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ORIGINAL RESEARCH ARTICLE

Evidence that variation at the serotonin transporter gene influences susceptibility to attention deficit hyperactivity disorder (ADHD): analysis and pooled analysis

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Reduced central serotonergic activity has been implicated in poor impulse regulation and aggressive behaviour in animals, adults and also young children.[1,2] Two recently published studies have implicated variation at a polymorphism in the promoter of the serotonin transporter (5HTT; hSERT) in influencing susceptibility to ADHD.[3,4] Consistent with these results we have also found a trend for the long allele of the promoter polymorphism to influence susceptibility to ADHD in a sample of 113 ADHD parent proband trios (65 transmissions *vs* 49 non-transmissions, $\chi^2 = 2.25$, $P = 0.13$). A pooled analysis of our, and these published results demonstrated a significant over representation of the long allele of the promoter in ADHD probands compared to controls ($\chi^2 = 7.14$, $P = 0.008$). We have also examined two other 5HTT polymorphisms (the VNTR in intron 2 and the 3′ UTR SNP). TDT analysis demonstrated preferential transmission of the T allele of the 3′ UTR SNP ($\chi^2 = 4.06$, $P = 0.04$). In addition, ETDT analysis of haplotypes demonstrated significant preferential transmission of haplotypes containing the T allele of the 3′ UTR SNP with the long allele of the promoter polymorphism ($\chi^2 = 13.18$, 3 df, $P = 0.004$) and the 10 repeat of the VNTR ($\chi^2 = 8.77$, 3 df, $P = 0.03$). This study provides further evidence for the possible involvement of the serotonin transporter in susceptibility to ADHD.
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Hyperkinetic disorder or attention deficit hyperactivity disorder (ADHD) affects 3–9% of school aged children with more boys diagnosed than girls.[5] ADHD is characterised by marked and pervasive inattention, overactivity and impulsiveness[6] and causes significant social, educational and psychological problems. Quantitative genetic research over the last decade, from family, twin and adoption studies has firmly established that ADHD has a significant genetic contribution.[7,8] As a result, considerable effort has been directed towards identifying susceptibility genes for ADHD. The most effective treatments for ADHD are stimulant medications which act to inhibit the dopamine transporter eg methylphenidate (Ritalin). As a result, a hyperdopaminergic hypothesis has been postulated and is supported by an animal model: a knockout mouse for the dopamine transporter gene (DAT1) exhibits extreme hyperactivity.[9] Of particular interest is the finding that the calming effect of psychostimulants in this mouse appears to be influenced by serotonergic neurotransmission.[10]

Reduced central serotonergic activity has been implicated in poor impulse regulation and aggressive behaviour in animals, adults and also young children.[1,2] The actions of the neurotransmitter serotonin (5-HT) are terminated by reuptake via a sodium-dependent serotonin transporter (5-HTT). Antidepressants such as the selective serotonin reuptake inhibitors (SSRIs) and tricyclics (TCAs), bind to and inhibit the presynaptic serotonin transporter, thereby increasing the concentration of serotonin in the synaptic cleft.

The gene encoding the human serotonin transporter is located at 17q11.2. Three common polymorphisms associated with the transporter have been described: an insertion/deletion in the promoter region,[11] a variable number tandem repeat (VNTR) in intron 2[12,13] and recently a 3′ untranslated region (UTR) G/T single nucleotide polymorphism (SNP).[14] The 44-base pair promoter polymorphism displays two allelic forms, a long and a short variant. The short variant is associated with reduced transcription of the gene and subsequent low functional expression of the transporter.[15] The intronic VNTR has three alleles consisting of either 9, 10 or 12 repeats. Variation at the VNTR can also influence expression of the transporter with the polymorphic VNTR regions acting as transcriptional regulators.[16] The 3′ UTR SNP is within a putative polyadenylation signal for one of the commonly used polyadenylation sites.[14] Although the functional significance of this polymorphism is not known, it is possible that abnormal polyadenylation may interfere with stability of mRNA and facilitation of its transport into the cytoplasm.

The promoter polymorphism has recently been investigated in ADHD. One study[3] reported a positive association in a case-control sample with the long variant and hyperkinetic disorder. A further study,[4] employing the haplotype relative risk (HRR) method,

Table 1 Parental allele frequencies for each polymorphism

Promoter		*VNTR*		*3′ UTR SNP*	
Allele	*Allele frequency*	*Allele*	*Allele frequency*	*Allele*	*Allele frequency*
long	0.6	9 repeat	0.01	G	0.43
short	0.4	10 repeat	0.43	T	0.57
		12 repeat	0.56		

Frequencies in parents of 113 trios. Promoter: long allele = 44-bp insertion; short allele = 44-bp deletion.

reported 'a significant decrease in the short/short genotype' of the promoter in ADHD probands compared to the HRR-derived control group (consisting of untransmitted parental genotypes).

We have attempted to replicate these recently published findings and have also examined other polymorphisms within the serotonin transporter gene in a family-based association study of 113 ADHD probands.

Parental allele frequencies for the 113 trios are shown in Table 1. Genotype frequencies in parents did not demonstrate any significant departure from Hardy–Weinberg equilibrium and were similar for previously published studies: promoter polymorphism[3,4] and the VNTR.[17] Evidence for linkage disequilibrium (LD) between markers was examined by χ^2 analysis in addition to calculating D′, the normalised disequilibrium coefficient.[18] Each possible polymorphism pair demonstrated highly significant moderate LD: Promoter/VNTR $\chi^2 = 17.8$, 1 df, $P < 0.0001$, $D' = 0.45$, Promoter/3′ UTR $\chi^2 = 9.3$, 1 df, $P < 0.005$, $D' = 0.41$ and VNTR/3′ UTR $\chi^2 = 25.5$, 1 df, $P < 0.0001$, $D' = 0.44$. The Transmission Disequilibrium Test (TDT)[19] was employed to test for association. Table 2 demonstrates the TDT results for each of the three individual polymorphisms individually. Consistent with the literature that suggests *a priori* that the long allele of the promoter polymorphism may be associated with ADHD, we have demonstrated a trend in the expected direction (one-tailed $P = 0.07$) but this does not reach statistical significance. Results for the independent trios demonstrate significant preferential transmission of the T allele of the 3′ UTR SNP ($\chi^2 = 4.06$, 1 df, $P = 0.04$). Haplotypes between marker pairs were constructed where phase could be determined and haplotype transmission was examined using the extended TDT (ETDT).[20] Table 2 demonstrates the allele-wise ETDT results for each haplotype pair. For haplotypes containing the VNTR, only one parent had the 9-repeat allele and for simplicity of analysis this was amalgamated with the group containing the 10-repeat allele of the VNTR. There was nominally significant preferential transmission of two haplotypes containing the 3′ UTR T allele: the 10-repeat VNTR/T allele of the 3′ UTR haplotype (χ^2 for the individual transmission = 3.90, uncorrected $P = 0.05$) and the long promoter/T allele of the 3′ UTR haplotype (χ^2 for the individual transmission = 11.58, uncorrected $P = 0.0007$). Significant preferential transmission of the 10-repeat VNTR/long promoter haplotype was demonstrated (χ^2 for the individual transmission = 5.09, uncorrected $P = 0.02$), but the overall allele-wise TDT result for this haplotype was not significant. Given that three possible haplotypes were examined, corrections of the allele-wise TDT results for multiple testing are appropriate. Following this correction, the promoter/3′ UTR haplotype transmission remains significant.

Constructing haplotypes only where phase can be determined may introduce bias.[21] We therefore also analysed our data employing the maximum likelihood method, HAPMAX (http://archive.uwcm.ac.uk/uwcm/mg/download/) which confirmed the significant associations at this locus. This additional method of analysis also allowed us to examine transmission of haplotypes consisting of all three polymorphisms. The haplotype consisting of the long promoter allele-10 repeat VNTR allele-T allele 3′ UTR was preferentially transmitted ($\chi^2 = 19.86$, 7 df, $P = 0.006$).

As described in the Methods section, we also analysed a subset of 90 trios who had completed a more detailed phenotype assessment. TDT and ETDT results were similar to those of the whole sample: individual polymorphisms demonstrated significant preferential transmission for the 3′ UTR T allele only ($\chi^2 = 5.28$, 1 df, $P = 0.02$) and haplotype analysis demonstrated preferential transmission for the same two haplotypes containing the 3′ UTR T allele: the 10-repeat VNTR/T

Table 2 TDT and ETDT results for the promoter, VNTR and 3′ UTR polymorphisms and haplotypes

Individual polymorphism	*Trios (n) for which full information was available*	*No. of informative transmissions*	*Allele-wise χ^2 TDT*	*df*	*P*
Promoter	111	114	2.25	1	ns
VNTR	111	126	0.73	2	ns
3′ UTR	106	120	4.06	1	0.04
Haplotypes					
Promoter/VNTR	80	114	6.22	3	ns
Promoter 3′ UTR	86	123	13.18	3	0.004
VNTR/3′ UTR	81	118	8.77	3	0.03

allele of the 3′ UTR haplotype (65 trios haplotyped, χ^2 for the individual transmission = 5.23, uncorrected $P = 0.02$, overall allele wise $\chi^2 = 12.18$, 3 df, $P = 0.007$) and the long promoter/T allele of the 3′ UTR haplotype (70 trios haplotyped, χ^2 for the individual transmission = 8.47, uncorrected $P = 0.004$, overall allele wise $\chi^2 = 11.7$, 3 df, $P = 0.008$).

In an attempt to replicate the findings of Manor *et al*,[4] the HRR method[22] was also employed to examine the promoter polymorphism in DSM-IV combined type ADHD probands only. In this method, parents' untransmitted alleles are used as the control genotypes. Manor *et al* reported a decrease in the short/short promoter genotype and the short allele in the combined type ADHD probands compared to the HRR-controls in their study (likelihood ratio = 9.62, $P = 0.008$ for genotypes and likelihood ratio = 3.81, $P = 0.07$ for alleles). Although we found a trend in the same direction as the findings of Manor *et al* for both genotypes ($\chi^2 = 2.02$, 2 df, $P = 0.36$) and alleles ($\chi^2 = 1.80$, 1 df, $P = 0.18$), these did not reach statistical significance. Collapsing the long/long and long/short genotypes into one group to compare with the short/short genotype group did not demonstrate any significant effect ($\chi^2 = 1.66$, 1 df, $P = 0.20$).

Finally, we performed a pooled analysis[23] of results for the promoter polymorphism from our study, and the studies by Seeger *et al*[3] and Manor *et al*.[4] To ensure greatest sample homogeneity, we used probands with ADHD (all subtypes), including those with comorbid conduct disorder from ours and Manor *et al*'s studies and employed the probands from Seeger *et al* who fulfilled ICD-10[24] criteria for either hyperkinetic disorder (F 90.0) or hyperkinetic disorder with conduct disorder (F 90.1). The controls used were either HRR controls (ours and Manor *et al*'s) or healthy screened controls (Seeger *et al*). Pooled genotype and allele frequencies are demonstrated in Table 3. There was no significant heterogeneity between samples ($\chi^2 = 0.4$, $P = 0.8$). This pooled analysis demonstrated a significant over representation of the long/long genotype in the ADHD population, and under representation of the short/short genotype with an overall odds ratio of 1.33 (95% CI = 1.06–1.66, $P = 0.01$). Allele frequencies also demonstrate an over representation of the long allele in the probands ($\chi^2 = 7.14$, 1 df, $P = 0.008$).

In conclusion, we have demonstrated preferential transmission of the T allele of the 3′ UTR polymorphism in the serotonin transporter in a sample of 113 ADHD trios in addition to preferential transmission of haplotypes containing the T allele of the 3′ UTR, as well as the long allele of the promoter polymorphism (a finding also supported by the pooled analysis). Given that reduced central serotonergic activity has been implicated in poor impulse regulation, the association of ADHD with the long allele of the promoter polymorphism is biologically reasonable. Of interest, in the Manor *et al* study, the most significant finding for a decrease in short/short homozygosity in the ADHD probands compared to HRR controls was observed when only the combined and impulsive subtype ADHD probands were examined, ie inattentive subtype was excluded so all selected probands had some degree of impulsivity.

This study provides further evidence for the possible involvement of the serotonin transporter in influencing susceptibility to ADHD. Our power to replicate the odds ratio of 1.46, from the comparable Seeger *et al* sample implicating the long allele of the promoter polymorphism in ADHD, was 60%. This may be one reason why our finding, although consistent with the literature may not have reached statistical significance. It is also possible that the 3′ UTR SNP is the functional variant. Previously reported significant association for the promoter polymorphism and ADHD may be by virtue of the LD between the promoter and 3′ UTR SNP

Table 3 Individual study and pooled promoter polymorphism genotype and allele frequencies and odds ratios

Study		*Genotype frequencies*			*Odds ratio for alleles (95% CI)*
		long/long (%)	*long/short (%)*	*short/short (%)*	
Kent *et al* (this paper)	Probands	43 (38)	56 (50)	14 (12)	1.29 (0.89–1.89)
	HRR-Controls	36 (32)	56 (50)	21 (18)	
Manor *et al*[4]	Probands	27 (28)	56 (57)	15 (15)	1.23 (0.83–1.83)
	HRR-Controls	28 (28)	44 (44)	26 (26)	
Seeger *et al*[3]	Probands	35 (44)	25 (31)	20 (25)	1.46 (1.00–2.14)
	Controls	40 (25)	83 (50)	40 (25)	
Pooled	Probands	105 (36)	137 (47)	49 (17)	1.33 (1.06–1.66)
	Controls	104 (28)	183 (49)	87 (23)	
		Pooled allele frequencies			
		long	*short*		
Probands		347 (60)	235 (40)		
Controls		391 (52)	357 (40)		

with the promoter acting as a marker. It will be useful for future studies of the serotonin transporter in ADHD to examine the 3′ UTR SNP.

Methods

Sample

This collaborative study was approved by the relevant research ethics committees. Written informed consent was obtained from parents, and children either provided written informed consent or assent. The sample was recruited from child psychiatry clinics in Birmingham, UK and Ireland and consisted of 113 (60 from Ireland, 53 from Birmingham) parent-proband trios. All probands fulfilled DSM-IV[6] diagnostic criteria for Attention Deficit Hyperactivity Disorder (ADHD). All of the Birmingham sample and 62% of the Irish sample were interviewed employing the Child and Adolescent Psychiatric Assessment (CAPA).[25] Consistent interview procedures were employed across the two centres, with researchers from each centre receiving a common training in the use of the CAPA. The remaining 38% of the Irish sample met criteria for DSM-IV ADHD following consensus diagnosis according to procedures published previously.[26] Both the full sample of 113 trios and the subset of 90 trios who had been interviewed with the CAPA were analysed. Probands were Caucasian, born in the UK or Ireland and were aged 5–16 years (mean age = 11.1, SD = 3.0). Of the 113 probands, 103 (91%) were male and 10 (9%) were female. Of the 113 probands, 91 (81%) were combined type, nine (8%) were inattentive subtype and 13 (11%) were hyperactive/impulsive subtype. In terms of comorbidity 44 (39%) were comorbid for oppositional defiant disorder, and 17(15%) for conduct disorder. Children with a significant learning disability, autistic spectrum disorder or significant medical condition such as epilepsy were excluded. Up to 20 ml of venous blood or a cheek swab was obtained from each subject.

Genotyping

Genomic DNA was extracted from either venous blood or cheek swabs according to standard procedures. For the promoter polymorphism (primer pair 5′ GGCGTTGCCGCTCTGAATGC 3′ and 5′ GAGGGACT GAGCTGGACAACCAC 3′), touchdown PCR amplification was performed with an initial denaturation at 95°C for 4.5 min, followed by 28 cycles with denaturation at 95°C for 30 s, annealing temperatures dropping from 63°C to 55°C for 30 s and extension at 72°C for 30 s. Final extension was at 72°C for 5 min. The PCR products were visualised on 2% agarose gel: a short allele of 484 bp and a long allele of 528 bp.

The VNTR polymorphism (primer pair 5′ GTCAGTATCACAGGCTGCGAG 3′ and 5′ TGTTCC TAGTCTTACGCCAGTG 3′) amplification was performed with an initial denaturation at 94°C for 4.5 min followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 61°C for 30 s and extension at 72°C for 30 s. Final extension was at 72°C for 10 min. The PCR products were visualised on 2.5% agarose gel: a 9-repeat allele of 250 bp, a 10-repeat allele of 267 bp and a 12-repeat allele of 300 bp.

The 3′ UTR G/T SNP was amplified (primer pair 5′ CATCAGGAAAGGAAGA TGTAAGAAGCTTAA 3′ and 5′ GTACCCTTCCAATAATAACCTCCATAC 3′) with a touch down procedure with an initial denaturation at 94°C for 3.5 min, followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 58°C dropping by one degree each cycle to 48°C for 30 s and extension at 72°C for 30 s. Final extension was at 72°C for 5 min. The product (208 bp) was digested for 3 h with *Mse I* and the resulting polymorphic fragments were separated on a 3% sea agarose gel into alleles G (27 bp and 181 bp) and T (27 bp, 52 bp and 129 bp).

Statistical analysis

Genotype frequencies for each polymorphism were compared to frequencies expected under Hardy–Weinberg equilibrium using a χ^2 goodness-of-fit test. The Transmission Disequilibrium Test[19] and the extended Transmission Disequilibrium Test (ETDT)[20] was employed to test for evidence of distorted transmission. Haplotypes between marker pairs were constructed where phase could be determined. Evidence for linkage disequilibrium between markers was examined by χ^2 analysis in addition to calculating D, the coefficient of disequilibrium and D′, the normalised disequilibrium coefficient.[18] Pooled analysis was performed according to the method described by Woolf.[23]

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