
DSM-IV hyperactivity	*10.8* (8.3)	1.692	24	0.1	1.4
DSM-IV attention deficiency	*17.5* (15.6)	0.966	24	0.344	1.8
DSM-IV impulsivity	*10.5* (7.3)	2.684	24	0.013	1.6

Analysis of the DRD5 polymorphism
The results of the TDT analyses for DRD5*151 are shown in Tables 2–4. Out of 71 parents heterozygous for DRD5*151, derived from 57 trios, there was excess transmission of DRD5*151 (42 *vs* 29, $\chi^2 = 2.38$, df = 1, one-tailed $P = 0.061$), which was not significant. As with the DRD4 polymorphism we found a significant interaction with MPH response ($P = 0.035$). TDT analysis of 52 trios in which the probands were all MPH responders gave rise to a more significant result than in the total sample (42 *vs* 24, $\chi^2 = 4.90$, df = 1, one-tailed $P = 0.013$). The odds ratios are listed in Table 5. Further analyses with the *t*-test and logistic regression failed to find any relationship between transmission of the DRD5*151 and symptom scales, comorbid conditions, sex of transmitting parent, sex of proband or family history.

Discussion

The aim of this study was to examine a sample of Turkish children with the combined subtype of ADHD for associations with DRD4*7 and DRD5*151, both reported in previous studies to be associated with an increased risk for ADHD. Using the TDT, we found suggestive evidence for DRD4*7 and a trend for DRD5*151 in the sample overall and more significant evidence for both, when only probands who were known to be responsive to MPH were considered. Further analyses designed to search for possible relationships between clinical variables and transmission of either allele found an inverse relationship between measures of clinical severity across several rating scales and subscales of hyperactive and impulsive behaviour with DRD4*7, but not with DRD5*151. Transmission of DRD4*7 was also negatively related to ratings of conduct disorder/oppositional defiant disorder and to anxiety/depression. Finally, DRD4*7 showed excess transmission to ADHD females but not males in this sample. These exploratory tests must be treated with caution, since the results fall below conventional significance levels when the number of different tests employed is taken into account.

The main findings are therefore supportive of the previously reported associations with DRD4*7 and DRD5*151, although they do not provide definitive evidence. The size of the effects is small and the sample size we have employed is not large. We have quoted one-tailed *P*-values, which are appropriate since we set out to test specific directional hypotheses based on previous findings. We considered whether to quote *P*-values adjusted for the number of markers we have tested to date, but rejected this on two counts. First, we plan many more extensive tests of association in this sample, so that a reasonable adjustment would take into account many thousands of loci. If we employ a genome-wide significance level as suggested by Rhisch and Merikangas[49] (alpha level of 5×10^{-8}), then clearly we fall well below any significant level. Second, it is not clear what an appropriate adjustment should be for *low* prior odds of association. This may be high, since there are plausible biological explanations for the involvement of both dopamine receptor genes and previous reports of associations with ADHD. Of these DRD4 is the most studied and there are now a total of six reports (including this one) that support the association with DRD4*7. Although there are some published and unpublished reports from groups that do not find this association, none of these are able to exclude the association due to limited sample size and the possibility of phenotypic heterogeneity resulting from differences in the ascertainment and assessment of clinical samples.

Additionally, presence of a locus in the same cytogenic band as DRD4 (11p15.5) exhibiting transmission ratio distortion (INS/IGF2)[50] raises the possibility of spurious findings due to linkage disequilibrium between the two loci. However a previous significantly positive study employing case control design of DRD4 and ADHD[18] suggests an argument against it.

The general level of consensus for the DRD4*7 association is unusual for molecular genetic studies of psychiatric disorders, where reported candidate gene associations have been followed by a majority of negative reports and only a few supporting reports. This has been well documented for schizophrenia where large

meta-analyses have been required to confirm, but not yet firmly establish, associations with variants of the D3 dopamine receptor and the serotonin 5HT2a receptor genes.[51] The main explanation for this is the very low odds ratios, in the order of 1.2, that have been estimated from these data, so that very large sample sizes are required to establish such findings. In contrast, the consistency of the DRD4 findings in ADHD are very encouraging, reflecting the higher odds ratios (1.7 in this study). The association with DRD5*151 remains more speculative.

One of the key issues that genetic studies of ADHD hope to address is the question of phenotypic and aetiological heterogeneity and it is therefore worth speculating on these issues. For example, heterogeneity may be reflected in the pattern of positive association findings reported to date, where some groups that report the association with DRD4*7 do not report association with (DAT1) and vice versa. The data reported here may also demonstrate the importance of heterogeneity since both the DRD4*7 and DRD5*151 associations are stronger among MPH responsive probands. DRD4*7 also shows an inverse relationship with anxiety and depression symptoms, which is interesting since some authors suggest that MPH response is much lower among ADHD probands comorbid for internalising disorders.[52] The relationship with MPH response in this sample may have two possible causes that are interesting. First, functional variation of the D4 receptor may give rise to different MPH response rates among ADHD probands. Second, MPH response may be a key feature of *core* ADHD, similar to the ICD-10 definition of hyperkinetic disorder (HKD); hyperactivity co-morbid with anxiety and depression (and less likely to respond to MPH) is considered to have an independent set of aetiologies.[52] A possible relationship with HKD has already been suggested by the findings of Swanson *et al*[19] who demonstrated the association of DRD4*7 in a sample with the combined subtype of ADHD and no significant comorbidity. On the other hand this may be merely a chance finding, since several groups have reported association between DRD4*7 and broadly defined ADHD (see below).

The finding of an inverse relationship between DRD4*7 and clinical severity, measured as the score on several rating scales for ADHD and disruptive child behaviour, is less easy to explain. The direction of this relationship is unexpected and is not supported by previous investigators who find evidence for increased severity among probands with DRD4*7. A speculative possibility is that hyperactivity is continuously distributed in the population, with DRD4*7 associated with high scores on dimensional ratings. ADHD associated with very extreme scores may however be made up of a number of other specific genetic and environmental causes. This model is comparable to the situation with cognitive ability. The relationship of DRD4*7 with dimensional ratings of hyperactivity has not been well studied since most groups have used full clinical assessments to select ADHD diagnosed probands. Some support for this hypothesis comes from the positive report of Rowe and colleagues[21] using maternally rated DSM-IV criteria checklists to derive ADHD categories. When the authors performed tests with dimensional scores they found association and linkage with inattentive symptoms but not hyperactive-impulsive symptoms. In addition, Castellanos *et al*[26] who studied a group of very severe ADHD cases, defined by willingness of parents to allow their children to leave regular school to enter a research day program for 3 months, failed to find any support for the DRD4*7 association.

On the other hand, a more likely explanation for these data, is a simple bias in the analysis of behavioral rating scales, since excess transmission of DRD4*7 is found only among girls with ADHD and not among boys in this sample. Due to the small sample size and relatively low number of girls this may have occurred by chance. Since boys are more likely than girls to score high on behavioral measures, there is an apparent association between DRD4*7 and low severity ratings.

Finally, there is the issue of power and the size of effect for both DRD4*7 and DRD5*151. For both polymorphisms we tested a single directional hypothesis with a specific allele, and as a result were able to gain additional power by the use of one-tailed tests of significance. The odds ratio (OR) for DRD4*7 = 1.77 (1.10–3.35), which is comparable with those from other studies: OR = 2.07 from Swanson *et al*,[19] OR = 1.99 from Rowe *et al*,[21] OR = 3.2 from La Hoste *et al*[18] and OR = 1.73 from Smalley *et al*.[22] The OR for DRD5*151 in this study is 1.28 when all subjects are considered and 1.47 when only MPH responders are considered, which is lower than the original OR = 2.52 from Daly *et al*.[30]

Cumulative data from independent studies suggest strongly that functional variation of the dopamine D4 receptor, probably the dopamine transporter and perhaps the dopamine D5 receptor influence risk for ADHD. The next stage is to explore the strength of these associations and their phenotypic boundaries. Further molecular analyses will now be required to confirm the functional role of the DRD4 repeat polymorphism demonstrated by Asgahri and colleagues[37] and move towards genetic animal models of DRD4 variants, which may be required to fully understand the mechanisms involved in ADHD pathogenesis. For both DRD5 and DAT1 it is not yet clear which, if any, genetic variants are functional. While the repeat polymorphism in the 3′ untranslated region of DAT1 remains a candidate, the microsatellite repeat for DRD5 is unlikely to be functional and significant variation has yet to be detected despite extensive studies of the coding regions and initial studies of regulatory regions.[53,54]

Acknowledgements

We would like to acknowledge the European Molecular Biology Organisation (EMBO), the UK Medical Research Council (MRC), TUBITAK, and Eczacibasi Scientific Research and Award fund for their financial

support. We also thank the families who participated in this research.

References

1 Taylor E, Chadwick O, Heptinstall E *et al*. Hyperactivity and conduct problems as risk factors for adolescent development. *J Am Acad Child Adolesc Psychiatry* 1996; **35**: 1213–1226.
2 Swanson J, Castellanos FX, Murias M *et al*. Cognitive neuroscience of attention deficit hyperactivity disorder and hyperkinetic disorder. *Curr Opin Neurobiol* 1998; **8**: 263–271.
3 Cantwell DP. Psychiatric illness in families of hyperactive children. *Arch Gen Psychiatry* 1972; **27**: 728–738.
4 Biederman J, Faraone SV, Keenan K *et al*. Family-genetic and psychosocial risk factors in DSM-III attention deficit disorder. *J Am Acad Child Adolesc Psychiatry* 1990; **29**: 526–533.
5 Biederman J, Faraone SV, Keenan K *et al*. Further evidence for family-genetic risk factors in attention deficit hyperactivity disorder: patterns of comorbidity in probands and relatives in psychiatrically and pediatrically referred samples. *Arch Gen Psychiatry* 1992; **49**: 728–738.
6 Stevenson J. Evidence for a genetic aetiology in hyperactivity in children. *Behav Genet* 1992; **22**: 337–344.
7 Goodman R, Stevenson J. A twin study of hyperactivity I and II. An examination of hyperactivity scores and categories derived from Rutter teacher and parent questionnaires. *J Child Psychol Psychiat* 1989; **30**: 671–689.
8 Thapar A, Holmes J, Poulton K *et al*. Genetic basis of attention deficit and hyperactivity. *Br J Psychiatry* 1999; **174**: 105–111.
9 Levy F, Hay DA, McStephen M *et al*. Attention-deficit hyperactivity disorder: a category or a continuum? Genetic analysis of a large-scale twin study. *J Am Acad Child Adolesc Psychiatry* 1997; **36**: 737–744.
10 Eaves LJ, Silberg JL, Meyer JM *et al*. Genetics and developmental psychopathology: 2. The main effects of genes and environment on behavioral problems in the Virginia Twin Study of Adolescent Behavioral Development. *J Child Psychol Psychiatry Allied Discip* 1997; **38**: 965–980.
11 Shekim WO, Javaid J, Davis JM *et al*. Urinary MHPG and HVA excretion in boys with attention deficit disorder and hyperactivity treated with d-amphetamine. *Biol Psychiatry* 1983; **18**: 707–714.
12 Shen YC, Wang YF. 3-methoxy-4-hydroxyphenylglycol sulfate excretion in 73 schoolchildren with minimal brain-dysfunction syndrome. *Biol Psychiatry* 1984; **19**: 861–870.
13 Zametkin AJ, Karoum F, Rapoport JL. Phenylethylamine excretion in attention deficit disorder. *J Am Acad Child Psychiatr* 1984; **23**: 310–314.
14 Arnold LE, Abikoff HB, Cantwell DP *et al*. National Institute of Mental Health Collaborative Multimodal Treatment Study of Children with ADHD (the MTA): design challenges and choices. *Arch Gen Psychiatry* 1997; **54**: 865–870.
15 Gainetdinov RR, Wetsel WC, Jones SR *et al*. Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science* 1999; **283**: 397–401.
16 Shaywitz BA. CSF amine metabolites in children with minimal brain dysfunction (MBD): evidence of involvement of brain dopamine. *J Pediatrics* 1977; **90**: 67–71.
17 Goldman-Rakic PS. Topography of cognition: parallel distribution networks in primate association cortex. *Ann Rev Neurosci* 1992; **11**: 137–156.
18 Lahoste GJ, Swanson JM, Wigal S *et al*. Dopamine D4 receptor gene polymorphism is associated with attention-deficit hyperactivity disorder. *Mol Psychiatry* 1996; **1**: 121–124.
19 Swanson JM, Sunohara GA, Kennedy JL *et al*. Association of the dopamine receptor D4 (DRD4) gene with a refined phenotype of attention deficit hyperactivity disorder (ADHD): a family-based approach. *Mol Psychiatry* 1998; **3**: 38–41.
20 World Health Organisation. *International Classification of Diseases: 10th edn*. WHO: Geneva, 1999.
21 Rowe DC, Stever C, Giedinghagen LN *et al*. Dopamine DRD4 receptor polymorphism and attention deficit hyperactivity disorder. *Mol Psychiatry* 1998; **3**: 419–426.
22 Smalley SL, Bailey JN, Palmer CG *et al*. Evidence that the dopamine D4 receptor is a susceptibility gene in attention deficit hyperactivity disorder. *Mol Psychiatry* 1998; **3**: 427–430.
23 Faraone SV, Biederman J, Weiffenbach B *et al*. Dopamine D4 gene 7-repeat allele and attention deficit hyperactivity disorder. *Am J Psychiatry* 1999; **156**: 768–770.
24 Asherson P, Virdee V, Curran S *et al*. Association study of DSM-IV attention deficit hyperactivity disorder (ADHD) and monoamine pathway genes. *Neuropsychiatric Genet* 1998; **81**: 549.
25 Gill M. Unpublished data, 1999.
26 Castellanos FX, Lau E, Tayebi N *et al*. Lack of an association between a dopamine-4 receptor polymorphism and attention-deficit/hyperactivity disorder: genetic and brain morphometric analyses. *Mol Psychiatry* 1998; **3**: 431–434.
27 Ebstein RP, Novick O, Umansky R *et al*. Dopamine D4 receptor (D4DR) exon III polymorphism associated with the human personality trait of Novelty Seeking. *Nature Genet* 1996; **12**: 78–80.
28 Benjamin J, Li L, Patterson C *et al*. Population and familial association between the D4 dopamine receptor gene and measures of Novelty Seeking. *Nature Genet* 1996; **12**: 81–84.
29 Paterson AD, Sunohara GA, Kennedy JL. Dopamine D4 receptor gene: novelty or nonsense? *Neuropsychopharmacology* 1999; **21**: 3–16.
30 Daly G, Hawi Z, Fitzgerald M *et al*. Mapping susceptibility loci in attention deficit hyperactivity disorder: preferential transmission of parental alleles at DAT1, DBH and DRD5 to affected children. *Mol Psychiatry* 1999; **4**: 192–196.
31 Cook JE, Stein MA, Krasowski MD *et al*. Association of attention-deficit disorder and the dopamine transporter gene. *Am J Hum Genet* 1995; **56**: 993–998.
32 Gill M, Daly G, Heron S *et al*. Confirmation of association between attention deficit hyperactivity disorder and a dopamine transporter polymorphism. *Mol Psychiatry* 1997; **2**: 311–313.
33 Waldman ID, Robinson BF, Feigon SA. Linkage disequilibrium between the dopamine transporter gene (DAT1) and bipolar disorder: Extending the transmission disequilibrium test (TDT) to examine genetic heterogeneity. *Genet Epidemiol* 1997; **14**: 699–704.
34 Bailey JN. DRD4 gene susceptibility to attention deficit hyperactivity disorder: differences in familial and sporadic cases. *Am J Med Gen (Neuropsych Gen)* 1997; **74**: 623 (abstract).
35 Tahir E. No association between ADHD and polymorphisms in DAT1 and DBH. Unpublished data, 1999.
36 Sunohara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG *et al*. Cloning of the gene for a human dopamine D5-receptor with higher affinity for dopamine than D1. *Nature* 1991; **350**: 614–619.
37 Asghari V, Sanyal S, Buchwaldt S *et al*. Modulation of intracellular cyclic AMP levels by different human dopamine D4 receptor variants. *J Neurochem* 1995; **65**: 1157–1165.
38 Asghari V, Schoots O, Van Kats S *et al*. Dopamine D4 receptor repeat: analysis of different native and mutant forms of the human and rat genes. *Mol Pharmacol* 1994; **46**: 364–373.
39 Kaufman J, Birmaher B, Brent D *et al*. Schedule for affective disorders and schizophrenia for school-age children–present and lifetime version (K-SADS-PL): initial reliability and validity data. *J Am Acad Child Adolesc Psychiatry* 1997; **36**: 980–988.
40 Conners CK, Barkley RA. Rating scales and checklists for child psychopharmacology. *Psychopharmacol Bull* 1985; **21**: 809–843
41 Achenbach TM. *Manual for the Child Behaviour Checklist*. 1991. Department of Psychiatry, University of Vermont, Burlington.
42 Greenhill LL, Abikoff HB, Arnold LE, Cantwell DP, Conners CK, Elliott G *et al*. Medication treatment strategies in the MTA Study: relevance to clinicians and researchers. *J Am Acad Child Adolesc Psychiatry* 1996; **35**: 1304–1313.
43 Lichter JB, Barr CL, Kennedy JL *et al*. A hypervariable segment in the human dopamine receptor D4 (DRD4) gene. *Hum Mol Genet* 1993; **2**: 767–773.
44 Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993; **52**: 506–516.
45 Schaid DJ, Sommer SS. Genotype relative risks: methods for design and analysis of candidate-gene association studies. *Am J Hum Genet* 1993; **53**: 1114–1126.
46 Sham PC, Curtis D. An extended transmission/disequilibrium test (TDT) for multiallele marker loci. *Ann Hum Genet* 1995; **59**: 323–336.

47 Waldman ID, Robinson BF, Rhee SH. A logistic regression extension of the transmission disequilibrium test for continuous traits: application to linkage disequilibrium between alcoholism and the candidate genes DRD2 and ADH3. *Genet Epidemiol* 1999; **17** Suppl 1: S379–S384.
48 Curtis D, Sham PC. Population stratifications can cause false positive linkage results if founders are untyped. *Annal Hum Genet* 1996; **60**: 261–263.
49 Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science* 1996; **273**: 1516–1517.
50 Eaves IA, Bennett ST, Forster P, Ferber KM, Ehrmann D, Wilson AJ *et al*. Transmission ratio distortion at the INS-IGF2 VNTR. *Nature Genet* 1999; **22**: 324–325.
51 O'Donovan MC, Owen MJ. Candidate-gene association studies of schizophrenia. *Am J Hum Genet* 1999; **65**: 587–592.
52 Pliszka SR. Comorbidity of attention-deficit/hyperactivity disorder with psychiatric disorder: an overview. *J Clin Psychiatry* 1998; **59** Suppl 7: 50–58.
53 Sobell JL, Lind TJ, Sigurdson DC *et al*. The D5 dopamine receptor gene in schizophrenia: identification of a nonsense change and multiple missense changes but lack of association with disease. *Hum Mol Genet* 1995; **4**: 507–514.
54 Asherson P, Mant R, Williams N *et al*. A study of chromosome 4p markers and dopamine D5 receptor gene in schizophrenia and bipolar disorder. *Mol Psychiatry* 1998; **3**: 310–320.