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Association study of a dopamine transporter polymorphism and attention deficit hyperactivity disorder in UK and Turkish samples

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Molecular genetic studies in attention deficit hyperactivity disorder (ADHD) have focussed on candidate genes within the dopamine system, which is thought to be the main site of action of stimulant drugs, the primary pharmacological treatment of the disorder.[1] Of particular interest are findings with the dopamine transporter gene (DAT1), since stimulant drugs interact directly with the transporter protein.[2,3] To date, there have been eight published association studies of ADHD with a 480 base-pair allele of a variable number tandem repeat (VNTR) polymorphism in the 3′-untranslated region of the gene, five[4–8] that support an association and three[9–11] against. We have analysed the same VNTR marker in a dataset of UK Caucasian children and an independent dataset of Turkish Caucasian children with DSM-IV ADHD, using the transmission disequilibrium test (TDT).[12] Results from the UK (χ^2 = 8.97, P = 0.001, OR = 1.95), but not the Turkish sample (χ^2 = 0.93, P = 0.34) support association and linkage between genetic variation at the DAT1 locus and ADHD. When considered alongside evidence from other published reports, there is only modest evidence for the association, consistent with a very small main effect for the 480-bp allele (χ^2 = 3.45, P = 0.06, OR = 1.15), however we find significant evidence of heterogeneity between the combined dataset (χ^2 = 22.64, df = 8, P = 0.004). *Molecular Psychiatry* (2001) **6,** 425–428.

In this study, we have taken a family-based association design to investigate the DAT1 VNTR marker in a dataset of UK Caucasian children and an independent dataset of Turkish Caucasian children. Cases were included if they had a diagnosis of ADHD under DSM-IV criteria and DNA from both parents available for genotyping. They were excluded if they had neurological disease or damage or congenital disorders known to cause hyperactivity. The UK sample consisted of 59 cases with the combined subtype, six the hyperactive/impulsive subtype and one the inattentive subtype of ADHD. Axis 1 co-morbidity other than oppositional defiant disorder and conduct disorder (ODD/CD) consisted of two cases with an affective disorder. The Turkish sample consisted of 111 complete trios with DSM-IV-ADHD combined type. Comorbid diagnoses other than ODD/CD were Tourette's syndrome and/or tics (TS/tics) in 34% and anxiety/depression in 8% of probands.

Analysis was performed using the transmission disequilibrium test (TDT) of linkage in the presence of association.[12] In order to further evaluate the evidence, we included data from other published reports and performed a combined analysis. The results of this study are shown in Table 1. When considered alone, data from the UK sample (χ^2 = 6.12, P = 0.0.01, OR = 1.95), but not the Turkish sample (χ^2 = 0.93, P = 0.335), support association and linkage between the DAT1 locus and ADHD.

We are not alone in finding differences between datasets. Among previous published reports there have been five providing evidence for the association and three against. The reasons for this are unclear and require further investigation, but may relate to the statistical power of individual samples. To address this issue we combined available published data on the VNTR polymorphism and applied the TDT. Because the TDT is primarily a test of linkage, it is valid to analyse the combined data by adding the number of transmitted and non-transmitted alleles across different studies. As shown in Table 1, combined analysis provides evidence for association and linkage at an alpha-level of 0.06 and odds ratio of 1.15.

Although diagnostic differences between the studies are likely to exist, these are minimised by the application of either DSM-III-R[4,11] or DSM-IV[4–11] criteria following a standardised research interview with one of the parents. The exception to this is Waldman *et al*[5] who applied DSM-IV criteria using data gathered from a DSM-IV criteria checklist and made diagnoses at three different levels of symptom severity. For the purposes of this analysis we have been inclusive by using their data for the least severe of these categories. If however, we use data from their most severe category

Table 1 Results of the transmission disequilibrium test (TDT) for the 480-bp repeat allele in 3′ untranslated region of DAT1

Study	*NITs*	*T*	*NT*	*Chi-square*	*P-value*	*Odds ratio*
UK—IOP/UB	59	39	20	6.119	0.013	1.95
Turkish—UM	87	39	48	0.931	0.335	0.81
Waldman *et al*[5]	63	39	24	3.571	0.059	1.63
Cook *et al*[4]	25	19	6	6.760	0.009	3.17
Daly *et al*[6,7]	79	48	31	3.658	0.056	1.55
Barr *et al*[8]	100	58	42	2.560	0.110	1.38
Holmes *et al*[10]	85	40	45	0.294	0.588	0.89
Swanson *et al*[9]	26	10	16	1.385	0.239	0.63
Palmer *et al*[11]	173	81	92	0.699	0.403	0.88
Total	664	356	308	3.470	0.063	1.16

NITs = number of informative transmissions; that is, the number of transmitting parents who are heterozygote for the 480-bp allele. T = number of transmitted alleles, NT = number of non-transmitted alleles. Findings from Waldman[3] and Gill/Daly[4,5] do not reach nominal significance values <0.05 in the TDT tests presented here, although their original papers provide stronger nominal evidence for the DAT1 association.

(22 transmitted *vs* 8 non-transmitted, $\chi^2 = 6.53$, $P = 0.01$) in the combined analysis, we find only a marginal change in overall significance ($\chi^2 = 3.47$, 1 df).

Finally, a homogeneity test of the data in Table 1 was significant ($\chi^2 = 22.64$, df = 8, $P = 0.004$), suggesting that systematic differences in findings between samples may exist, giving rise to conflicting results with regard to the DAT1 association.

The significance level observed in the combined analysis is modest and does not amount to convincing evidence for the DAT1 association. However, the finding of heterogeneity among the nine independent datasets suggests that the studies may divide into two groups; those in which the 480-bp allele has a main effect and those in which the allele does not. In this case, failure to replicate in some studies may not result simply from low power (due to small sample size) to detect small genetic effects, but may result from variation in the strength of the genetic influence in different populations. For example, the size of the genetic influence observed with a single marker may vary with differing exposures to interacting genes (epistasis), interacting environments or the degree of linkage disequilibrium (LD) between marker and functional variant. Phenotype definition and the way in which cases are recruited may also play an important role in producing heterogeneity, but this is hard to quantify. For example, we can not rule out systematic differences in the way diagnostic criteria have been applied in the UK and Turkey, although major differences are unlikely. A more apparent difference is case ascertainment, since the Turkish sample comprises over one third with TS/tics, compared with no cases with similar comorbidity in the UK sample. Finally, it remains possible that the positive findings reported so far have arisen by chance alone and the test of homogeneity may be sensitive to publishing bias.

Assuming that DAT1 is associated with ADHD, we do not yet know whether the VNTR itself or a functional variant in linkage disequilibrium (LD) with the VNTR is implicated. Barr *et al*[8] addressed this issue in a recent report in which they analysed the VNTR plus two single nucleotide polymorphisms in intron 9 and exon 9, in a sample of 102 nuclear families. They applied the TDT test to haplotypes of these three polymorphisms and found significant evidence of biased transmission for one haplotype and biased non-transmission for two other haplotypes. These findings were more significant for the haplotypes than for the VNTR alone, suggesting that the VNTR may be acting as a marker for a functional variant in LD with itself. Further information on the possible location of a putative functional variant comes from a recent presentation by Kelsoe and colleagues.[13] They analysed a number of polymorphisms spanning DAT1 and found two regions of strong LD encompassing exons 1–8 and 9–15. These two regions did not appear to be associated with each other and they concluded that a recombination hotspot lies between them. The implication from this is that any functional variants of DAT1 that are in LD with the 480-bp allele must lie within the region spanning exons 9–15 (including introns and 3′-untranslated region). Although a few of the identified polymorphisms bring about amino-acid changes, these have very low allele frequencies and are unlikely to explain the VNTR or haplotype associations with ADHD.

Other lines of evidence suggest a role for DAT1 in ADHD. Knockout mice lacking DAT1 (DAT-KO) share certain behavioural and pharmacological characteristics with individuals with ADHD.[14–16] DAT-KO exhibit novelty-driven hyperactivity, deficits in learning and memory processes, perseverative errors and reduction of hyperactivity in response to MPH. However, there are caveats to the DAT-KO model of hyperactivity. First, it is unlikely that complete functional absence of DAT occurs in ADHD patients and, consequently, DAT-KO mice represent an extreme case of DAT dysfunction. Second, genetic influences on ADHD are most likely to result from alterations in the function of several genes and DAT-KO mice model only one such potential influence. Third, the calming effects of

MPH require substantially higher doses in DAT-KO mice than that used in children with ADHD.[16] Fourth, while MPH is known to mediate a reduction in overactive behaviour in DAT-KO, it has not been determined whether improvements extend to areas of cognitive performance relevant to ADHD, such as attention. Fifth, a simple hypo-dopaminergic hypothesis for ADHD has been proposed on the basis that MPH increases intracellular DA levels and low extracellular DA has been reported in ADHD patients.[17,18] In contrast, DAT-KO have high extracellular levels of DA. The apparent paradox may be explained by the observation that in DAT-KO, striatal stores of DA are low and DA autoreceptors are down-regulated, resulting in overall hypo-dopaminergicity. However, the discrepancy may be better explained by extending the simple unitary hypo-dopaminergic hypothesis of ADHD. For example, Castellanos[19] proposed that different abnormalities may exist in two dopamine regions: underactivity in anterior cingulated (cortical) resulting in cognitive deficits, and overactivity in a caudate nucleus (sub-cortical) resulting in motor overactivity.

More direct evidence for the involvement of DAT in ADHD comes from two studies using single photon emission computed tomography (SPECT) in adult ADHD cases.[20,21] Both show an age-corrected increase in the density of striatal DAT in ADHD cases compared to controls. Furthermore, DAT density was shown to decrease to control levels following 4 weeks treatment with MPH.[21] While such studies suggest that changes in the regulation of DAT density occur in individuals with ADHD, they do not distinguish between primary causative factors and secondary changes. Preliminary evidence for primary causation comes from *in vivo* expression studies using SPECT, in which individuals with the 9-repeat/10-repeat genotype at the DAT1 VNTR had a mean 22% reduction of DAT availability in the putamen compared with 10-repeat homozygous individuals, suggesting that the VNTR polymorphism is associated with expression of the DAT protein.[22]

In conclusion, a role for DAT in the aetiology of ADHD is suggested by a number of lines of evidence. Molecular studies provide some support for this hypothesis, although combined analysis of available data is not significant and we have failed to establish a definite link between the VNTR polymorphism in the 3′-untranslated region of DAT1 and ADHD. Nevertheless, some evidence for association and linkage remains. The finding of heterogeneity between datasets suggests that further work is required to investigate differences in diagnostic and ascertainment procedures, identify interacting genetic and environmental risk factors, search for haplotype associations in different populations using markers in the region of the DAT1 VNTR and identify functional variants of DAT1.

Methods

Clinical samples

The clinical samples used in this study have been described elsewhere.[23,24] The UK cases were collected at the Institute of Psychiatry (IOP) and the University of Birmingham (UB) following referral by child behavioural clinics in Southern and Mid-England. Parents of referred cases were interviewed with a modified version of the Child Assessment Parent Interview (CAPA)[25] and information on ADHD symptoms at school were obtained using the Teacher Conners[26] questionnaire. Following the IOP assessments, HYPESCHEME data sheets were completed using data gathered from the research interview, teacher's questionnaire and where necessary review of case notes. HYPESCHEME is an operational criteria checklist for ADHD and hyperkinetic disorders, which summarises and applies DSM-IV and ICD-10 operational criteria.[27] HYPESCHEME diagnoses were checked against researcher applied DSM-IV criteria and discrepancies reviewed by two researchers (PA and SR). Where consensus could not be reached, cases were brought to case conference and final consensus agreement made with a senior clinical researcher (ET). In UB DSM-IV criteria were applied directly by the researcher (LK) and consensus diagnosis agreed at case conference.

The Turkish sample was collected at the Marmara University Medical School in Istanbul (UM) from cases attending a Neuropsychiatric clinic. Diagnoses in Turkey were made following a research interview using the Kiddie-Schedule for Affective Disorders and Schizophrenia (K-SADS)[28] with one of the child's parents and review of behavioural questionnaires including Connors parent and teacher rating scales and the Child Behavioural Checklist (CBCL) with the associated Teacher Report Form (TRF).[29] DSM-IV criteria were applied directly by the researcher (YY) and consensus diagnosis agreed at case conference.

Genotyping

The 3′ UTR VNTR was amplified on an MJ PTC-225 thermal cycler (MJ Research, Waltham, MA, USA) in a hot-start protocol involving an initial 5-min denaturing step at 95°C, followed by 38 cycles of 93°C for 1 min and 72°C for 1 min. The primers used were 5′-TGT GGT GTA GGG AAC GGC CTG AG-3′ and 5′-CTT CCT GGA GGT CAC GGC TCA AGG-3′. The reaction mix included 75 ng of genomic DNA, 1.5 mM $MgCl_2$, 20 mM dNTPs, 10 mM 10 × PCR buffer (PE Applied Biosystems, Foster City, CA, USA) and 1 unit of Taq polymerase (added separately 30 s into the denaturing step). PCR products were run out on a 2% agarose gel.

Statistical analysis

Association of the 480-bp allele of the DAT1 VNTR with ADHD was investigated using the transmission disequilibrium test (TDT).[12] For these analyses, we chose to test the single hypothesis generated from the original report of Cook and colleagues.[2] That is, that there is excess transmission of the 480-bp allele from parents who are heterozygote for that allele, to their offspring with ADHD.

We combined available published data on the VNTR polymorphism and performed a combined TDT analysis. Transmission data suitable for the TDT were

obtained from published reports[4,5,8–11] or directly from the original authors.[6,7] For the combined analysis we restricted analysis to complete trios to avoid the possibility of an ascertainment bias when using parent-offspring duos.[30] Finally we performed a test of homogeneity by applying the χ^2-statistic to the table of studies against the number of transmitted and untransmitted alleles (2×9 table; see Table 1).

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