Replication of a rare protective allele in the noradrenaline transporter gene and ADHD

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Abstract

Objective—Replication is a key to resolving whether a reported genetic association represents a false positive finding or an actual genetic risk factor. In a previous study screening 51 candidate genes for association with ADHD in a multi-centre European sample (the IMAGE project), two single nucleotide polymorphisms (SNPs) within the norepinephrine transporter (*SLC6A2*) gene were found to be associated with attention deficit hyperactivity disorder (ADHD). The same SNP alleles were also reported to be associated with ADHD in a separate study from the Massachusetts General Hospital in the US.

Method—Using two independent samples of ADHD DSM-IV combined subtype trios we attempted to replicate the reported associations with SNPs rs11568324 and rs3785143 in *SLC6A2*.

Results—Significant association of the two markers was not observed in the two independent replication samples. However, across all four datasets the overall evidence of association with ADHD was significant (for SNP rs11568324 *P*=0.0001; average odds ratio=0.33; for SNP rs3785143 *P*=0.008; average odds ratio=1.3).

Conclusions—The data were consistent for rs11568324, suggesting the existence of a rare allele conferring protection for ADHD within the *SLC6A2* gene. Further investigations should focus on identifying the mechanisms underlying the protective effect.

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Introduction

The gene encoding the norepinephrine transporter (*SLC6A2*, Gene ID: 6530, formerly known as *NET1*) is a strong candidate for genetic association with attention deficit hyperactivity disorder (ADHD). The norepinephrine neurotransmitter system is thought to play a key role in attentional processes and potentially in ADHD. Atomoxetine, a specific noradrenergic reuptake inhibitor is an effective treatment for ADHD, although the slow time course of action suggests that indirect mechanisms are involved in the therapeutic response. Atomoxetine leads to an overall increase in norepinephrine release in the brain especially in the pre-frontal cortex with no reported increases found in other areas of the brain implicated in ADHD such as the striatum. Furthermore the norepinephrine transporter appears to mediate the main part of the reuptake of synaptic dopamine in the frontal cortex.

Several groups have investigated the association of ADHD with genetic variants of the *SLC6A2* gene, which is located on the long arm of chromosome 16. The initial association studies proved negative although only a limited number of SNPs were investigated and these studies failed to screen common genetic variation spanning the entire gene. Xu et al. 2005 were the first to provide a more comprehensive screen of *SLC6A2*. Using a DNA pooling approach to scan 21 SNPs spanning the gene they identified a SNP rs3785157 showing nominal significance for association with ADHD in 180 ADHD probands from the UK (nominal *P*=0.04). They also found a trend association for SNP rs998424 (*P*=0.07). A subsequent report also reported association of ADHD with rs3785157 and rs998424 (Bobb et al., 2005), however the associated alleles were found to be the opposite of that reported in the previous study. An additional study reported a novel promoter SNP (-3081A/T) to be associated with ADHD (Kim et al., 2006).

More recently the gene was investigated in a study of 51 candidate genes in a European collaborative sample collected by the International Multi-centre ADHD Genetics (IMAGE) project (Brookes et al., 2006). A total of 23 SNPs in *SLC6A2* were investigated and two novel SNP associations were identified showing nominal significance for association with ADHD: rs3785143 located in intron 1 and rs11568324 located in intron 5. Further support for association with the same two SNPs has subsequently come for the analysis of 24 SNPs in a sample of 474 ADHD probands collected at the Massachusetts in the United States (Kim et al., 2007). Only the two SNP alleles associated with ADHD in the IMAGE sample showed significance in this study, constituting a direct replication.

To establish whether this finding could be further replicated we have genotyped the two associated markers in two additional datasets from the IMAGE collaborative project sample and from Dublin using a within family test of association. We further report on the combined analysis of all four independent samples that have analysed the association between ADHD and the two SNPs to date.

Method

IMAGE ST2 Sample

European Caucasian subjects were recruited from twelve specialist clinics in eight countries: Belgium, Germany, Holland, Ireland, Israel, Spain, Switzerland and United Kingdom. Ethical approval for the study was obtained from National Institute of Health registered ethical review boards for each centre. Detailed information sheets were provided and informed consent obtained from the majority of children and from all of their parents. All ADHD probands and their siblings were aged 5 to 17 at the time of entry into the study and access was required to one or both biological parents for DNA collection. Entry criteria for probands were a clinical diagnosis of DSM-IV combined subtype ADHD and having one or more full siblings available

for ascertainment of clinical information and DNA collection. Exclusion criteria applying to both probands and siblings included autism, epilepsy, IQ < 70, brain disorders and any genetic or medical disorder associated with externalising behaviours that might mimic ADHD. Inclusion criteria included white European ancestry and living at home with at least one biological parent.

The diagnosis of ADHD was made following a parent interview with the Parental Account of Child Symptoms (PACS; Taylor et al., 1986) that asks about ADHD symptoms in various settings. An algorithm was used to derive each of the DSM-IV ADHD symptoms from the PACS interview data and these were combined with items that scored 2 or more from teacher ratings of DSM-IV items taken from the long version of the Conners' Teacher Rating Scale (Conners, 2003). The diagnosis of ADHD was made if sufficient items were identified to fulfil DSM-IV criteria, and both impairment (based on severity of symptoms identified in the PACS interview) and pervasiveness (based on the presence of ADHD symptoms in more than one setting from PACS and scoring more than one item on the teacher Connors) were present. In 28 cases where no Connors data was present pervasiveness was defined on the basis of PACS data alone. More information on the clinical evaluation of the ADHD probands and the stage 1 (ST1) sample can be found in Brookes et al. 2006.

In this report we include new data from the IMAGE stage 2 sample (ST2) that consists of 435 combined type ADHD cases, including 376 probands and 59 of their affected siblings, with both parents present for 290 of the ADHD probands and only the mother included for 77 of the probands. Following the accumulation of further clinical information and a comprehensive audit of the entire clinical and DNA datasets completed in April 2007, the final set of individuals with combined type ADHD and fulfilling all inclusion and exclusion criteria in ST1 and ST2 was amended. The final combined ST1 and ST2 dataset used in this report consists of 1,147 individuals with DSM-IV combined subtype ADHD cases consisting of 988 affected probands and 159 of their affected siblings.

Dublin sample

Two hundred and twenty two families with clinically diagnosed ADHD children were recruited from child guidance clinics and ADHD support groups around Ireland. The sample selection and clinical procedures for this sample are described in detail in Kirley et al., 2004. The ages of ADHD probands were between 4 and 17. Exclusion criteria included epilepsy, fragile X syndrome, foetal alcohol syndrome, pervasive developmental disorder, Tourettes syndrome and psychosis, IQ <70). To confirm DSM-IV diagnoses of ADHD, one or both parents of each child were interviewed using the child and adolescent psychiatric assessment (CAPA) (Angold et al., 1995). To fulfil ICD-10 and DSM-IV ADHD criteria for symptom pervasiveness, information about ADHD symptoms at school was obtained from teachers using a semi-structured teacher telephone interview which involved asking the class teacher about DSM-IV symptoms of ADHD and impairment shown in class. DSM-IV diagnostic criteria were then applied using the parent and teacher interview data to derive the ADHD diagnostic subtypes.

Control sample

In this study 382 controls of Northern European ancestry were genotyped for rs11568324. The controls were collected from southeast England as part of a separate study of neurocognitive endophenotypes (Kuntsi et al., 2006) and consisted of the parents of child twin pairs who were unselected for phenotype and fulfilled the same age, IQ and exclusion criteria as the IMAGE clinical sample.

Genotyping and statistical analysis

The SNP markers rs11568324 and rs3785143 were included in a 48-SNP multiplex designed by Applied Biosystems (ABI, Foster City, US) as part of their SNPlex chemistry for genotyping. Additional genotyping was performed using the ABI TaqMan protocol under standard conditions provided by ABI with a pre-designed TaqMan® SNP genotyping Assay (Assay ID: C_43668146_10). Genotyping for the samples from Dublin were performed on a commercial platform by KBiosciences (Hoddesdon, UK). Genotype data were analysed using the UNPHASED software (www.mrc.cam.ac.uk/personal/frank/software/unphased) with implementation of the option to handle missing parental genotypes for the ST1 and ST2 samples. The Dublin samples included analysis of complete trios only.

Results

The transmission disequilibrium test results are presented in Table 1. For rs11568324, genotyping was successful for 98.3% of the ST2 sample and 93% of the Dublin sample. The T allele for this marker was previously found to have a low minor allele frequency (MAF) of around 1% and a similar MAF was observed in both samples with genotypes also in Hardy Weinberg Equilibrium (HWE). In both the IMAGE ST2 and Dublin samples the T allele was under-transmitted from heterozygote parents to their affected offspring with similar odds ratios to that observed in the previous two studies; although neither findings were significant on their own (ST2: χ^2=2.57, P=0.10; Dublin: χ^2=2.0, P=0.20).

For rs3785143 genotyping was successful in 88% of the ST2 sample and 89% of the Dublin sample. Despite the relatively low call rates the data were included because the MAF was similar in the ST2 sample to that seen in the ST1 sample (around 10%) and genotypes were in HWE. In the Dublin sample the minor allele frequency was 9% and the genotypes were also in HWE. The results for rs3785143 did not replicate the original data reported, with no evidence of over transmission of the previously identified risk allele in either sample.

Combined analysis

To clarify the evidence for association from the four available studies we completed a combined TDT analysis for the two markers. For rs11568324 each of the four studies found similar odds ratios with the rare T allele being transmitted in 17 out of 49 informative transmissions, indicating moderately strong evidence of association with an overall odds ratio of 0.34 (nominal P= 8×10^{-5}). We further completed analysis using only the complete parent-proband trios from the ST1 and ST2 samples. The data were comparable (ST1: 3T:15NT, p<0.005, OR=0.20; ST2: 4T:9NT, p<17, OR=0.44) with an overall significance in the combined analysis of 6.1×10^{-5}.

Due to the possibility of transmission distortion at this locus or the recognised bias for association with major alleles that results from genotyping errors when using the TDT (Adele et al., 2003) we genotyped an additional set of Northern European controls ascertained from southeast England and compared these to allele frequency data that we had for the two IMAGE samples and the Dublin sample. These data are presented in Table 2 and show that for each of the three samples as well as the overall combination there was evidence for the association in the case control data that was comparable to that obtained from the TDT analysis.

The more frequent rs3785143 did not show consistent effect sizes across the four studies although overall there was a nominally significant transmission of the minor allele to affected offspring with an average odds ratio of 1.3 (P=0.008).

Finally we considered haplotype analysis. The two markers are part of the same haplotype block according to the D' statistic (D'=1.00 for transmitted alleles, D'=0.65 for non-transmitted

alleles). However, due to the marked difference in their allele frequencies they were not strongly correlated with each other ($r^2=0.01$ for transmitted alleles, $r^2=0.03$ for non-transmitted alleles). The two markers are therefore not tagging each other and due to the very low MAF of rs11568324 no additional information was gained from the analysis of the haplotype compared to the single marker data (data available upon request).

Discussion

The data from SNP rs11568324 in *SLC6A2* provides good evidence for association with ADHD. SNP rs11568324 is located in intron 5 and is relatively rare with a minor allele frequency of 1%. Although the rare allele was not found to be significantly under transmitted in the ST2 and Dublin samples the estimated odds ratios were very close to those previously observed in the ST1 and MGH studies. Furthermore, when the data from the four datasets were combined the overall significance for this finding was considerable with an overall significance level of 1×10^{-4} and combined odds ratio of 0.33. Confirmation of this finding requires a significance level that reaches genome-wide levels in the region of 7×10^{-8}, requiring the analysis of additional datasets (Dudbridge and Gusnanto, 2008). Nevertheless, the data presented here indicate that the rare T allele of rs11568324 is likely to confer protection for ADHD. While this finding suggests an important role for the noradrenergic transporter in the neuronal processes underlying ADHD, the very low frequency of the minor allele means that the association has limited relevance to clinical practice.

Since the T allele has a frequency of no more than 1% in the population it is particularly important to exclude potential biases in the data that could lead to a misleading set of findings. A chief candidate for such problems is genotyping error since it would only take a limited number of changes to the transmission ratio to have a dramatic impact on the significance value and effect size for such a rare allele. This could occur for example if the genotype assays failed to observe the rare T allele in some individuals (Adele et al., 2003). This is however thought to be unlikely in this case since all four studies used very different genotyping platforms, did not find Mendelian errors in evaluating parent-offspring relationships and each reported similar effect sizes for the association with odds ratio of 0.28, 0.40 0.31 and 0.33 in the respective studies. The allele frequency observed in each of the four studies is also very similar to that reported in the CEU HapMap panel of 0.008 (http://www.hapmap.org/). Furthermore, the findings were supported in the comparison of case with control allele frequencies, although the allele frequency of 1.44% was higher than that reported in the HapMap panel and the relatively small sample size for the controls indicates that some caution must be taken drawing firm conclusions from this analysis. Finally, in both the IMAGE ST1 sample (Brookes et al., 2006) and the MGH study (Kim et al., 2007) rs11568324 was one of only two nominally associated findings from the analysis of 40 and 24 SNPs spanning *SLC6A2* in the respective studies. The next steps are to further substantiate the evidence for association of rs11568324 with ADHD and investigate the functional mechanisms involved in the protective effect of the rare T allele.

In addition to the association with rs11568324 in intron 5 we also investigated the association of ADHD with rs3785143 located in intron 1. The evidence for association with this SNP is less striking since the two new studies presented here did not replicate the previous findings reported in the IMAGE ST1 and MGH studies. Nevertheless, the combined analysis did not exclude the association of the T allele of rs3785143 ($p<0.01$) and this remains a potential candidate requiring further data to confirm or refute this finding.

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Table 1

Summary of TDT data from the IMAGE Stage I (ST1) and Stage II (ST2); and the Massachusetts General Hospital (MGH) and Dublin samples. T=transmitted alleles from heterozygous parents; NT=non-transmitted alleles from heterozygous parents; OR=odds ratio.

SAMPLE	N trios	rs11568324					rs3785143				
		ALLELE	T	NT	P VALUE	OR	ALLELE	T	NT	P VALUE	OR
ST1	752	T	5	19	0.004	0.26	T	133	95	0.02	1.40
ST2	435	T	6	11	0.22	0.54	T	28	30	0.80	0.93
MGH	474	T	4	13	0.03	0.31	T	93	64	0.02	1.45
Dublin	222	T	2	6	0.16	0.33	T	29	34	0.62	0.85
TOTAL	1,843	T	17	49	0.00008	0.34	T	283	223	0.008	1.27

Table 2

Case-control comparison for genotypes and allele counts for rs11568324.

	N probands	Genotype counts			Allele counts		
		TT	TC	CC	T-allele	C-allele	*p-value	Odds ratio (95% CI)
IMAGE ST1 probands	650	0	4	646	4	1296	0.003	0.21 (0.06 − 0.72)
IMAGE ST2 probands	350	1	4	345	6	694	0.30	0.59 (0.19 − 1.74)
Dublin probands	212	0	2	210	2	422	0.12	0.32 (0.05 − 1.55)
Total probands	1,354	1	10	1201	12	2412	0.007	0.34 (0.14 − 0.83)
Control sample	382	1	9	372	11	753		

*
Each of the samples investigated in this study (ST1, ST2, Dublin) and the combined dataset are compared to a single set of control data. One affected individual used from sibships containing two or more affected cases. Odds ratios are listed for the tests of allelic association. The frequency of the rare protective allele is 0.50% in the combined cases and 1.44% in the controls.