

## ORIGINAL RESEARCH ARTICLE

# Haplotype analysis of SNAP-25 suggests a role in the aetiology of ADHD

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Several lines of evidence suggest a role for SNAP-25 (synaptosomal-associated protein of 25 kDa) in the genetic aetiology of ADHD. Most notable is the *coloboma* mouse mutant, which displays spontaneous hyperactivity and is hemizygous for a deletion spanning this gene. We have screened the SNAP-25 gene using denaturing high-performance liquid chromatography and sequencing, and genotyped six polymorphic single-nucleotide polymorphisms and two microsatellites in a clinically ascertained sample of 188 probands. Several markers were found to show association with ADHD, both individually and in combination with other markers to form multimarker haplotypes. Analyses of transmission by parental sex suggested that the association of SNAP-25 with ADHD is largely due to transmission of alleles from paternal chromosomes to affected probands, suggesting that this locus may be subject to genomic imprinting. Overall our data provide some evidence for a role of this gene in ADHD, although the precise causal functional variant is yet to be ascertained.

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SNAP-25 (synaptosomal-associated protein of 25 kDa) is a presynaptic plasma membrane protein with an integral role in synaptic transmission. It forms a complex with syntaxin and the synaptic vesicle proteins (synaptobrevin and synaptotagmin), which mediates the  $\text{Ca}^{2+}$ -mediated exocytosis of neurotransmitter from the synaptic vesicle into the synaptic cleft. Expression studies suggest that SNAP-25 is differentially expressed throughout the brain, and present primarily in the neocortex, hippocampus, anterior thalamic nuclei, substantia nigra, and cerebellar granule cells.<sup>1</sup> During development, SNAP-25 appears to be involved in synaptic plasticity and axonal growth,<sup>2</sup> but in the mature nervous system expression is generally only seen in presynaptic terminals.<sup>1</sup>

Animal studies, in particular, provide strong evidence to suggest that this gene may be involved in the aetiology of attention-deficit hyperactivity disorder (ADHD), a common and highly heritable pervasive disorder with onset in early childhood. The *coloboma* mouse mutant was produced by neutron irradiation and is only viable in the heterozygous form. It demonstrates a number of behavioural deficits that resemble some of the deficits seen in ADHD,

including a marked increase in the level and variability of spontaneous locomotor activity.<sup>3</sup> Hyperactivity in these animals is alleviated by low doses of D-amphetamine, mirroring the use of stimulants to treat ADHD in children, although methylphenidate appears to increase motor activity in a similar way to controls. The extreme hyperactivity exhibited by these mice results from a 2-cM deletion on mouse chromosome 2, in a region containing the mouse SNAP-25 gene (*Snap*).<sup>4</sup> Hess *et al*<sup>3</sup> replaced the deleted *Snap* gene with a homologous transgene and observed amelioration of the hyperlocomotion normally exhibited by *coloboma* mice, suggesting it was the cause of the *coloboma* phenotype. A recent paper by Jones *et al*<sup>5</sup> reports abnormal presynaptic catecholamine regulation in the *coloboma* mouse, a finding that may be very important given the probable role of abnormal dopamine regulation in ADHD.

There is mounting evidence to suggest that polymorphisms within SNAP-25 may play a role in susceptibility to ADHD. While an early study found no linkage between ADHD and markers in the chromosome 20p11–12 region containing SNAP-25,<sup>6</sup> to date there are no negative association-based studies of SNAP-25 markers and ADHD. In a previous study, we found evidence for an association with a microsatellite marker in intron 1 of the SNAP-25 gene.<sup>7</sup> Barr *et al*<sup>8</sup> investigated a couple of single-nucleotide polymorphisms (SNPs) in the 3' untranslated region (UTR) of SNAP-25 and found evidence to suggest a specific haplotype was associated with ADHD. This

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finding was partially supported by Kustanovich *et al*,<sup>9</sup> who found evidence for the same haplotype, but only when paternal transmissions were considered. Finally, Brophy *et al*<sup>10</sup> also looked at these SNPs, and while they found no evidence to support biased transmission of the haplotype nominated by Barr *et al*,<sup>8</sup> they did find that one of the SNPs was individually associated with ADHD as well as evidence for increased transmission of the risk allele from paternal chromosomes.

The aim of this study was to scan the SNAP-25 gene for additional polymorphic variants and to build on our previous association with the intron 1 microsatellite repeat in a larger clinical sample. We used denaturing high-performance liquid chromatography (DHPLC) and sequencing to find novel sequence variants in the promoter and coding regions of the gene for use in a family-based association study. In total, eight polymorphic markers were genotyped across the SNAP-25 gene and haplotype analyses used to test for associations with clinical ADHD. As two of the previous studies of SNAP-25 have suggested that the association of this gene with ADHD is largely due to the transmission of paternal alleles or haplotypes to cases,<sup>9,10</sup> we also explored the potential role of genomic imprinting in SNAP-25 by examining maternal vs paternal transmissions.

## Methods

### *Sample for mutation screen*

According to Risch and Merikangas,<sup>11</sup> one of the major factors in determining the success of association studies in complex traits, such as ADHD, is the frequency of disease-associated alleles in the population. Screening studies on several genes have shown that the majority of variants discovered are actually relatively infrequent occurring at frequencies less than 1%, for example, Cargill *et al*<sup>12</sup> and Halushka *et al*.<sup>13</sup> Detecting and utilising these variants would probably not add much information in the hunt for susceptibility loci for common complex disorders. In this study, it was decided to screen 32 individuals which gives a probability of >0.95 for the detection of a variant with a minor allele frequency of 0.05. Increasing the sample size obviously increases this probability, but also dramatically increases the likelihood of detecting extremely rare variants that may not add any extra information for association studies. DNA was obtained from volunteers using buccal swabs, and extracted using the method outlined in Freeman *et al*.<sup>14</sup>

### *Mutation screen using DHPLC and sequencing*

Primers were designed to span the immediate 5' putative promoter region of SNAP-25 and the eight exons using Primer Express software (PE Applied Biosystems, Foster City, CA, USA). Oligo primer sequences used for the mutation screen can be seen in Table 1. All PCRs were optimised to work under standard conditions, with an initial 5 min denaturing

step at 95°C followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension phase of 72°C for 10 min. Heteroduplex molecules were formed by denaturing the PCR products in a thermal cycler for 3 min at 95°C, and then gradually reannealing them from 95 to 65°C for 30 min. Heteroduplexed PCR products were analysed using the WAVE™ DNA Fragment Analysis System (Transgenomic, Oak Ridge, TN, USA) using column gradient conditions determined by software provided with the WAVE system. The run temperature(s) for each amplicon were determined primarily by using the DHPLC Melt program (<http://insertion.stanford.edu/melt.html>). In a number of cases, alternative melting temperatures were also determined using Transgenomic's WAVEMAKER software. Column temperatures can be seen in Table 1. Individual DNA fragment elution profiles were compared by visual inspection, and overlaid via a Postscript algorithm (Schalkwyk, personal communication) to allow for the easy detection of variants. Samples containing probable mutations were amplified by PCR and sequenced in both directions using the same primers as were used for DHPLC. Dye terminator sequencing was carried out using the ABI PRISM BigDye system (PE Applied Biosystems, Foster City, CA, USA) and samples run on either an ABI 377 or 3100 machine (PE Applied Biosystems, Foster City, CA, USA). Sequencing traces were analysed using Sequencer software (Gene Codes Corporation, Ann Arbor, MI, USA), and multiple samples aligned to aid mutation detection.

### *Genotyping SNAP-25 SNPs*

SNPs detected by DHPLC and sequencing were tested for heterozygosity in a small number of test DNA samples. Six SNPs with a frequency >0.05 were selected to be genotyped in this study. Genotyping was performed using the ABI PRISM SNaPshot chemistry (PE Applied Biosystems, Foster City, CA, USA), a primer-extension methodology based upon allele-specific nucleotide incorporation. Primer extension oligos were designed using an automated in-house primer-design program, and are listed in Table 2. Reactions were performed on the DHPLC amplicon templates using standard SNaPshot conditions with an annealing temperature of 55°C. Products were separated on an ABI 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) and analysed using GENOTYPER (PE Applied Biosystems, Foster City, CA, USA) software.

### *Amplification of microsatellite repeats*

The intron 1 (TAAA)*n* microsatellite was amplified and genotyped as described in Mill *et al*.<sup>7</sup> The promoter-region (TG)*n* repeat was amplified using the primers PRM2-F and PRM2-R (see Table 1) and the PCR reaction conditions described above. The 5'-end of PRM2-F was labelled with the FAM fluorophore. Fluorescently tagged products for both amplification reactions were separated on an ABI

**Table 1** Oligo primer sequences and DHPLC column temperatures used for DHPLC analysis of SNAP-25

DHPLC assay	Amplicon	Features	Primer sequences	DHPLC temp(s) (°C)
PRM1	–2301 to –1809 (493 bp)	Putative promoter	F: ATCAGCAGTTGAAAACCTCAGAATACC R: GCATGTTGCTGAAAATTTGTTTACTTAA	57, 52
PRM2	–1828 to –1400 (429 bp)	Putative promoter	F: AACAAATTTTCAGCAACATGCCTT R: AAACCATTTAGAGCTGGGTTTCG	61, 56
PRM3	–1420 to –973 (468 bp)	Putative promoter	F: GAACCCAGCTCTAAATGGTTTCTC R: AGGACGGCTGGGACACG	64, 59
PRM4	–973 to –494 (480 bp)	Putative promoter	F: CGGCCGTGTCCCAGC R: GCGATTTGTTTTCTCCGAAGAG	62
PRM5	–525 to –99 (427 bp)	Putative promoter	F: CCTACGGACCCCTCTTCGGAG R: GCGGCGCGCTTGACT	61, 56, 51
PRM6	–240 to 157 (398 bp)	Putative promoter	F: ATGCAGTTGCGGGATGAAC R: GTTCTTACCTGGGCTTCTCGG	64
EX1	–3 to 452 (456 bp)	Exon1 (5'UTR)	F: CGGCCATCTTTGATGAGGG R: CGCAAAATGGAAGAAAGAGGG	62, 57
EX2	56450 to 56819 (370 bp)	Exon2 (1st coding exon)	F: GGCTTTCCCTACCTGAACCTG R: GCACAATTCTGTTGACCCCTG	62, 57
EX3	58547 to 59117 (571 bp)	Exon3	F: CTACAGAGGTGGCTTGGGCT R: TCATGCATTCTGAGCATGCA	59, 54
EX4	65716 to 66031 (316 bp)	Exon4	F: AGCGACCATCTGCGTATGC R: AGCTGGCCCATTTCTTCTATCC	56
EX5	73809 to 74311 (503 bp)	Exon5	F: TCCCCACAGAATGAAGCAGAT R: GAATCAAACAGGCCTTCCACC	58
EX6	78064 to 78420 (357 bp)	Exon6	F: ACCACCAGACAAGTGAATGGG R: CTGTGCCTTGTCACTCACCT	61, 56
EX7	80346 to 80699 (354 bp)	Exon7	F: AGTGCCTTGAATCCAAGG R: CCCAGAGGACGACAGATTTCC	60
EX8-1	87466 to 87716 (251 bp)	Exon8 (mainly 3'UTR)	F: ACACATCAGTCCACCCCAT R: GAACAATACTGTGGATTGAAAGAATCA	58
EX8-2	87544 to 88110 (567 bp)	Exon8 (mainly 3'UTR)	F: TCTCTATATATTGACAGGAAAAAAGTGACA R: CTCTCTTGGTCTCTTTCTTTCCAAA	57, 52
EX8-3	88108 to 88609 (502 bp)	Exon8 (mainly 3'UTR)	F: AAATTAAGCAGATCATCTGGAGTCTATAA R: AGACTTCTAAATCATAATCATCCTTTTAAAA	55, 50

**Table 2** Primer extension oligo sequences used to genotype six polymorphic SNPs across SNAP-25

Assay (amplicon)	Primer sequence <sup>a</sup>	Size (bp) <sup>a</sup>	Orientation
–2015 A/T (PRM1)	(A)12 GTG TCA CAC TTT GGA AAA	30	F
–900 G/T (PRM4)	CCA CGG AGG CAC AGA	15	R
80609 G/A (EX7)	(A)21 TGA CTC GCA GTA GGG A	37	R
87610 T/G (EX8-2)	TGG CTC TAA CTC CTT GA	17	F
87614 T/C (EX8-1)	GAG AGA AAA TGA AAA ATG AAA CTC A	25	R
88268 T/C (EX8-3)	(A)21 AAG ATT GCT CTC TCC AAA AC	41	R

<sup>a</sup>In order to allow multiplexing of SNaPshot reactions, poly-A tails were added to certain primer extension oligos.

3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) and analysed using GENOTYPER (PE Applied Biosystems, Foster City, CA, USA) software.

#### Sample

In total, DNA from 188 probands and their families were used for this study. DNA was available from both parents for 121 of the families, and from only the mother in 64 families. In all, 113 of the affected

families had at least one sibling who was also genotyped. 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 comorbidity apart from oppositional defiant disorder and conduct disorder. Parents of referred cases were interviewed with a modified version of the Child and Adolescent Psychiatric Assessment (CAPA).<sup>15</sup>

Information on ADHD symptoms at school were obtained using the long form of the Conners questionnaire.<sup>16</sup> Following 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 summarises and applies DSM-IV and ICD-10 operational criteria.<sup>17</sup> HYPEScheme diagnoses were checked against researcher applied DSM-IV criteria and discrepancies reviewed by two researchers (PA and SR). Where consensus could not be reached, cases were brought to case conference and final consensus agreement made with a senior clinical researcher (ET). All the subjects used in this study had an IQ above 70, were free of neurological disease and damage, and did not have any congenital disorders known to cause hyperactivity. Cases were included in this study if they had a diagnosis of ADHD under DSM-IV criteria. Out of 188 cases included in this study, 176 had the combined subtype, eight had the hyperactive/impulsive subtype, and four the inattentive subtype. DNA was obtained using buccal swabs and extracted as described in Freeman *et al.*<sup>14</sup>

### Analysis

Family genotype data were primarily analysed using UNPHASED, written by Frank Dudbridge, and available freely online at <http://www.hgmp.mrc.ac.uk/Registered/Option/unphased.html>. UNPHASED comprises of a suite of programmes for association analysis of multilocus haplotypes from unphased genotype data. Several tests were performed on the SNAP-25 genotype data: the extended transmission disequilibrium test (ETDT),<sup>18</sup> the haplotype-based haplotype relative risk test (HHRR),<sup>19</sup> and the pedigree disequilibrium test (PDT),<sup>20</sup> which utilises extra genotype data (eg from siblings). David Clayton's program TRANSMIT (version 5.4), which is available for download from the web at <http://www-gene.cimr.cam.ac.uk/clayton/software/>, was also used to analyse this data. TRANSMIT tests for association between genetic markers and disease by examining the transmission of markers from parents to affected offspring. The main features of TRANSMIT, which differ from other similar programmes are that as well as dealing with the transmission of multilocus haplotypes, even if phase is unknown, it can cope with unknown parental genotypes. The tests are based on a score vector, which is averaged over all possible configurations of parental haplotypes and transmissions consistent with the observed data. Data from unaffected siblings (or siblings whose disease status is unknown) may be used to narrow down the range of possible parental genotypes that need to be considered, thus maximising the power of our sample to detect an association with any of the SNAP-25 markers tested. Linkage disequilibrium (LD) between the eight SNAP-25 markers was assessed in the parental samples using the program

2LD, written by Jing Hua Zhao, and available online at <http://www.iop.kcl.ac.uk/iop/Departments/PsychMed/GEpiBSt/software.shtml>.

## Results

### Mutation screen

In total, 11 SNPs were detected by DHPLC and sequencing, and of these six were at a frequency high enough (MAF > 0.05) to be considered for genotyping in the clinical ADHD sample. Two of these SNPs (87610 T/G and 87614 T/C), located in the 3'UTR of SNAP-25, have been previously reported by Barr *et al.*<sup>8</sup> In addition to the intron 1 (TAAA)*n* microsatellite repeat (previously reported in Mill *et al.*<sup>7</sup>), a further repeat polymorphism was detected in the PRM2 amplicon. This (TG)*n* dinucleotide was found to be highly polymorphic in a test control sample, and was thus also genotyped in the clinical ADHD sample. While none of the polymorphisms have a nonsynonymous effect on protein sequence, several occur in the UTR and putative promoter regions of the gene, and the (TAAA)*n* repeat is located very close to the first translated exon of SNAP-25. Future studies will focus on ascertaining the functional implications of these polymorphisms. Table 3 lists all the novel polymorphisms detected in this study.

### Individual marker analysis

TDT, HHRR, PDT, and TRANSMIT results, along with allele frequencies, for each of the individual SNPs can be seen in Table 4. Similar data for the promoter-region microsatellite is displayed in Table 5, and for the intron 1 (TAAA)*n* repeat in Table 6. Evidence for

**Table 3** Polymorphisms detected by DHPLC and sequencing in this study

Polymorphism	Amplicon	Position in PCR amplicon (bp)	Relative position in SNAP-25 (bp)
A/T SNP	PRM1	287	-2015
T/C SNP <sup>a</sup>	PRM2	157	-1661
(TG) <i>n</i> RPT	PRM2	313-313 (+ 2n)	-1515 to -1515(+ 2n)
G/T SNP	PRM4	73	-900
T/G SNP <sup>a</sup>	EX2	15	56465
T I/D <sup>a</sup>	EX5	411	74220
C/T SNP <sup>a</sup>	EX6	83	78147
G/A SNP	EX7	263	80609
G/A SNP <sup>a</sup>	EX8-1	116	87582
T/G SNP	EX8-1	144	87610
T/G SNP	EX8-2	66	87610
T/C SNP	EX8-1	148	87614
T/C SNP	EX8-2	70	87614
T/C SNP	EX8-3	160	88268

<sup>a</sup>At very low frequency (minor allele frequency < 5%) and not used for genotyping. Position 1 corresponds to the start of transcription of SNAP-25 mRNA.

**Table 4** TDT, HHRR, PDT, and TRANSMIT analysis of SNAP-25 SNP markers and allele frequencies in parental samples

	-2015 A/T			-900 G/T			80609 G/A			87610 T/G			87614 T/C			88268 T/C		
	A	T		G	T		G	A		G	T		C	T		C	T	
TDT	Allele frequency (%)																	
	T	41	59	64	36		81	19		40	60		22	78		60	40	
	NT	70	47	87	80		48	29		56	68		31	43		61	54	
	$\chi^2$ (P-value)	4.55 (0.03)		0.29 (0.59)	87		4.74 (0.03)	48		1.35 (0.24)	56		43	31		54	61	
HHRR	T	117	145	174	85		251	41		102	180		52	231		173	103	
	NT	94	168	167	92		232	60		115	167		64	219		167	109	
	$\chi^2$ (P-value)	4.20 (0.04)		0.42 (0.52)			4.34 (0.04)			1.26 (0.26)			1.56 (0.21)			0.28 (0.60)		
PDT	z	2.09	-2.09	0.57	-0.57		2.11	-2.11		-1.19	1.19		-1.38	1.38		0.54	-0.54	
	P	0.04	0.04	0.57	0.57		0.03	0.03		0.23	0.23		0.17	0.17		0.59	0.59	
	$\chi^2$ (P-value)	4.37 (0.04)		0.33 (0.57)			4.46 (0.03)			1.42 (0.23)			1.90 (0.17)			0.30 (0.59)		
TRANSMIT	T	167	203	231	137		321	51		144	230		81	293		228	140	
	Exp	154	218	233	135		304	68		147	223		87	287		224	144	
	$\chi^2$ (P-value)	3.68 (0.06)		0.06 (0.81)			11.36 (0.001)			0.27 (0.60)			1.33 (0.25)			0.35 (0.55)		

association using TDT, HHRR, PDT, and TRANSMIT analyses was found for three SNAP-25 polymorphisms: the -2015 A/T SNP, the intron 1 microsatellite, and the 80609 G/A SNP. We have previously reported a significant association for the intron 1 repeat in a subsection of this sample combined with a sample from the University of Birmingham,<sup>7</sup> although the level of significance is higher in this expanded clinical sample. The overall global significance of this polymorphism is mediated almost entirely by two alleles—allele 2 (137bp), which has a protective effect and is significantly untransmitted to affected probands and allele 5 (149bp), which appears to be a risk allele and is significantly overtransmitted to affected probands. We also found significant associations for two of the SNPs with increased transmission of the A allele of the -2015 promoter-region SNP, and the G allele of the 80609 intron 7 SNP. None of the other five markers was individually associated with ADHD in this sample. Figure 1 shows the location of the eight individual polymorphisms and summarises the association data for each. Repeating the analyses with the 12 noncombined subtype probands removed made little difference to the data.

#### LD relationships between SNAP-25 markers

Two standardised measures of LD were calculated— $D'$  and  $R^2$ .  $D'$  is the most widely used measure of LD, and is a standardised, pairwise disequilibrium value that has the advantage of being independent of allele frequency.  $R^2$  takes into account allele frequency and gives more information about how well a genotype at one marker location predicts that at another. Two markers can be in strong LD, as shown by a high  $D'$  value, but still be uninformative about each other's genotype, because of large differences in allele frequency. The LD relationships between the eight markers genotyped in this study can be seen in Figure 2. LD appears to be fairly significant across the gene, and although the most 5' marker (-2015 A/T) is not in significant LD with the SNPs at the extreme 3'-end of the gene, disequilibrium does seem to extend up to ~80 000bp. As would be expected from the short physical distances between them, the three SNPs at the 3'-end of the gene are all in strong LD with each other ( $D' > 0.95$ ).

#### Haplotype analyses

Our initial haplotype analysis involved taking three-marker haplotype windows and moving along the SNAP-25 gene in a 5'→3' direction. Figure 3 shows the -logP values for the TDT analyses of the six haplotype windows moving sequentially across the SNAP-25 gene. It can be seen that several of these haplotype analyses gives a positive global significance value, with the most significant associations being for the third and fourth haplotype windows. For the third haplotype window, the most significant haplotype was G-4-G (TDT: 48T, 31NT,  $P=0.05$ ); for the fourth window, the most significant haplotype was 4-G-T (TDT: 46T, 28NT,  $P=0.04$ ); for the fifth

**Table 5** ETDT, HHRR, PDT, and TRANSMIT analysis of the promoter region microsatellite and allele frequencies in parental samples

		<i>Allele (size in bp)</i>														
		1	3	5	6	7	8	9	10	11	12	13	14	15	16	19
		415	419	423	425	427	429	431	433	435	437	439	441	443	445	451
<i>Allele frequency (%)</i>		0.5	1.6	0.5	0.5	3.5	8.1	20.2	25.7	7.7	10.7	10.7	7.2	1.8	1.2	0.2
ETDT	T	2	6	2	1	11	20	45	48	21	21	25	25	2	3	1
	NT	1	3	1	1	5	24	43	51	19	33	28	13	8	3	0
	$\chi^2$ ( <i>P</i> -value)	16.54, 14 df (0.28)														
HHRR	T	2	6	2	1	12	21	60	72	23	24	28	27	2	3	1
	NT	1	3	1	1	6	25	58	75	21	36	31	15	8	3	0
	$\chi^2$ ( <i>P</i> -value)	15.56, 14 df (0.34)														
PDT	z	1.4	2.0	0.6	0	1.3	1.2	0.2	0.1	0.2	1.7	0.1	1.6	1.4	0	1
	$\chi^2$ ( <i>P</i> )	16.92, 14 df (0.26)														
TRANSMIT	T	2	7	2	4	16	27	78	93	26	32	40	32	6	4	1
	Exp	2	5	2	3	13	30	76	96	26	38	41	27	8	4	1
	$\chi^2$ ( <i>P</i> -value)	12.043, 14 df (0.60)														

window, the most significant haplotype was G-T-T (TDT: 47T, 29NT  $P=0.04$ ); and for the sixth haplotype window, the most significant haplotype is T-T-C (50T, 30NT,  $P=0.03$ ). Analyses for the same haplotypes using HHRR, PDT, and TRANSMIT produced very similar results and are not presented here to conserve space but are available on request from the authors.

We also investigated a number of other multimarker haplotypes. A haplotype made up of the three markers that gave individual significant evidence for association with ADHD gave a significant result (global TDT  $P$ -value = 0.001), as did a whole range of other haplotype configurations. Although such haplotype analysis is, by definition, multiple testing, it is striking that the transmission data for numerous haplotype configurations is consistently significant. There is no room to present a complete list of possible haplotypes in this paper, and any that are not reported here are available on request from the authors.

Two of the 3'UTR SNPs identified in this study (87610 T/G and 87614 T/C) have been investigated in relation to ADHD in several previous studies,<sup>8–10</sup> and so we decided to investigate haplotypes of these markers. Allele and haplotype frequencies did not differ significantly from those reported by Barr *et al.*<sup>8</sup> Overall, there is some suggestive evidence for haplotypes of these SNPs being involved in ADHD with the T-T haplotype being over-transmitted to ADHD probands (TDT: 54T, 31NT,  $\chi^2=6.22$ ,  $P=0.01$ ), although the global significance values are not as high as when other haplotype configurations were considered. The data for individual haplotypes of these two markers can be seen in Table 7.

#### *Effect of maternal vs paternal transmission*

Each of the three individually associated SNAP-25 markers was more significantly associated when

paternal transmissions were analysed compared to maternal transmissions: –2015A/T (maternal TDT  $\chi^2=1.73$ , 1 df,  $P=0.19$ ; paternal TDT  $\chi^2=4.76$ , 1 df,  $P=0.03$ ), (TAAA)*n* (maternal TDT  $\chi^2=6.18$ , 5 df,  $P=0.29$ ; paternal TDT  $\chi^2=15.13$ , 5 df,  $P=0.01$ ), and 80609G/A (maternal TDT  $\chi^2=1.79$ , 1 df,  $P=0.18$ ; paternal TDT  $\chi^2=4.28$ , 1 df,  $P=0.04$ ). For each of these markers, evidence for biased paternal transmission is at least as strong as evidence from both maternal and paternal transmissions taken together, despite the smaller sample size. Although the paternal data are not actually significantly different from maternal transmissions, this is not surprising given the small number of actual transmissions analysed in each group, and a trend towards significance is apparent in each case.

#### **Discussion**

In this study, we report on 10 novel variants identified across SNAP-25 and detect some evidence for association of this gene with ADHD using eight of the markers that had minor allele frequencies greater than 5%. We found significant association with three individual polymorphisms in SNAP-25: the –2015 A/T SNP, the intron 1 microsatellite, and the 80609 G/A SNP. Stronger evidence for association resulted from the analysis of multimarker haplotypes. These findings extend our previous report of an association with the tetranucleotide repeat in intron 1 of the gene,<sup>7</sup> and concur with reports from other groups linking this gene with ADHD.<sup>8–10</sup> Although an earlier study failed to detect linkage between ADHD and markers in the chromosome 20p11–12 region containing SNAP-25,<sup>6</sup> the analysis performed lacked power to detect the size of gene effects described subsequently.

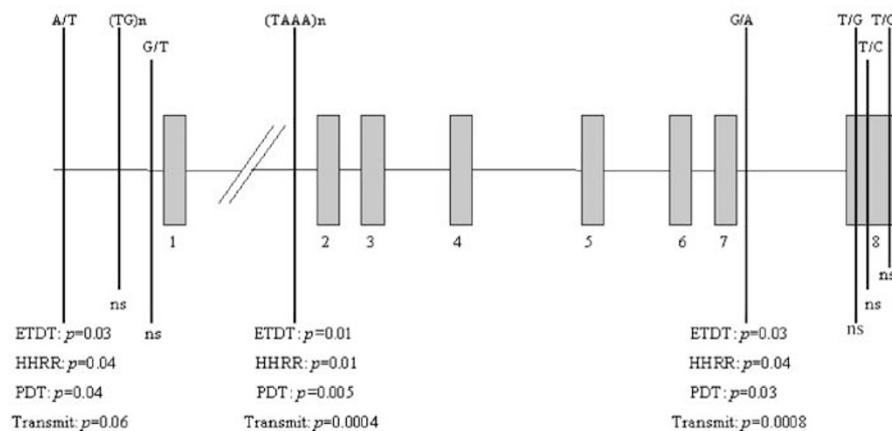
**Table 6** ETDT, HHRR, PDT, and TRANSMIT analysis of the intron 1 microsatellite and allele frequencies in parental samples

		Allele (size in bp)						
		2	3	4	5	6	7	
		137	141	145	149	153	157	
Allele frequency ETDT	T	14	2	20	54	9	1	
	NT	23	8	40	89	26	2	
		51	6	48	55	25	3	
	$\chi^2$ (P-value)	14.80, 5 df (0.01)						
HHRR	T	25	8	52	173	26	2	
	NT	53	6	60	139	25	3	
		15.07, 5 df (0.01)						
	$\chi^2$ (P-value)							
PDT	z	-2.94	1.27	-1.39	2.72	0	-0.58	
	P	0.003	0.21	0.17	0.007	1	0.56	
		16.56, 5 df (0.005)						
	$\chi^2$ (P-value)							
TRANSMIT	T	35	11	70	228	30	2	
	Exp	53	9	74	203	33	2	
		21.66, 5 df (0.001)						
	Global $\chi^2$ (P-value)							

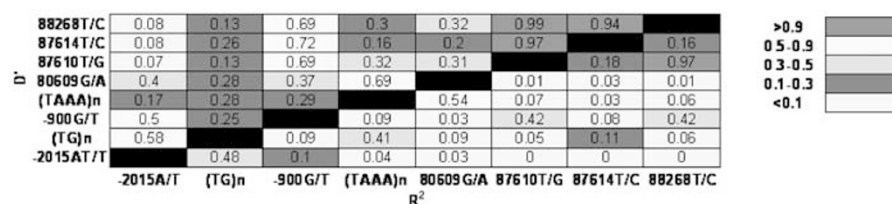
It should be noted, however, that the previous studies of Barr *et al*,<sup>8</sup> Kustanovic *et al*,<sup>9</sup> and Brophy *et al*,<sup>10</sup> which have focused on two SNPs in the 3'UTR of SNAP-25, are not entirely consistent with each other, or with the data presented for these two SNPs here. Barr *et al* found no significant evidence of association in a Canadian Caucasian sample when each of these SNPs was analysed individually, but observed biased transmission of the T-C haplotype in a family-based analysis.<sup>8</sup> They also found near-significant nontransmission of the T-T haplotype. Kustanovic *et al*<sup>9</sup> found evidence for biased transmission of the T-C haplotype in a US Caucasian sample, although this was only significant when paternal transmissions were considered. Brophy *et al*,<sup>10</sup> using an Irish ADHD sample, found an association with the T allele of the 87614 SNP and evidence for paternal transmission, but no evidence for an association with any particular haplotype or the 87610 SNP. Our data for these two markers suggest that neither SNP is individually associated with ADHD, but that the T-T haplotype is overtransmitted to affected probands. Taken together, these data suggest the involvement of SNAP-25 genetic variation in ADHD susceptibility, although they do not supply a clear picture of how this association operates. The overlapping, but ultimately disparate results for these two SNPs suggest that they are unlikely to be the mediating functional variants causing the SNAP-25 association, but are likely to be associated with other functional markers in or near to this gene. This conclusion is supported by the analysis of additional markers spanning SNAP-25 and the multimarker haplotypes reported here and discussed below.

Of the other markers individually associated with ADHD in this sample, the strongest association was obtained with the intron 1 (TAAA)<sub>n</sub> microsatellite repeat. Allele 5 is significantly overtransmitted to affected probands and allele 2 appears to be a protective allele. The location of this polymorphic repeat motif very close to the first translated exon of SNAP-25 suggests it may have a role in the expression or function of this gene. The G/A SNP in intron 7 is also individually strongly associated with ADHD, with the G allele being overtransmitted to affected probands. Finally, a number of rare variants (MAF <5%) were discovered in SNAP-25, but not genotyped in the full sample. It is possible, however, that these polymorphisms may have a role in susceptibility to ADHD, for example, by influencing splicing.

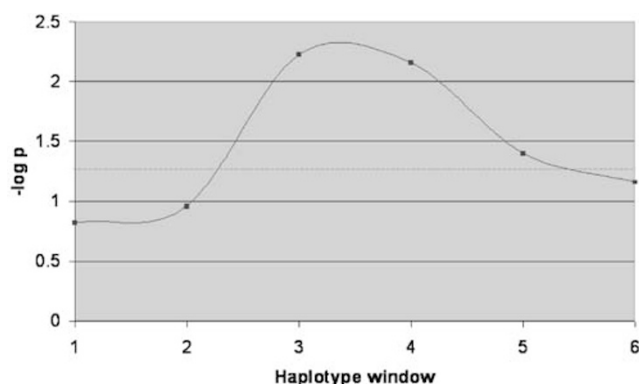
Haplotype analyses also gave evidence for association of SNAP-25 with ADHD. Moving along the gene sequentially, haplotype windows comprising of three markers at a time gave some evidence for association in all but the first two windows (see Figure 3). A number of the individual significant haplotypes within each of these four haplotype windows are significantly associated with ADHD. The strongest individual association is for the T-T-C haplotype of the final three SNPs, which is significantly overtransmitted to affected probands. The consistently



**Figure 1** Global significance values for the eight individual polymorphisms across SNAP-25.



**Figure 2** LD relationships between SNAP-25 markers.



**Figure 3** Sequential haplotype analysis of SNAP-25 using three-marker haplotype windows (1 = -2015A/T, (TG)n, -900G/T; 2 = (TG)n, -900G/T, (TAAA)n; 3 = -900G/T, (TAAA)n, 80609G/A; 4 = (TAAA)n, 80609G/A, 87610T/G; 5 = 80609G/A, 87610T/G, 87614T/C; 6 = 87610T/G, 87614T/C, 88268T/C). Data shown are the  $-\log P$  values for the global TDT analyses on the six three-marker haplotype windows. The dotted line corresponds to  $P = 0.05$ . Data for specific individual haplotypes are available on request from the authors.

high level of association for most haplotype combinations away from the 5'-end of SNAP-25 (ie those encompassing markers after the large first intron) does not really help in locating the precise functional variant, but does suggest that some polymorphism within, or close to, SNAP-25 is increasing susceptibility to ADHD in this sample.

As the polymorphisms across this region are in significant LD with each other, it is possible that any of the reported associated markers, or an as yet uncharacterised variant in this region, is the true risk polymorphism. Given the relative strength of association of multimarker haplotypes compared to individual markers, it is unlikely that any of the individual markers is the true risk variant, although we cannot rule out the possibility that more than one functional variant is involved.

An interesting observation from our data is that the association of these SNAP-25 polymorphisms with ADHD appears to be largely due to paternal transmissions. Brophy *et al*<sup>10</sup> and Kustanovic *et al*<sup>9</sup> also report biased paternal transmission of SNAP-25 risk alleles in ADHD. Taken together, these data suggest that genomic imprinting may operate at the SNAP-25 locus, and may be an important factor in the molecular aetiology of ADHD. As far as we know, there is no data on imprinting in the syntenic region of the mouse genome. Furthermore, we could find no examples of other genes in 20p11-12 that are imprinted. Further work is required to test directly the hypothesis that genomic imprinting is acting in this region of chromosome 20.

While none of the polymorphisms have a nonsynonymous effect on protein sequence and are thus unlikely to directly effect function, several occur in the UTR and putative promoter regions of the gene, and the (TAAA)n repeat is located very close to the first translated exon of SNAP-25. Future work should focus on ascertaining the effect of these



**Table 7** Haplotype analysis for the two 3'UTR SNPs implicated by Barr *et al*,<sup>8</sup> Kustanovich *et al*,<sup>9</sup> and Brophy *et al*<sup>10</sup>

		Haplotype			
		G-C	G-T	T-C	T-T
ETDT	T	0	35	20	53
	NT	2	43	32	31
	$\chi^2$ (P-value)	7.18, 3 df, $P=0.07$			
HHRR	T	0	102	50	115
	NT	3	110	60	94
	$\chi^2$ (P-value)	5.92, 3 df, $P=0.11$			
PDT	z	-1.385	-0.851	-1.296	1.985
	P	0.17	0.39	0.20	0.05
	Global $\chi^2$ (P-value)	6.196, 3 df, $P=0.10$			

polymorphisms—both individually and in haplotype combinations—on the expression of SNAP-25. It is possible that the direct functional risk variant within, or near, the SNAP-25 gene has yet to be characterised. The majority of the intronic regions of the SNAP-25 gene have yet to be screened for polymorphisms and so future work should also focus on an investigation of these regions. One of the major limitations of this study is that by concentrating solely on the promoter and coding regions of the gene, there is a large (~55 kb) gap that encompasses intron 1, for which no association data are available. Furthermore, while no common polymorphisms were found within the protein-coding region of SNAP-25 in our mutation screen, it is possible that such variants evaded detection, although it is reassuring that our screen detected the variants reported by Barr *et al*.<sup>8</sup>

To conclude, we have detected a number of novel polymorphic variants across the SNAP-25 gene. There is some evidence that this gene is associated with ADHD in our clinical ADHD sample, although the precise functional risk variant is yet to be ascertained. Analyses stratified by paternal sex suggests that the association of these SNAP-25 variants is largely due to the transmission of paternal alleles suggesting that genomic imprinting may be operating at this locus. Future work will focus on further characterising variation across this gene and examine functional effects of associated polymorphisms and haplotype blocks.

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