American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 134B:115–118 (2005)

DNA Pooling Analysis of 21 Norepinephrine Transporter Gene SNPs With Attention Deficit Hyperactivity Disorder: No Evidence for Association

Xiaohui Xu, Jo Knight, Keeley Brookes, Jonathan Mill, Pak Sham, Ian Craig, Eric Taylor, and Philip Asherson*

MRC Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, King's College, London

The norepinephrine system is known to play a role in attentional and cognitive-energetic mechanisms and is thought to be important in attention deficit hyperactivity disorder (ADHD). Stimulant medications are known to alter the activity of norepinephrine as well as dopamine in the synapse and the highly selective norepinephrine reuptake inhibitor, atomoxetine, is an effective treatment for ADHD symptoms. This study set out to investigate whether common polymorphisms within the norepinephrine transporter gene (NET1) are associated with DSM-IV ADHD combined subtype, using a sample that has previously shown association with genes that affect the synaptic release and uptake of neurotransmitters; DAT1 and SNAP-25. We identified 21 single nucleotide polymorphisms (SNPs) from publicly available databases that had minor allele frequencies ≥5% and span the NET1 genomic region, including those analyzed in previous studies of ADHD. DNA pooling was used to screen for associations using two case pools (n = 180 cases) and four control pools (n = 334 controls). We identified three SNPs that showed suggestive evidence for association using either case-control or within family tests of association, however, none of these were significant after adjustment for the number of markers analyzed. We conclude that none of the markers show significant evidence of association with ADHD although we cannot rule out small genetic effects. © 2005 Wiley-Liss, Inc.

KEY WORDS: norepinephrine; polymorphisms; ADHD; DNA pooling; association study

Attention deficit hyperactivity disorder (ADHD) is one of the most prevalent, stable, and heritable conditions of childhood. Current estimates indicate that 3%–6% of school age children are diagnosed with ADHD [Swanson et al., 2000]. The disorder is known to be both familial and heritable and polymorphic variations within several genes that regulate dopamine neurotransmitter pathways have been found to be associated with the ADHD in several studies [reviewed in Asherson, 2004]. The norepinephrine system is another interesting candidate pathway where genetic variation might influence risk for ADHD. Norepinephrine transmission is known to play a role in attention and behavioral flexibility [Aston-Jones et al., 1999] and a causal role for dysregulation of these pathways has therefore been suspected as an underlying factor in ADHD [Pliszka et al., 1996; Biederman and Spencer, 1999]. Direct pharmacological evidence for the potential role of the norepinephrine transporter comes from the demonstration that Atomoxetine, a specific norepinephrine reuptake inhibitor, is an effective and specific treatment for ADHD.

The norepinephrine transporter gene (NET1) maps to chromosome 16q12.2 [Bruss et al., 1993], consists of 14 exons and spans approximately 45 kb [Porzgen et al., 1995]. To date there have been two studies reporting on the association of NET1 polymorphisms and ADHD. Barr et al. [2002] examined three SNPs located in exon 9, intron 9, and intron 13 in a sample of 122 families with a total of 155 children with ADHD but found no evidence for the association with ADHD. In the second study, McEvoy et al. [2002] examined two SNPs located in intron 7 and intron 9 using nuclear families from Ireland but again their results showed no evidence for the association with ADHD. The aim of this study was to provide a more comprehensive screen of NET1 by investigating all available single nucleotide polymorphisms (SNPs) in a sample that had previously shown association with genes that affect the synaptic release and reuptake of neurotransmitters; DAT1 [Curran et al., 2001] and SNAP-25 [Mill et al., 2002, 2004].

DNA samples were collected from 180 DSM-IV ADHD combined subtype probands, from both parents for 116 of the ADHD probands and from the mother alone for 64 of the probands. Cases were referred for assessment if they were thought by experienced clinicians to have a diagnosis of the combined subtype of ADHD under DSM-IV criteria, with no significant Axis I co-morbidity apart from oppositional defiant disorder (ODD) and conduct disorder (CD). Parents of referred cases were interviewed with a modified version of the Child and Adolescent Psychiatric Assessment (CAPA) [Angold et al., 1995]. Information on ADHD symptoms at school was obtained using the long form of the Conners' questionnaire [Conners, 1995]. Following research assessments HYPESCHEME data sheets were completed using data gathered from the research interview, questionnaire, and where necessary review of case notes. HYPESCHEME is an operational criteria checklist for ADHD and hyperkinetic disorders, which summarizes and applies DSM-IV and ICD-10 operational criteria [Curran et al., 2000]. HYPESCHEME diagnoses were checked against researcher applied DSM-IV criteria and discrepancies reviewed by two researchers (P.A. and S.R.). Where consensus could not be reached, cases were brought to case conference and final consensus agreement made with a senior clinical researcher (E.T.). All probands were of European-Caucasian origin.

A series of 334 unrelated controls from the same ethic background were selected from an UK population sample of

Grant sponsor: The Medical Research Council; Grant sponsor: Wellcome Trust.

*Correspondence to: Philip Asherson, MRC Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, King's College, London. E-mail: p.asherson@iop.kcl.ac.uk

Received 1 July 2004; Accepted 20 October 2004

DOI 10.1002/ajmg.b.30160

© 2005 Wiley-Liss, Inc.

8- and 9-year old twins [Trouton et al., 2002]. Controls were selected on the basis of low ADHD symptom scores, defined as the bottom 20% of the distribution of an index of ADHD-symptoms. The ADHD index was derived as the average of parent rated Strength and Difficulties Questionnaire scores [SDQ; Goodman, 1997] for hyperactivity/inattention at ages 2, 3, and 4 years [Price et al., 2001].

We identified 35 SNPs spanning the NET1 genomic region from publicly available databases that had reported minor allele frequencies $\geq$5% or were coding region polymorphisms with slightly lower heterozygosity. Published heterozygosity values were validated in a test pool of 40 DNA samples from Caucasian control subjects, prior to use for association analysis. Three SNP assays that failed are not reported here. Of the remaining 32 SNPs, 21 were selected for association analysis in the final set, since 14 markers (34% of the SNP set) were insufficiently polymorphic (see Table I).

In the first stage of the analysis, SNP markers were screened for association using a DNA pooling approach. DNA pools were constructed by mixing equal quantities of DNA quantified to a final concentration of 5 ng/μl (±0.5 ng) prior to mixing. The concentration of each DNA sample was measured using the PicoGreen dsDNA Quantitation Reagent (Cambridge Biosciences, Cambridge, UK) in a Fluorimeter (Thermo Life Sciences, Hampshire, UK). DNA pools constructed consisted of two case pools (n = 90, n = 90) and four control pools (n = 90, n = 88, n = 77, n = 79). Each genotype assay was analyzed in quadruplicate on each pool using the SNaPshot^{TM} method (ABI, Foster City). Allele frequencies were estimated from the DNA pool images by averaging across each set of quadruplicate data and adjusting for the unequal peak height observed in heterozygote samples using the method described by Hoogendoorn et al. [2000]. In order to account for both technical error and sampling error in estimating an appropriate significance value for observed allele frequency differences between case and control pools, we adopted a meta-regression method (MRM) for the analysis of multiple pools (Knight and Sham, unpublished method). For each pool we derived an estimate of the effect size, the variance of the estimate and an independent variable relating to the phenotype of the individuals in each pool. Effect size of each pool is taken to be the average allele frequency over the measurements from the

TABLE I. List of dbSNP Markers That Were Analyzed in Test Pools With Their Estimated Heterozygosity Rates

dbSNP rs no.	Contig position (NT_010498)	Gene location	Published heterozygosity	Estimated heterozygosity	Estimated allele frequency differences	MRM *P*-value estimates	χ^2 statistic *P*-value (TDT *P*-value)
rs2242446	4414331	5′ sequence	0.46	0.38	0.01	ns	
rs1610905	4415736	Intron 2	0.50	0.49	0.02	ns	
rs3785143	4419012	Intron 2	0.32	0.20	0.02	ns	
rs747107	4419627	Intron 2	0.29	0.23	0.05	ns	
rs747106	4419630	Intron 2	0.10	NP	—	—	
rs40434	4423431	Intron 2	0.47	0.48	0.01	ns	
rs192303	4424130	Intron 2	0.44	0.43	0.01	ns	
rs187714	4430403	Intron 4	0.48	0.49	0.02	ns	
rs3785151	4436422	Intron 4	0.17	0.25	0.01	ns	
rs3785152	4440453	Intron 4	0.17	0.20	0.01	ns	
rs2270935	4442787	Intron 4	0.25	NP	—	—	
rs40616	4445190	Intron 5	0.50	0.49	0.02	ns	
rs5563	4449924	Exon 6 (non-synonymous)	0.08	NP	—	—	
rs3785156	4450377	Intron 6	0.10	NP	—	—	
rs47958	4450465	Intron 6	0.50	0.49	0.05	ns	
rs1861647	4452409	Intron 7	0.35	0.47	0.04	ns	
rs2279805	4453127	Intron 7	0.33	0.50	0.02	ns	
rs5565	4453236	Exon 8 (non-synonymous)	0.08	NP	—	—	
rs5567	4453294	Exon 8 (non-synonymous)	0.08	NP	—	—	
rs3785157	4453839	Intron 8	0.33	0.46	0.11	0.001	0.034 (0.04)
rs5568	4454127	Intron 8	0.46	0.44	0.00	ns	
rs1566652	4455578	Intron 9	0.44	0.45	0.01	ns	
rs36010	4455671	Intron 9	0.35	0.07	0.02	ns	
rs5570	4455939	Exon 10 (non-synonymous)	0.18	NP	—	—	
rs998424	4455949	Intron 10	0.42	0.48	0.13	0.01	0.066 (ns)
rs5558	4457562	Exon 12	0.08	NP	—	—	
rs5559	4458105	Exon 13 (non-synonymous)	0.09	NP	—	—	
rs5560	4459798	Exon 14 (synonymous)	0.03	NP	—	—	
rs2242447	4459915	Intron 15	0.50	0.43	0.011	0.004	ns (0.07)
rs5561	4460237	Exon 15 splice site	0.18	NP	—	—	
rs15534	4460530	3′ sequence	0.40	0.30	0.02	ns	
rs42460	4461661	3′ sequence	0.30	0.16	0.05	ns	

Markers were selected if minor allele frequency was >5% (9.5% heterozygosity). In addition, we selected six coding region variants with slightly lower minor allele frequencies. Estimated allele frequency differences are calculated from differences in the means of the case versus control pool comparisons. *P*-values are estimated using the meta-regression method (MRM). True *P*-values were calculated from χ^2-analysis of the individual case-control genotypes and TDT using parental genotypes.

TABLE II. Individual Genotype Counts and Frequencies for the Three Markers That Provided Some Evidence for Association

SNP marker	rs3785157		rs998424		rs2242447	
Allele	1	2	1	2	1	2
Control allele counts (frequency)	433(0.652)	231 (0.348)	437 (0.658)	227 (0.342)	212 (0.318)	454 (0.682)
Probands allele counts (frequency)	257(0.714)	103 (0.357)	250 (0.698)	108 (0.302)	135 (0.375)	225 (0.625)
Allele frequency difference (%)	6.2		4.0		5.7	
Odds ratio (95% CI)	1.3 (1.0–1.8)		1.2 (0.9–1.6)		1.17 (1.0–1.4)	

replicate pools. The measurement variance for each marker (j), within a pool, is calculated using data from the replicate pools:

$$\sigma_j^2 = \frac{\sum_{i=1}^{n} (p_{ij} - \bar{p}_j)^2}{n-1}$$

with n the number of replicates; p_j the average allele frequency across the replicates; p_{ij} the allele frequency for each replicate.

An average measurement of experimental variance for a marker across the different pools is calculated. The total variance for each pool is then calculated:

$$Variance = \frac{\bar{p}_j(1-\bar{p}_j)}{2y} + \frac{\hat{\sigma}^2}{n}$$

Sampling variance — Measurement variance

where p_j is average allele frequency; y is number of individuals; n is number of pools.

From the 21 SNPs selected for screening in the test pools, we identified six that showed evidence of association between case and control pools using a lax criterion ($P < 0.1$) when analyzed using a chi-square comparison method of the estimated allele counts within each pool. Three of these remained significant following estimation of the P-value using the MRM method; rs3785157 $P = 0.001$; rs998424 $P = 0.01$; rs2242447 $P = 0.004$.

In the second stage of the analysis we analyzed the six SNPs identified by the chi-square comparisons by individual genotyping of DNA samples. These data confirmed the non-significance by MRM of three negative markers and trends for association with rs3785157 ($P = 0.04$) and rs2242447 ($P = 0.07$), but not rs998424. Haplotypes of the six markers analyzed using WHAP [Purcell et al., http://statgen.iop.kcl.ac.uk] did not provide additional evidence for the association. Family trio data (probands plus parents) was subsequently analyzed for the three markers using TDTPHASE (http://www.hgmp.mrc.ac.uk) and showed trends for association with rs3785157 ($P = 0.034$) and rs998424 ($P = 0.066$). All haplotype combinations were analyzed using the-EM option of TDTPHASE to include both phase known and unknown haplotype data and trimming low frequency haplotypes (<2%) from the analysis, however these tests did not provide additional evidence of association. The data from individual genotyping of the three putatively associated markers are summarized in Tables I and II.

Marker–marker linkage disequilibrium (LD) was evaluated using the program 2LD [Zhao et al., 2000]. Significant LD was observed over the region of the three associated SNP markers, which was very strong between rs3785157 and rs998424 ($D' = 0.97$, $R^2 = 0.95$), and strong for rs3785157 and rs2242447 ($D' = 0.59$, $R^2 = 0.30$), and rs998424 and rs2242447 ($D' = 0.61$, $R^2 = 0.32$).

The potential role of norepinephrine pathways in ADHD has long been suspected and NET1 is therefore an interesting candidate gene to investigate. To date there have been two association studies of NET1 and ADHD in children and one study in a sample of adults with ADHD, all of which have failed to find evidence for an association [Barr et al., 2002; McEvoy et al., 2002; De Luca et al., 2004]. However these studies used a relatively small number of available polymorphic markers. Here we screened 32 SNPs spanning NET1 of which only 21 were sufficiently polymorphic for the detection of a putative common functional allele associated with ADHD. Our analyses identified three SNPs that showed suggestive evidence for association from case-control and within family tests of association, although only one marker (rs3785157) was significant under both methods using a nominal P-value of 0.05. Haplotype and LD analysis using either the case-control data or the proband-trio data provided no additional evidence for the association, but did suggest that the associated markers were co-segregating together in the population due to their close proximity; 6.4 kb between the two outer associated markers).

The use of DNA pooling in the initial screening stage provided an efficient and cost-effective approach to screening multiple SNPs for allelic association. However, the estimated significance levels were less than those calculated from individual genotyping suggesting that technical difficulties were inflating the evidence for association. The cause of these difficulties is unclear, although accurate DNA quantification and variability of a few SNP assays are problems that have the potential to generate type I errors. The possibility of type II errors has not been evaluated in this study.

Overall, we conclude that none of the markers analyzed show significant evidence of association with ADHD since the significance values are marginal and do not survive adjustment for the number of markers tested. Nevertheless, we cannot rule out the possibility of small genetic effects. When considered alongside the previously published datasets using NET1 markers in ADHD, there is currently no good evidence for the association of ADHD with NET1 polymorphisms.

ACKNOWLEDGMENTS

The Medical Research Council funded the sample collection and the Wellcome Trust the genotyping for this study. We thank Dr. Margaret Thompson, Dr. Ann York, Dr. Quentin Spender, Dr. Saama El Abd, Dr. Mark Berlowitz, Dr. Fiona McNicholas, Dr. Mary Cameron, Sandra Richards, Jonathan Sharp, Claire Batten, and Shamira Fernando for their assistance in recruiting the clinical sample. We thank the families that took part in this research.

REFERENCES

Angold A, Prendergast M, Cox A, Harrington R, Simonoff E, Rutter M. 1995. Child and Adolescent Psychiatric Assessment (CAPA). Psychol Med 25: 739–753.

Asherson P. 2004. Image Consortium. Attention deficit hyperactivity disorder in the post-genomic era. Eur Child Adolesc Psychiatry 13(Supp 1): 150–170.

Aston-Jones G, Rajkowski J, Cohen J. 1999. Role of Locus Coeruleus in attention and behavioral flexibility. Biol Psychiatry 46:1309–1320.

Barr CL, Kroft J, Feng Y, Wigg K, Robert W, Malone M, Ickowicz A, Schachar R, Tannock R, Kennedy J. 2002. The norepinephrine transporter gene and attention-deficit hyperactivity disorder. Am J Med Genet 114:255–259.

Biederman J, Spencer T. 1999. Attention-deficit/hyperactivity disorder (ADHD) as a norepinephrine disorder. Biol Psychiatry 46:1234–1242.

Bruss M, Kunz J, Lingen B, Bonisch H. 1993. Chromosomal mapping of the human gene for the tricyclic antidepressant-sensitive norepinephrine transporter. Hum Genet 91:278–280.

Conners CK. 1995. The Conners rating scales: Instruments for assessments of childhood psychopathology. Durham, NC: Duke University.

Curran S, Taylor E, Asherson P. 2000. HYPESCHEME: An operational criteria checklist and minimum data set for molecular genetic studies of attention deficit hyperactivity disorders. Am J Med Genet 96:244–250.

Curran S, Mill J, Tahir E, Kent L, Richards S, Gould A, Huckett L, Sharp J, Batten C, Fernando S, Ozbay F, Yazgan Y, Simonoff E, Thompson M, Taylor E, Asherson P. 2001. Association study of a dopamine transporter polymorphism and attention deficit hyperactivity disorder in UK and Turkish samples. Mol Psych 6:425–428.

De Luca V, Muglia P, Jain U, Kennedy JL. 2004. No evidence of linkage or association between the norepinephrine transporter (NET) gene MnlI polymorphism and adult ADHD. Am J Med Genet 124B:38–40.

Goodman R. 1997. The strengths and difficulties questionnaire: A research note. J Child Psychol Psychiatry 38:581–586.

Hoogendoorn B, Norton N, Kirov G, Williams N, Hamshere ML, Spurlock G, Austin J, Stephens MK, Buckland PR, Owen MJ, O'Donovan MC. 2000. Cheap, accurate and rapid allele frequency estimation of single nucleotide polymorphisms by primer extension and DHPLC in DNA pools. Hum Genet 107:488–493.

Knight J, Sham PC. 2004. DNA pooling analysis methods. Am J Med Genet, Part B (Abstracts for the XIIth World Congress of Psychiatric Genetics 130B:7.

McEvoy B, Hawi Z, Fitzgerald M, Gill M. 2002. No evidence of linkage or association between the norepinephine transporter (NET) gene polymorphisms and ADHD in the Irish population. Am J Med Genet 114: 665–666.

Mill J, Curran S, Kent L, Gould A, Huckett L, Richards S, Taylor E, Asherson P. 2002. Association study of a SNAP-25 microsatellite and attention deficit hyperactivity disorder. Am J Med Genet 114:269–271.

Mill J, Richards S, Knight J, Curran S, Taylor A, Asherson P. 2004. Haplotype analysis of SNAP-25 suggests a role in the aetiology of ADHD. Mol Psychiatry 9:801–810.

Pliszka SR, McCracken JT, Mass JW. 1996. Catecholamines in attention-deficit hyperactivity disorder: Current perspectives. J Am Acad Child Adolesc Psychiatry 35:264–272.

Porzgen P, Bonisch H, Bruss M. 1995. Molecular cloning and organization of the coding region of the human norepinephine transporter gene. Biochem Biophys Res Commun 215:1145–1150.

Price TS, Simonoff E, Waldman I, Asherson P, Plomin P. 2001. Hyperactivity in preschool children is highly heritable. J Am Acad Child Adolesc Psychiatry 40:1362–1364.

Purcell SM, Daly MJ, Sham PC. 2003. SNP haplotype analysis for qualitative and quantitative traits in unrelated individuals and parent-offspring trios. Am J Med Genet, Part B (Abstracts for the XIth World Congress of Psychiatric Genetics) 122B:105.

Swanson JM, Flodman P, Kennedy J, Spence MA, Moyzis R, Schuck S. Murias M, Barr C, Smith M, Posner M. 2000. Dopamine genes and ADHD. Neurosci Behav Rev 24:21–25.

Trouton A, Spinath FM, Plomin R. 2002. Twins early development study (TEDS): A multivariate, longitudinal genetic investigation of language, cognition and behavior problems in childhood. Twin Res 5:444–448.

Zhao JH, Curtis D, Sham PC. 2000. Model-free analysis and permutation test for allelic associations. Hum Hered 50:133–139.