ORIGINAL ARTICLE

A Common Haplotype of the Dopamine Transporter Gene Associated With Attention-Deficit/Hyperactivity Disorder and Interacting With Maternal Use of Alcohol During Pregnancy

Keeley-Joanne Brookes, BSc; Jon Mill, PhD; Camilla Guindalini, BSc; Sarah Curran, MRCPsych, PhD; Xiaohui Xu, MD; Jo Knight, PhD; Chih-Ken Chen, MD, PhD; Yu-Shu Huang, MD; Vaheshta Sethna, BSc; Eric Taylor, FRCP, FRCPsych, PhD; Wai Chen, MRCPsych; Gerome Breen, PhD; Philip Asherson, MRCPsych, PhD

Context: Attention-deficit/hyperactivity disorder (ADHD) is a common heritable childhood behavioral disorder. Identifying risk factors for ADHD may lead to improved intervention and prevention. The dopamine transporter gene (*DAT1*) is associated with ADHD in several studies, with an average 1.2 odds ratio and evidence of heterogeneity across data sets.

Objective: To investigate sources of heterogeneity by refining the *DAT1* association using additional markers and investigating gene-environment interaction between *DAT1* and maternal use of alcohol and tobacco during pregnancy.

Design: Prospective study.

Setting and Patients: Children with ADHD from child behavior clinics in the southeast of England and in the Taipei area of Taiwan.

Interventions: Within-family tests of association using 2 repeat polymorphisms in the 3′ untranslated region and intron 8 plus additional markers in the English sample.

Main Outcome Measures: Transmission ratios of risk alleles from heterozygote parents to affected offspring and comparison of the transmission ratios in high- and low-exposure groups for the environmental variables.

Results: A novel association was identified between ADHD, the intron 8 polymorphism, and a specific risk haplotype in both English and Taiwanese samples. The risk haplotype showed significant interactions with maternal use of alcohol during pregnancy.

Conclusions: The identification of a common haplotype in 2 independent populations is an important step toward identifying functionally significant regions of *DAT1*. Interaction between *DAT1* genotypes and maternal use of alcohol during pregnancy suggests that *DAT1* moderates the environmental risk and has implications for the prevention of ADHD. Further studies are required to delineate the precise causal risk factor involved in this interaction.

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Author Affiliations: Medical Research Council Social Genetic Developmental Psychiatry Centre, Institute of Psychiatry, London, England (Mss Brookes, Guindalini, and Sethna and Drs Mill, Curran, Xu, Knight, W. Chen, Taylor, Breen, and Asherson); and Department of Psychiatry, Chang Gung Memorial Hospital, Taipei, Taiwan (Drs C.-K. Chen and Huang).

ATTENTION-DEFICIT/HYPERactivity disorder (ADHD) is one of the most prevalent and heritable childhood behavioral disorders.[1-3] The disorder is characterized by an onset of age-inappropriate hyperactivity, impulsivity, and inattentiveness before the age of 7 years (*DSM-IV*). Familial risk is established, with an estimated sibling risk ratio (λ_s = [Risk to Siblings of ADHD Probands]/[Population Risk]) for broadly defined ADHD of around 3-fold to 4-fold.[4] Twin studies suggest that genetic factors are the major influence on familial risk, with heritability estimates for ADHD symptom scores consistently reported to be in the region of 60% to 90%.[5] In general, these studies find little evidence of shared environmental influences on familial occurrence, although the role of environment may still be pivotal, acting through mechanisms of gene-environment interaction.[6]

Progress in identifying some of the genes involved in ADHD susceptibility has been relatively fruitful during the past decade by screening genetic variants that lie within or close to genes that regulate neurotransmitter systems, particularly dopamine pathways.[7] Of particular interest are reports of association between ADHD and the 10-repeat allele of a 40–base pair (bp) variable number tandem repeat polymorphism (VNTR) in the 3′-untranslated region (UTR) of *DAT1*. This association has been reported in several studies and meta-analyses, although the net effect across studies appears to be small, with an average odds ratio around 1.2.[8-19] There are in addition

recent reports of quantitative trait locus associations with ADHD-trait scores in general population samples,[20,21] interaction with maternal smoking during pregnancy,[22] association with poor performance on a sustained attention task and an increase in neuronal activity in response to treatment with methylphenidate hydrochloride,[23] and treatment response to stimulant medication.[24-27]

Despite the number of studies supporting the 10-repeat allele association with ADHD, it is still not clear whether the 3′ UTR VNTR has direct functional significance. Meta-analysis finds significant evidence of heterogeneity across the various published data sets,[15] although the source of this variation is currently unknown. One possible source of heterogeneity could arise if the 3′ UTR VNTR was not the functional site itself but was acting as a tagging marker for a nearby functional site, or if the VNTR sequence interacted with a second functional polymorphic site. In this case, differences in the strength of association between the 3′ UTR VNTR and an alternative *DAT1* functional site, or differences in the frequency of interacting genetic variants, could influence the size of main effects observed with the 10-repeat allele. There are some published data addressing this issue. Barr et al[16] reported significant evidence of increased transmission of a haplotype of the 10-repeat allele with single-nucleotide polymorphism (SNP) alleles in exon 9 and intron 9, and Galili-Weisstub et al[28] reported a similar finding with an exon 15 SNP. Hawi et al[29] also reported haplotype associations involving the 10-repeat allele, but this time in association with alleles of simple sequence repeat markers flanking the gene. These studies indicate that the 10-repeat allele is most likely acting as a tagging marker for an alternative functional site.

A different potential source of heterogeneity is differing background levels of interacting environmental risks that act to moderate the genetic effects of *DAT1* variants on ADHD. This is plausible since, although ADHD is highly heritable, the heritability coefficient indexes not only the direct effect of genes but also effects of interactions between genes and environments.[6] The importance of gene-environment interactions in behavioral disorders has been highlighted by recent studies demonstrating that some genes show effects only in groups of individuals subjected to specific environmental stressors.[30-32] Gene-environment interactions have considerable relevance for developmental health disorders such as ADHD, where environmental risks have been identified and include maternal smoking and alcohol use during pregnancy[33] and critical expressed emotion.[34]

In this study we provide evidence that there is an alternative functional site in the dopamine transporter gene that is associated with increased risk of ADHD by using 2 independent samples from England and Taiwan. We further investigate whether, in the English population, the associated *DAT1* haplotype interacts with maternal use of alcohol and tobacco during pregnancy.

METHODS

ENGLISH SAMPLE

Samples of DNA were collected from 180 probands with a *DSM-IV* diagnosis of ADHD combined subtype, from both parents of 116 of the probands, and from the mother alone for 64 of the probands. Cases were recruited from child behavior clinics in southeast England and 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 comorbidity apart from oppositional defiant disorder and conduct disorder and IQ greater than 70. Only individuals fulfilling the recruitment criteria after completion of research assessments were included in the study. Of the 180 probands, 96% were male. The age range was 5 to 15 years at the time of assessment (mean±SD age, 10.41±2.34 years).

Parents of referred probands were interviewed with a modified version of the Child and Adolescent Psychiatric Assessment.[35] Information on ADHD symptoms at school was obtained with the long form of the Conners questionnaire[36] and scored as present if rated greater than 1. HYPESCHEME, a computerized operational criteria checklist and diagnostic algorithm for *DSM-IV* and *International Classification of Diseases, 10th Revision*,[37] was used to confirm the diagnosis. HYPESCHEME data sheets were completed with the data from the parent and school questionnaires plus review of case notes. The HYPESCHEME diagnoses were checked against researcher-applied *DSM-IV* criteria, and discrepancies were reviewed by 2 researchers. Situational pervasiveness was operationalized as the presence of 1 or more ADHD symptoms at home and school plus descriptions from the parent of impairment from ADHD symptoms in more than 1 setting. Where consensus could not be reached, cases were brought to case conference and final consensus agreement was made with a senior clinical researcher (E.T.). All probands were of white European origin.

Environmental screening questions were included in our analysis of the English sample. These were simple yes/no response questions about maternal behavior while pregnant with the child with ADHD. Maternal alcohol use during pregnancy was defined by a single question, "*Did you give up alcohol during pregnancy?*" and maternal smoking during pregnancy by the question, "*Did you smoke at least 20 cigarettes a day for 3 months of the pregnancy?*" No other information relating to these 2 environmental risk factors was gathered in this sample. All subjects provided informed consent, and ethical approval was obtained from the local ethical committee of the Institute of Psychiatry, London, England, and the South London and Maudsley NHS Trust, Beckenham, England.

TAIWANESE SAMPLE

Samples of DNA were collected from 216 probands with a *DSM-IV* diagnosis of ADHD, from both parents for 135 of the probands, and from the mother alone for 35 of the probands. Cases of ADHD were ascertained from child psychiatric clinics in the Chang Gung Memorial Hospital in the Taipei area of Taiwan. After completion of a standard maternal interview, the Schedule for Affective Disorders and Schizophrenia for School-Age Children–Present and Lifetime Version,[38] and completion of parent and teacher Conners revised rating scales,[36] diagnosis was applied according to *DSM-IV* criteria. Pervasiveness of symptoms was determined by clinical judgment and was not operationalized in this sample; however, teachers reported at least 3 ADHD symptoms in all cases. Of the 216 probands, 78% had the combined type and 22% the inattentive subtype of ADHD, with no comorbid disorders apart from oppositional defiant disorder, conduct disorder, and possible Tourette syndrome in 4 cases. Of the sample, 89% were male, 13% had an IQ between 50 and 69 (87% were >69), and the age range was 5 to 15 years (mean±SD age, 8.96±2.60).

GENOTYPING AND MARKER SELECTION

In the English sample, we investigated the 3′ UTR VNTR, 3 SNPs within the 3′ end of the gene (*rs40184, rs1042098,* and

Table 1. *DAT1* Markers Genotyped in the White English Sample

Marker Name	Marker No.	Location in Gene	Chromosome 5-bp Position (UCSC Map)
AC repeat	1	Upstream (5′) of *DAT1*	1 586 044
30-bp VNTR	2	Intron 8	1 464 372
AAAC repeat	3	Intron 14	1 450 285
rs40184	4	Intron 14	1 447 815
rs1042098	5	3′ untranslated region	1 447 553
rs27072	6	3′ untranslated region	1 447 260
40-bp VNTR	7	3′ untranslated region	1 446 435
D5S678	8	Downstream (3′) of *DAT1*	1 418 468
D5S2005	9	Downstream (3′) of *DAT1*	1 394 813
D5S1981	10	Downstream (3′) of *DAT1*	1 207 167

Abbreviations: bp, base pair; *DAT1,* dopamine transporter gene; UCSC, University of California, Santa Cruz; VNTR, variable number tandem repeat polymorphism.

rs27072), and 4 microsatellites (AC repeat, *D5S678, D5S2005,* and *D5S1981*) that lie outside of the gene and had previously been reported to show haplotype associations with ADHD.[28] In addition, we investigated an intron 8, 30-bp VNTR and tetranucleotide repeat (AAAC-rpt) that were identified by in silico analysis. In the Taiwanese sample, we genotyped the 2 VNTR markers. The marker locations are listed in **Table 1**.

Standard genotyping assays were followed for the VNTRs, involving 30 cycles of annealing 64°C (intron 8 VNTR) or 60°C (3′ UTR VNTR) for 1 minute and extension 72°C for 1 minute. Polymerase chain reaction products were genotyped on 2% agarose gel, checked, and repeated whenever the band pattern was not clear. The SNPs were analyzed with a detection system (Amplifluor; Serologicals Corp, Norcross, Ga) by Kbiosciences (http://www.kbioscience.co.uk) and microsatellite markers by means of capillary electrophoresis.

ANALYSIS OF GENOTYPE DATA

Within-family tests of association using single and multiple marker-haplotype transmission disequilibrium tests (TDTs) and haplotype-based haplotype relative risk analysis, as well as D′ and Cramer V (r^2) measures of linkage disequilibrium between genetic markers, were calculated by means of UNPHASED (available at http://www.litbio.org). Confirmatory analyses were performed with WHAP (available at http://statgen.iop.kcl.ac.uk). Gene-environment interactions were analyzed by stratifying the probands into groups based on the binary environmental risk measures. The TDT haplotype associations were then recalculated by means of UNPHASED to determine the odds ratios of the haplotypes in each environmental exposure group. A χ^2 comparison of the transmitted vs nontransmitted alleles from parents to their affected offspring tested the significance of the gene-environment interaction. The presence of gene-environment correlation was tested by comparison of maternal haplotype frequencies for the environmental groups with the RUNGC routine of GENECOUNTING (available at http://www.smd.qmul.ac.uk/statgen/dcurtis/software.html).

RESULTS

LINKAGE DISEQUILIBRIUM

We found evidence of extensive linkage disequilibrium, the nonrandom association of alleles at different genetic markers, across the 3′-region of *DAT1* in the English sample (**Table 2**). This concurs with previous observations in a white population of European origin.[39] Allele frequency differences between SNP alleles and the 2 common repeat alleles of the VNTRs give rise to discrepancies between the D′ measure of linkage disequilibrium (which describes the extent of association relative to the maximum that would be possible given the allele frequencies at the 2 sites) and the Cramer V (r^2) measure (which makes no adjustment for allele frequency differences).[40] Linkage disequilibrium between the 2 VNTRs gives rise to the same D′ estimate of 0.4 in both the English and Taiwanese populations and slightly different estimates of Cramer V (0.25 in the English sample and 0.34 in the Taiwanese sample).

ASSOCIATION ANALYSIS OF THE 3′ UTR AND INTRON 8 VNTRS

The TDT data for the 2 VNTR markers are summarized in **Table 3**. As expected from our previous work on subsets of these samples, both the English and Taiwanese samples provide global evidence of association and linkage between the 3′ UTR VNTR and the ADHD phenotype: $P=.003$ (English group) and $P<.001$ (Taiwanese group). The intron 8 VNTR is also associated in both populations with global significance: $P=.006$ (English group) and $P=.03$ (Taiwanese group).

To examine the effect of the risk alleles in combination, we conducted haplotype analysis. Briefly, different combinations of alleles are known as *haplotypes*, and a haplotype is defined as a set of linked or physically close alleles inherited as a unit, with each individual having 2 at any single location because of the diploid nature of the genome. Haplotype analysis programs use algorithms to estimate the probability of each haplotype existing in each individual given his or her genotype and look at how these are transmitted from parents to children. They allow us to look at interactions between loci and to extract information about other associations in the region covered by the haplotypes, which may contain many more variations than those tested.

Examination of the haplotype transmission ratios and haplotype-specific *P* values showed key new findings. In both populations, only the 10/3 haplotype was overtransmitted to probands with ADHD in comparison with all other haplotypes. The haplotype-specific significance transmission ratios were similar in the 2 populations, with odds ratios of 2.56 ($P=.003$) in the Taiwanese sample and 2.59 ($P=.01$) in the English sample.

An alternative haplotype analysis program, WHAP, was used to confirm the haplotype-specific association. WHAP also allows the user to drop 1 or more markers to test whether they contribute significantly to the haplotype association. A significant *P* value in this instance implies that the marker being dropped is contributing significantly to the haplotype association. The results of this analysis were slightly different for the 2 populations. In the Taiwanese sample, dropping both the 3′ UTR VNTR ($P=.02$) and the intron 8 VNTR ($P=.008$) provided a worse fit than using the 2 markers together. In the English sample, dropping the 3′ UTR VNTR gave a worse fit ($P=.02$), whereas there was only a trend for the intron 8 marker ($P=.1$). However, since there was a trend

Table 2. Pairwise LD Estimates in the English Sample Using D′ (LD as a Proportion of Maximum Possible LD) and Cramer V (r^2 Statistic of Absolute LD)

Cramer V by Marker	D′ Statistic by Marker: AC Repeat	Intron 8 VNTR	AAAC Repeat	*rs40184*	*rs1042098*	*rs27072*	3′ UTR VNTR	*D5S678*	*D5S2005*	*D5S1981*
AC repeat		0.19	0.86	0.27	0.29	0.38	0.29	0.22	0.25	0.20
Intron 8 VNTR	0.25		1.00	0.31	0.31	0.45	0.40	0.17	0.12	0.30
AAAC repeat	0.22	0.25		1.00	1.00	1.00	1.00	0.76	0.62	0.46
rs40184	0.36	0.23	0.24		1.00	1.00	0.92	0.24	0.18	0.13
rs1042098	0.34	0.25	0.21	1.00		1.00	0.91	0.22	0.20	0.19
rs27072	0.29	0.15	0.18	0.32	0.32		1.00	0.22	0.30	0.20
3′ UTR VNTR	0.62	0.34	0.24	0.88	0.88	0.26		0.27	0.21	0.14
D5S678	0.20	0.17	0.43	0.31	0.27	0.16	0.26		0.35	0.22
D5S2005	0.20	0.20	0.41	0.33	0.37	0.19	0.24	0.27		0.22
D5S1981	0.18	0.27	0.14	0.13	0.18	0.18	0.15	0.15	0.21	

Abbreviations: LD, linkage disequilibrium; UTR, untranslated region; VNTR, variable number tandem repeat polymorphism.
LD value: >0.80 | 0.06-0.79 | 0.40-0.59 | <0.40

Table 3. Transmission Disequilibrium Test Results for the 3′ UTR VNTR, Intron 8 VNTR, and Haplotypes of the 2 Markers in the Taiwanese and English Samples*

Marker	Repeat No.	Taiwanese Sample: T	NT	χ^2	*P* Value	OR	English Sample: T	NT	χ^2	*P* Value	OR
3′ UTR	(6/8)	2	0	2.00	.2	NA	1	1	0.0	>.99	1.00
	9	6	22	9.14	.003	0.27	30	64	12.3	<.001	0.47
	10	28	9	9.76	.002	3.11	65	32	11.2	<.001	2.03
	11	1	6	3.57	.06	0.17	1	0	1.0	.3	NA
Intron 8	2	27	48	5.88	.02	0.56	32	58	7.5	.006	0.55
	3	51	28	6.70	.01	1.82	58	32	7.5	.006	1.81
	4	1	3	1.00	.3	0.33					
Haplotype	(6/8)/3	2	0	2.00	.2	NA	0	1	1.0	.3	NA
	9/2	4	10	2.57	.1	0.40	10	27	7.8	.005	0.37
	9/3	2	9	4.45	.04	0.22	13	25	3.8	.05	0.52
	10/2	14	24	2.63	.1	0.58	8	14	1.6	.2	0.57
	10/3	46	18	12.25	<.001	2.56	57	22	15.5	<.001	2.59
	10/4	0	2	0.33	.6	0.50	1	0	1.0	.3	NA
	11/2	1	3	1.00	.3	0.33					
	11/3	0	3	3.00	.08	NA					

Abbreviations: LD, linkage disequilibrium; NA, not applicable; NT, allele-specific nontransmission; OR, odds ratio; T, allele-specific transmission; UTR, untranslated region; VNTR, variable number tandem repeat polymorphism.
*Allele-specific transmissions NTs from heterozygote parents are listed with nominal significance values and ORs. Global significance values provided in the text are significant for both the individual VNTR markers and the haplotype in both populations.

in the English sample and that sample was relatively small and lacked power for this test, we have interpreted these data as showing a similar pattern in the 2 populations.

The estimated allele frequencies of the risk allele in each population appear to be similar for the intron 8, 3-repeat allele (82% in the Taiwanese sample and 77% in the English sample). The 3′ UTR 10-repeat allele was found at a higher frequency in the Taiwanese sample (90%) than the English sample (73%). The 10/3 risk haplotype was the most common haplotype in both populations, with an estimated population frequency of 74% in the Taiwanese population and 59% in the English population. As expected, haplotype-based haplotype relative risk analysis of transmitted vs nontransmitted allele counts gave similar significance levels and odds ratios to those derived from the TDT (data not shown). The greatest allele frequency difference between transmitted and untransmitted haplotypes from parents and their affected offspring was for the 10/3 haplotype (15.1% difference in the English sample and 12.6% in the Taiwanese sample). These frequency differences were greater than those seen for the risk alleles analyzed separately for the individual loci.

EXTENDED HAPLOTYPE DATA IN ENGLISH SAMPLE

To investigate the extent of the region associated with ADHD, we examined transmission ratios for each of the 10 genotyped markers across the gene individually, then in pairwise combinations (**Table 4**). This suggested that the following hypothetical risk haplotype, going from left

Table 4. Four-Marker Haplotype Window for the 10 Markers Analyzed in the White English Sample*

AC Repeat	Intron 8 VNTR	AAAC Repeat	*rs40184*	*rs1042098*	*rs27072*	3′ UTR VNTR	*D5S678*	*D5S2005*	*D5S1981*	OR
289	2	194	2							2.0
	2	194	2	2						1.9
		194	2	2	1					1.5
			2	2	1	10				2.0
				2	1	10	250			1.7
				2	1	10	254			1.6
					1	10	250	167		3.3
						10	254	167	262	2.1
						10	254	169	260	1.6

Abbreviations: OR, odds ratio; UTR, untranslated region; VNTR, variable number tandem repeat polymorphism.
*Listed are all observed haplotypes that gave ORs (transmissions/nontransmissions) greater than 1 for the association with attention-deficit/hyperactivity disorder. The shading indicates a risk haplotype that spans the DAT1 region. The 3 microsatellites that lie downstream of the gene do not form part of this risk haplotype, indicated by alternative risk haplotypes when data from this end of the gene are included in the analysis.

Table 5. Transmission-Nontransmission Ratios From Heterozygote Parents to Their Offspring With ADHD for the 4 Common Haplotypes of the *DAT1* VNTR Markers in the 3′ UTR and Intron 8, by Maternal Alcohol Use Status During Pregnancy in the English Sample*

		No Alcohol Exposure					Alcohol Exposure				
Marker	Repeat No.	T	NT	χ^2	*P* Value	OR	T	NT	χ^2	*P* Value	OR
Haplotype	9/2	5	6	0.09	.8	0.83	4	21	11.56	<.001	0.19
	9/3	5	6	0.09	.8	0.83	9	16	1.96	.2	0.56
	10/2	3	2	0.20	.7	1.50	4	12	4.00	.05	0.33
	10/3	10	9	0.05	.8	1.11	45	13	17.65	<.001	3.46

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; NT, nontransmission; OR, odds ratio; T, transmission; UTR, untranslated region; VNTR, variable number tandem repeat polymorphism.
*There is a significant interaction between exposure to alcohol during pregnancy and the transmission ratios for the observed haplotypes ($P = .04$).

to right, was overtransmitted to probands with ADHD: 289-2-194-2-2-1-10. This haplotype was further tested with the use of 4-, 5-, and 6-marker haplotype windows, with similar results. The 4-marker haplotype window results presented in **Table 5** suggest that a single risk haplotype, defined as any haplotype with a TDT odds ratio greater than 1, spans the first 7 markers but breaks down for the last 3 markers. Because for the last 3 markers different alleles appear in alternative haplotypes, these markers do not appear to be part of a single risk haplotype. This was expected, since the last 3 markers are far apart and show low levels of association with the haplotype markers. Because of differences in marker type and low map density, these data cannot fully define the region that contains functional variants responsible for the observed association, but a region spanning at least 140 kilobase (kb) is implicated.

GENE × ENVIRONMENT

The screening questions on maternal behavior found that 57.8% of the mothers drank alcohol at some time during the pregnancy with the child with ADHD, while 28.6% of mothers smoked at least 20 cigarettes a day for 3 months of the pregnancy (12% of mothers did not return answers to these questions). No interactions with genotype were observed for maternal smoking during pregnancy; odds ratios (ORs) for the 10/3 haplotype for exposed (OR, 1.5; $P = .07$) vs nonexposed (OR, 2.27; $P = .07$) probands did not differ significantly ($P = .3$).

There was, however, evidence that the *DAT1* 10/3 haplotype mediates the effects of maternal alcohol use during pregnancy. The ORs estimated from the transmission ratios of heterozygote parents for the 10/3 risk haplotype was higher in the group exposed to the environmental risk (45 transmitted, 13 nontransmitted; OR, 3.46; haplotype-specific $P = .00003$) than the nonexposed group (10 transmitted, 9 nontransmitted; OR, 1.11; haplotype-specific $P = .8$). The difference in the ORs between the 2 exposure groups was significant ($P = .04$). The same comparisons completed with the use of haplotype frequencies from haplotype-based haplotype relative risk analysis also found differences in estimated ORs for the exposed and nonexposed group (OR, 2.7; 95% confidence interval, 1.6-4.5; $P = .00005$; and OR, 1.24; 95% confidence interval, 0.62-2.47; $P = .5$), with a similar level of significance found in a direct comparison of the 2 environmental exposure groups ($P = .04$).

Gene-environment correlation, where the 10/3 haplotype is associated with both mother's use of alcohol during pregnancy and offspring ADHD, was also investigated. The estimated frequencies of the 10/3 haplotype in the 2 groups was 65.9% among mothers who reported alcohol use during pregnancy and 58.9% in the group that did not (trend for association, $P = .07$).

HAPLOTYPE ASSOCIATION IN ASIAN AND EUROPEAN POPULATIONS

The association between the 10-repeat allele and ADHD has been previously reported in the 2 populations investigated,[15,18] and results of analyses of extended samples in both populations were consistent with the earlier reports. Despite reports of association between ADHD and *DAT1*, investigators have failed to identify alternative functional variants in the 3′ region, and it is widely assumed that the VNTR in this region confers altered gene regulation, although there is as yet no firm evidence for this conclusion.[7] Haplotype data from Barr et al,[16] Hawi et al,[29] and Galili-Weisstub et al[28] are, however, consistent with our data from English and Taiwanese samples in suggesting that 1 or more alternative functional sites exist, although the generalizability of these findings to other populations such as those of African or Hispanic descent has yet to be established.

Herein we report the association of ADHD with a novel 30-bp repeat polymorphism within intron 8 of *DAT1* that has 2 common alleles. The more frequent 3-repeat allele is associated with ADHD in both the English and Taiwanese populations. These data are interesting, because the size of the observed genetic effects are similar for both VNTR markers in the 2 populations, despite differences in the ethnicity, culture, diagnostic procedures, and subtype selection. This suggests that the data should be generalizable to other populations. The question arises about the relationship between the 2 associated markers and ADHD. There are several possibilities: (1) One of the 2 VNTRs is the functional site and the other is an associated tagging marker. (2) The VNTRs represent 2 interacting sequences, either as functional sites themselves or by tagging of adjacent functional sites. (3) Both VNTRs are acting as tagging markers for a single functional site not yet identified in the region.

To investigate these competing hypotheses, we used a model-fitting approach implemented within the WHAP program to test the contribution of each VNTR to the haplotype association. This analysis suggested that neither marker can be dropped without a significant change in model fit, so hypothesis 1 is unlikely. A striking observation is that in both populations only 1 of the 4 possible common haplotypes, consisting of the 10-repeat allele and the 3-repeat allele, is preferentially transmitted to affected offspring and is therefore a risk factor, whereas the 3 alternative haplotypes have neutral or protective effects. This is consistent with both hypotheses 2 and 3. The observation by 3 other groups of similar haplotype associations involving the 10-repeat allele[16,28,29] and our observation of an extended association spanning a region of at least 140 kb suggests that the 2 VNTRs are acting as tagging markers for a third alternative functional site. It is not possible, however, to rule out the existence of 2 interacting functional sites that could include 1 or both of the 2 VNTRs.

As yet there are no published data concerning the functional effects of the intron 8 VNTR. The 3′ UTR VNTR has been investigated in more depth, with preliminary evidence that the 10-repeat allele is associated with increased messenger RNA levels in postmortem brain tissue.[41] Evidence from in vivo single-photon emission computed tomography studies find that striatal dopamine transporter density is increased in probands with ADHD compared with controls.[42-44] Although in 1 study striatal dopamine transporter density was also associated with the 10-repeat allele, this was not confirmed in other reports.[45-48]

A clear pattern of differential function effects for the 9-repeat vs 10-repeat alleles has yet to be demonstrated. Fuke et al[49] examined the effect of the VNTR polymorphism on gene expression in human COS-7 cells and found significantly higher expression of a report gene for the 10-repeat than for the 7-repeat or 9-repeat alleles. In contrast, Miller and Madras[50] concluded that the 9-repeat allele was correlated with increased expression in human HEK-293 cells and that expression was further mediated by an SNP also located in the 3′ UTR of *DAT1*. Their findings were not, however, consistent between experiments, with the 9-repeat allele showing no or lower expression compared with the 10-repeat allele in several replications. Mill et al[51] found no differential effects on transcription of 2 alleles in human neuroblastoma and embryonic kidney cells, similar to data from Greenwood and Kelsoe[52] in human SN4741 cells. This last study did, however, report a 1.5-fold difference in regulatory activity between haplotypes of the promoter–intron 1 region and enhancer elements within introns 9, 12, and 14.

DAT1 INTERACTION WITH MOTHERS' USE OF ALCOHOL AND TOBACCO DURING PREGNANCY

Although genetic risk factors are prominent in the development of ADHD, environmental risks, including maternal use of alcohol and tobacco during the pregnancy, are also thought to be important.[53] However, genes are likely to moderate the effects of exposure to environmental risks, and herein we report evidence that the *DAT1* risk haplotype moderates the environmental risk associated with mothers' use of alcohol during pregnancy. In this sample, the association with *DAT1* is seen only in the subset whose mothers reported drinking alcohol during the pregnancy, with a significant difference in the TDT transmission ratios between the 2 environmental exposure groups.

We also investigated the influence of maternal smoking on ADHD, but did not show the interaction with *DAT1* suggested by the report of Kahn et al.[22] However, limitations in the study design preclude drawing firm conclusions on the negative association with maternal use of tobacco. Our study is underpowered to detect small interactional effects, and we had only retrospective accounts of broad screening questions for environmental risks. For smoking we can only exclude the potential effects of smoking more than 20 cigarettes a day during any 3-month period of the pregnancy. There is also substantial room for misclassification of exposed and unexposed groups, since the offspring of a mother who smoked only slightly less than 20 cigarettes a day throughout the pregnancy would be classified in the "tobacco-unexposed" group.

Although our data find an interaction between prenatal use of alcohol and *DAT1* genotypes, we do not have sufficiently detailed information to enable us to quantify the timing and amount of alcohol used by mothers. In addition, the offspring of a mother who had only a single drink in preg-

nancy might be classified within the "alcohol-exposed" group, further limiting interpretation of these data. The causal relationships need to be considered carefully, since maternal drinking may be correlated with parental behaviors that could act as more proximal risk factors, such as levels of critical comments, quality of parenting, and maternal psychopathology including ADHD. Furthermore, interactions with variables that reflect parental behavior may index genetic loading consistent with the increased cotransmission of interacting genes (gene-gene interaction). Although we found preliminary evidence of gene-environment correlation between the 10/3 haplotype and maternal prenatal use of alcohol, we have controlled for this in our analysis by using the TDT, since the test uses transmission of risk alleles from heterozygote parents to their offspring, which is independent of parental allele frequencies. However, this does highlight the complexity of interpreting gene-environment effects where genes cause change in parent as well as offspring behavior, and we cannot draw firm conclusions on the identity of the interacting risk factor.

The possibility of direct toxic effects of alcohol on fetal development is, however, an attractive hypothesis. A specific role for prenatal alcohol exposure has been postulated on the basis of the observation that children with fetal alcohol syndrome tend to be hyperactive, exhibit cognitive deficits, and are at increased risk of other psychiatric disorders.[54] Mick et al[33] investigated the effects of prenatal exposure to alcohol and found evidence of a 2-fold increase in the risk of ADHD, although in another study the association disappeared after controlling for familial risk of alcoholism, prenatal exposure to smoking, maternal current alcohol use, and parental psychopathology.[55] Other investigators reported associations between prenatal alcohol exposure and attention deficits and impulsivity on the Continuous Performance Test,[56] externalizing behaviors,[57] and attention deficits.[58]

The effects of prenatal alcohol on rodent dopamine systems have been known for some time. Reported changes include reduced ventral tegmental area dopamine neuron activity that was reversed with methylphenidate,[59] marked dopamine deficiencies in striatum and frontal cortex,[60] reduced reactivity of the dopamine D3 receptors,[61] and reduced activity of midbrain dopamine neurons.[62] Although the mechanisms for dopamine system changes in response to alcohol are not well understood, a series of recent studies found that ethanol increased dopamine transporter activity and the number of transporters expressed at the cell surface, indicating that the dopamine transporter may represent an important site of action for ethanol.[63] Subsequent studies have identified ethanol-sensitive sites on the dopamine transporter that modulate transporter activity.[64] From a behavioral perspective, prenatal alcohol exposure in rats is reported to cause increased reaction time variability and false alarms on a choice reaction time task, both characteristic cognitive impairments found in children with ADHD.[65]

CONCLUSIONS

Our investigation provides further evidence that genetic variants of *DAT1* confer increased risk of ADHD. The identification of a specific risk haplotype in 2 independent populations is consistent with other reports and helps to narrow the search for functional *DAT1* sites. Our findings concur with evidence from Caspi et al[30,31] that genetic risks may be overlooked if environmental variables that mark interacting factors are not measured. This study provides evidence that genetic variation of *DAT1* is involved in higher-order interactional effects that might include direct effects of alcohol or other drugs on the developing fetus, maternal (or paternal) behavior associated with maternal use of alcohol during pregnancy, or epistatic genes associated with maternal behavior and cotransmitted to offspring. Although these data do not support an interaction between *DAT1* and maternal use of tobacco, our data set has limited power to exclude this possible interaction.

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Correspondence: Philip Asherson, MRCPsych, PhD, Institute of Psychiatry, Kings College London, Room 222, SGDP Centre, PO 81, London SE5 8AF, England (p.asherson@iop.kcl.ac.uk).
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