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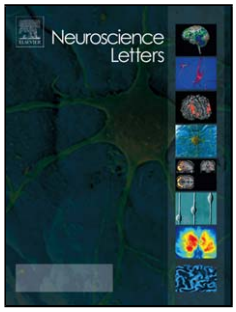

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PII: S0304-3940(09)00897-0
DOI: doi:10.1016/j.neulet.2009.06.084
Reference: NSL 26244

To appear in: *Neuroscience Letters*

Received date: 27-5-2009
Revised date: 18-6-2009
Accepted date: 27-6-2009

Please cite this article as: C. Doyle, K. Brookes, J. Simpson, J. Park, S. Scott, D.R. Coghill, Z. Hawi, A. Kirley, M. Gill, L. Kent, Replication of an Association of a Promoter Polymorphism of the Dopamine Transporter Gene and Attention Deficit Hyperactivity Disorder, *Neuroscience Letters* (2008), doi:10.1016/j.neulet.2009.06.084

Replication of an Association of a Promoter Polymorphism of the Dopamine Transporter Gene and Attention Deficit Hyperactivity Disorder

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N° pages: 10
N° tables: 1

ACKNOWLEDGEMENTS:

The sample collection for this project was partly funded by the Wellcome Trust (LK) and with generous support from the Health Research Board (Dublin (ZH, MB and EB). The authors declare that there are no competing interests. We would like to thank all the families involved in the study for their participation.

KEYWORDS: ADHD; DAT1; Association; Replication; Polymorphism

ABSTRACT:

Genetic associations for Attention Deficit Hyperactivity Disorder (ADHD), a common highly heritable childhood behavioural disorder, require replication in order to establish whether they are true positive findings. The current study aims to replicate recent association findings from the International Multi-centre ADHD Genetics (IMAGE) project in one of the most studied genes related to ADHD, the dopamine transporter (*DAT1*) gene. In a family-based sample of 450 ADHD probands, three single nucleotide polymorphism (SNP) markers have been genotyped using TaqMan assays. Transmission Disequilibrium Test analysis demonstrates that one of three SNP markers (rs11564750) in the 5' promoter region of the gene is significantly associated with ADHD (P=0.02). This provides further evidence that in addition to the well-known and investigated 3'UTR polymorphism associated with ADHD, there is potentially a further association signal emanating from the 5' promoter region of the gene. Further replication and functional studies are now required to fully understand the consequence of polymorphisms present at both the 5' and 3' ends of the *DAT1* gene and their role in ADHD pathophysiology.

INTRODUCTION:

Attention Deficit Hyperactivity Disorder (ADHD) is one of the most prevalent and heritable childhood psychiatric disorders [2], affecting an estimated 3-5% of school aged children [15]. The search for genetic variants that increase the risk for ADHD has focused mainly on the neurotransmitter systems involved in the response of ADHD symptoms to dopaminergic and noradrenergic medications. The dopamine transporter is the main site of action of stimulant medications, which provide a marked and rapid reduction in the level of ADHD symptoms. One of the most interesting findings replicated in several studies is the association between the 10-repeat allele of a Variable Number Tandem Repeat (VNTR) located within the 3'untranslated (3'UTR) region of the dopamine transporter gene (*DAT1).* The association is not present in all samples and indeed a recent meta-analysis found no overall association [19]. Despite this, the association remains of considerable interest due to the number of positive reports and the presence of significant evidence of heterogeneity across the datasets, indicating that a subset of datasets may show true association. One potential cause of heterogeneity could occur if only a subset of individuals carrying the 10-repeat allele is at risk for ADHD. This could arise if for example the *DAT1* risk allele interacts with additional genetic or environmental risk factors, which vary in frequency between the different studies [6, 18, 20]. An alternative explanation, is that the 10-repeat allele is not the causative allele itself, but rather 'tags' a nearby functional variant that is only partially correlated with the 10-repeat allele. Evidence for this comes from the observation in several studies that specific *DAT1* haplotypes containing the 10-repeat allele confer risk for ADHD [3, 4, 6, 13, 16, 17].

In addition, the International Multi-centre ADHD Genetics (IMAGE) project first stage (ST1) samples were screened for association with 32 Single Nucleotide Polymorphism (SNP) markers and two further VNTR markers spanning the *DAT1* gene, as part of a study that included the analysis of 51 candidate genes [5]. To summarise the data from the ST1 *DAT1* gene analysis; six SNP markers and three VNTR markers demonstrated nominal evidence of association with combined type ADHD with significance values of less than P=0.05 for each marker. The association

signal fell into two distinct groups of markers at the 3'and 5' ends of the gene. Genetic variants within each region were found to be in high linkage disequilibrium (LD), but there was little LD between the two regions, suggesting the presence of two independent loci associated with ADHD. Four SNP markers were then subsequently investigated using the second stage (ST2) samples from the IMAGE project [7]. Two of these SNP markers were located in the 5' promoter region of the gene (rs2550946 and rs11564750) with little LD between them; the third was located with intron 10 (rs3776153) and the fourth was located near the 3' end of intron 14 (rs40184), again displaying no LD with the two independent 5' promoter SNP markers. The ST2 sample size was much smaller than the original sample and the sample failed to find association with two of the markers (rs2550946 and rs40184); however the allele observed to be over transmitted to the ADHD probands in each case was the same as detected in the ST1 sample. When the two samples were combined, all four SNP markers were significantly associated with ADHD with P values of less than 0.01 [7]. This study attempts to replicate three of these findings by investigating the association of three SNP markers in the Intron 14 (rs40184 C-allele) and 5' promoter regions (rs11564750 G-allele and rs2550946 G-allele) that were associated with ADHD in samples collected by the IMAGE consortium [5, 7].

MATERIALS AND METHODS:

Sample

For this study 450 ADHD probands (inclusive of 12 affected siblings) and their parents were recruited from several child psychiatry clinics in the UK (N=183) and Ireland (N=267), following approval from the appropriate research ethics committees. Parents were interviewed by trained psychiatrists or psychologists employing the Child and Adolescent Psychiatric Assessment (CAPA) [1]. Consistent interview procedures were employed across the two centres with researchers from each centre receiving a common training in the use of the CAPA. In addition teacher ratings were obtained for children by the Conners Teacher Rating Scale (CTRS) [9]. This was to confirm that symptoms met the criterion of pervasiveness. Established cut-off points for possible and likely ADHD caseness on the CTRS were adhered to i.e. a T score above 55 was required. All probands were white and born in the UK or Ireland (Age range 4-15 years). The sample was predominantly male (90.1%) with no significant

difference in sex ratio between the two study recruitment centres. All probands fulfilled DSM-IV diagnostic criteria for Attention Deficit Hyperactivity Disorder (ADHD). Of these N=42 (9.3%) had the inattentive subtype, and N=38 (8.4%) had the hyperactive impulsive subtype, the rest had the combined subtype (82.3%). Children with an IQ below 70, autistic spectrum disorder or significant medical conditions such as epilepsy were excluded. Two hundred and eleven children (46.9%) had comorbid oppositional defiant disorder and 69 children (15.3%) fulfilled criteria for comorbid conduct disorder. Frequencies of subtype and comorbidity were similar across the two recruitment centres.

Genotyping

High molecular weight genomic DNA was extracted from either whole blood (Wizard Genomic DNA Purification A1620, Promega, Wisconsin) or cheek swab according to routine procedures [12]. Genotyping of the three SNP markers was carried out by TaqMan Genotyping Assays from Applied Biosciences (Applied Biosciences, Foster City, CA). Assays were conducted following the Applied Biosciences standard protocols and run on an Mx3005P (Stratagene, CA) with allele calls determined by MxPro Software version 4.0 (Stratagene, CA). Samples where allele calls were ambiguous were repeated.

Analysis

Transmission Disequilibrium Test (TDT) analysis of the sample was carried out using the UNPHASED software suite of programmes for the association analysis of multi-locus genotype data in families and unrelated subjects [11]. UNPHASED implements maximum-likelihood inference on haplotype and genotype effects while allowing for missing data such as uncertain phase or missing genotypes. TDT analysis observes allele transmissions from the heterozygote parent to the proband. In cases where affected siblings are present (N=12), the parental genotypes are recounted and treated as an independent trio for analysis. Evidence for association of the marker with ADHD was set at the 0.05 level of significance. Given that this study was testing a specific *a priori* hypothesis, aiming to replicate previous significant findings, results have not been corrected for multiple testing.

RESULTS:

Genotyping was successful for 97% or above for the samples for all three SNP markers investigated. Hardy-Weinberg equilibrium calculations were conducted on parent genotypes, and showed all SNP markers to be in equilibrium. Linkage disequilibrium (LD) analysis suggests that LD between all three markers is low (r^2<0.1), as previously observed [5, 7]. Allele frequencies observed in the current study were similar to those observed in the IMAGE study [5, 7] (Table 1).

The current study observed an association with one of the three SNP markers. The 5' promoter SNP marker rs11564750 displayed nominal statistical significance (P=0.02) with the G-allele over transmitted to ADHD probands (T:56 NT:34, OR1.65) (Table 1). TDT analysis for both the other SNP's, although not reaching statistical significance demonstrated OR's greater than 1. For rs2550946, this finding is in the same direction as the previous IMAGE finding with the G allele being over transmitted, but for rs41804, the opposite allele was over transmitted to that found in the IMAGE study.

INSERT TABLE 1 HERE

DISCUSSION:

Replication is essential for establishing the credibility of a genotype–phenotype association [8]. The current study partially replicates the findings identified in previous studies [5, 7], by observing a statistically significant association of the rs11564750 SNP marker with ADHD in an allele-specific manner; both studies found the G-allele to be over transmitted to ADHD probands.

There is gathering support for an association signal from the 5' end of the DAT1 gene, in addition to the association most frequently observed between the 3'VNTR and ADHD [10]. Two other studies have also identified association signals in the 5'

region of the *DAT1* gene with two further SNP markers [14, 21]. In 2006, it was reported that a SNP polymorphism located 67bp upstream to the translation start site was associated with ADHD in a small Iranian population [21]. Subsequently in 2007 another study found an association with a polymorphism further upstream (rs2652511) with ADHD in a Brazilian sample [14].

The rs11564750 polymorphism is located in the 5' region of the *DAT1* gene, and therefore may affect the functionality of the gene by mediating levels of transcription. This has not yet been established and therefore further work must be carried out to investigate this possibility.

The non-replication of an association of ADHD with two of the SNP markers investigated in this study may be due to a lack of power to detect the association, due to the sample size, despite the allele frequencies being comparable between the two studies [5, 19]. The over transmission of the same allele of rs2550946 to ADHD probands as the IMAGE findings, although not reaching statistical significance would support this. In addition it may be that these markers are not risk variants, but may simply be in LD with the true casual polymorphism/s and therefore the statistical association will not be observed in all samples. The IMAGE sample was recruited internationally, compared to the population studied here, which is of UK and Irish origin only. Allelic heterogeneity between different populations may account for the non-replication in our population. Risk variants may also be associated with different relative risks in different populations, which may impact on a study's ability to replicate previous findings. Additionally, all probands in the IMAGE study had combined type ADHD, unlike this current study which includes probands with all subtypes and a different diagnostic instrument was employed in the IMAGE collection compared to that used in this study. Both these factors could account for significant differences between the phenotypes employed in each study, which could also have led to non-replication of 2 of the markers.

Given the findings highlighted in this study and those of its predecessors it is important that future investigations of both the 5' and 3' regions of the *DAT1* gene are conducted in order to elucidate whether these associations both contribute risk to the ADHD phenotype.

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TABLE 1: TDT analysis of the three DAT1 SNP markers

SNP Marker	MAF	Risk Allele	T	NT	OR	P Value	Replicates IMAGE [5, 7]
rs2250946	0.41	G	130	116	1.12	0.38	No
rs11564750	0.13	G	56	34	1.65	0.02	Yes
rs40184	0.45	T	124	115	1.08	0.56	No