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ORIGINAL RESEARCH ARTICLE

Synaptosomal-associated protein 25 (SNAP-25) and attention deficit hyperactivity disorder (ADHD): evidence of linkage and association in the Irish population

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Several lines of evidence have suggested that ADHD is a polygenic disorder produced by the interaction of several genes each of a minor effect. Synaptosomal-associated protein 25 (SNAP-25) is a presynaptic plasma membrane protein which is expressed highly and specifically in the nerve cells. The gene encodes a protein essential for synaptic vesicle fusion and neurotransmitter release. Animal model studies showed that the coloboma mouse mutant has a hyperactive phenotype similar to that of ADHD. The hyperactive phenotype of this model has been shown to be the result of a deletion of the SNAP-25 gene. DNA variations within or closely mapped to the SNAP-25 gene may alter the level of expression and hence may have an effect on the function of synaptic vesicle fusion and neurotransmitter release. Using HHRR and TDT we analysed 93 ADHD nuclear families from Ireland and found increased preferential transmission of SNAP-25/*Dde*I allelel to ADHD cases; HHRR ($\chi^2 = 6.55$, $P = 0.01$) and linkage (TDT) ($\chi^2 = 6.5$, $P = 0.015$). In contrast to our findings, Barr *et al*[1] reported an increased transmission of allele 2 of the *Dde*I polymorphism though this was not statistically significant. However, they also reported a significantly increased transmission of a haplotype (made of allele 1 of *Mnl*I and allele 2 of the *Dde*I) in their Canadian ADHD sample. It is not clear what the role of SNAP-25 in ADHD is until these findings are either confirmed or refuted in other ADHD samples.
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Attention deficit hyperactivity disorder (ADHD) is a neuropsychiatric condition characterized by hyperactive-impulsive behaviour and persistent inattention. Individuals with this condition experience social and academic dysfunction. Although the aetiology of ADHD is not known, it is postulated that a developmental failure in the brain mechanism that underlies self-control and inhibition is involved.[2] ADHD affects approximately 3–6% of children and adolescents from different geographical regions worldwide.[3] Boys are affected more frequently than girls, with the ratio ranging from 3:1 to 9:1.[4] Family, twin and adoption studies have indicated a strong genetic component in susceptibility to ADHD. An approximate five- to six-fold increase in the frequency of ADHD among first-degree relatives when compared with the general population was reported.[5] A probandwise concordance rate of 72% monozygotic twins and 32% same-sex dizygotic twins was observed.[6] A number of twin studies have estimated the heritability of ADHD to range from 39% to 91% for various symptoms of the disorder.[7,8] Adoption studies have also supported a genetics component for ADHD. In a recent study Sprich *et al*[9] reported 6% of the adoptive parents of adopted ADHD probands had ADHD compared with 18% of the biological parents of non-adopted ADHD probands and 3% of the biological parents of the control.

The dopaminergic neurotransmission systems have been implicated in the pathology of ADHD. Genes of the dopamine system have been linked with ADHD for a number of reasons including: (1) the reduction in symptoms brought about by pharmacological agents (such as methylphenidate) that primarily act in the dopaminergic system;[10] (2) the results from recent imaging studies of an ADHD brain implicate brain structures with rich dopamine content, such as the fronto-striatal circuitary in the aetiology of ADHD;[3] (3) animal model studies such as the mouse model lacking dopamine transporter (ie DAT1 knockout mice) demonstrate a link between the dopamine system and ADHD. Mice lacking DAT1 have high extracellular striatal dopamine levels and display increased locomotor activity, a typical characteristic of the ADHD phenotype.[11] Recently, several association studies utilizing DNA variants at DAT1, DRD4, DRD5 and DBH have suggested the involvement of these loci as susceptibility variants (genes of minor effect) for ADHD.[12–14] However, absence of associations with some of the above variants and ADHD has also been reported.[15,16]

Synaptosomal-associated protein 25 (SNAP-25) is a presynaptic plasma membrane protein which is expressed highly and specifically in the nerve cells.[17] The gene encodes a protein essential for synaptic vesicle fusion and neurotransmitter release. SNAP-25 along with syntaxin 1a and VAMP-2 (synaptobrevin-2) make up the core complex essential for docking and holding synaptic vesicles at the presynaptic membrane in preparation for Ca^{2+}-triggered neurotransmitter exocytosis.[17] It forms a connection between the synaptic vesicles holding the transmitter and the plasma

Table 1 HHRR analysis of SNAP-25 (*Mnl*I and *Dde*I) polymorphisms in 93 ADHD nuclear families

Marker	*Allele*	*T*	*NT*	*RR*	χ^2	*P*
*Mnl*I	1	96	95	1.01	0.01	0.90
	2	57	58			
*Dde*I	1	131	113	1.51	6.55	0.01
	2	22	40			

T, transmitted; NT, not transmitted; RR, relative risk.

membrane at the site of fusion. The SNAP-25-containing complex may also assist in the assembly of the other complexes involved in Ca^{2+}-triggered membrane fusion, such as N-ethylmaleimide-sensitive fusion protein (NSF), synaptotagmin, α, β and γSNAPs. Animal model studies have shown that the coloboma mouse mutant has a hyperactive phenotype similar to that of ADHD. This model was shown to be the result of a deletion of the SNAP-25 gene.[18] The SNAP-25 gene maps to the human chromosome 20p11.2.[19] Mutations within the SNAP-25 gene may alter the level or function of the protein and hence may have an effect on the functions of synaptic vesicle fusion and neurotransmitter release, thus the SNAP-25 gene is considered a candidate gene for ADHD susceptibility.

In this study, we used the haplotype-based haplotype relative risk (HHRR) and transmission disequilibrium test (TDT) approaches to analyse two polymorphisms mapped to SNAP-25 (at positions 1065T-G and 1069T-C) for possible association or linkage with ADHD in a sample of 69 ADHD trios and 24 parent proband duos ascertained in Ireland. We also attempted to replicate the recently reported evidence of linkage and association between SNAP-25 and ADHD in a Canadian sample.[1] HHRR analysis of 93 ADHD nuclear families is presented in Table 1. A significant increase in the transmission of allele 1 of the *Dde*I polymorphism was observed ($\chi^2 = 6.55$, $P = 0.01$, RR = 1.51). Conversely and strikingly, no differences in the transmission of the alleles at the polymorphism *Mnl*I were observed though the two polymorphisms are mapped only 3 bp apart ($\chi^2 = 0.01$, $P = 0.90$, RR = 1.01). Similarly, transmission disequilibrium test (TDT) analysis of the two markers (Table 2) showed a significantly increased transmission of allele 1 of the *Dde*I polymorphism ($\chi^2 = 6.48$, $P = 0.015$, RR = 2.13). However, no distortions in the transmission of the alleles of the *Mnl*I polymorphism were observed ($\chi^2 = 0.06$, $P = 0.9$, RR = 1.06). Furthermore, when the transmission from parents was considered separately (Tables 3 and 4), the association of *Dde*I polymorphism observed in the total sample was also observed for paternal transmission (HHRR $\chi^2 = 5.47$, $P = 0.019$, RR = 2.0) and (TDT $\chi^2 = 7.1$, $P = 0.013$, RR = 6.0) but not in the maternal sample, (HHRR $\chi^2 = 2.64$, $P = 0.1$, RR = 1.45) and (TDT $\chi^2 = 2.46$, $P = 0.17$, RR = 1.9). However, with the small number of transmissions observed in each group the 95% confidence intervals for the relative risks overlapped suggesting that there is no significant difference between the transmissions by sex of parent. No significant differences in the transmission of the alleles at the *Mnl*I polymorphism were observed for paternal or maternal transmissions (Tables 3 and 4). Finally, linkage disequilibrium between the two markers was examined using the program EH which estimates linkage disequilibrium between different markers or between a disease locus and a marker. A significant disequilibrium for the distribution of the

Table 2 TDT analysis of SNAP-25 (*Mnl*I and *Dde*I) polymorphisms in 69 ADHD trios

Marker	*Allele*	*T*	*NT*	*RR*	χ^2	*P*
*Mnl*I	1	35	33	1.06	0.059	0.90
		33	35			
*Dde*I	1	34	16	2.13	6.48	0.015
	2	16	34			

T, transmitted; NT, not transmitted.

Table 3 HHRR analysis of parental transmission of SNAP-25 (*Mnl*I and *Dde*I) polymorphisms in 93 ADHD nuclear families

Marker	*Allele*	*T*	*NT*	*RR*	χ^2	*P*
Paternal transmission						
*Mnl*I	1	40	37	1.1	0.29	0.59
	2	24	27			
*Dde*I	1	59	49	2.0	5.47	0.019
	2	6	16			
Maternal transmission						
*Mnl*I	1	50	50	1.0	0.0	1.0
	2	26	26			
*Dde*I	1	67	59	1.45	2.64	0.10
	2	11	19			

T, transmitted; NT, not transmitted.

Table 4 TDT analysis of parental transmission of SNAP-25 (*Mnl*I and *Dde*I) polymorphisms in 69 ADHD trios

Marker	*Allele*	*T*	*NT*	*RR*	χ^2	*P*
Paternal transmission						
*Mnl*I	1	14	13	1.08	0.03	1.0
	2	13	14			
*Dde*I	1	12	2	6.0	7.1	0.013
	2	2	12			
Maternal transmission						
*Mnl*I	1	14	14	1.0	0.0	1.0
	2	14	14			
*Dde*I	1	17	9	1.9	2.46	0.17
	2	9	17			

T, transmitted; NT, not transmitted.

genotypes was observed ($\chi^2 = 29.7$, df = 3, $P = 0.000002$). However, using the program TRANSMIT which estimates the genetic associations from probabilities of haplotype transmission to affected offspring showed no preferential transmission of any particular haplotype (data not shown).

SNAP-25 is known to have an essential role in neurotransmission. Together with syntaxin and VAMP (synaptobrevin), SNAP-25 forms a core complex which is involved in synaptic vesicle fusion and neurotransmitter release. It is also thought to assist in the assembly of a number of other components, such as NSF, synaptotagmin, α, β and γ SNAPs, which are required for Ca^{2+}-triggered membrane fusion.[17] From this evidence, it is postulated by Wilson[20] that an alteration in the level of expression of the SNAP-25 gene would lead to an alteration in the level of neurotransmitter release as opposed to a mechanism which alters reuptake of neurotransmitter of receptor activation. Studies on the mouse mutant coloboma (congenital cleft in some part of the eye, commonly the iris, but may also occur in the lids) have produced a model for ADHD. The hyperactive phenotype has been attributed to the deletion of the SNAP-25 gene[1] and has been shown to be rescued by the transgene encoding SNAP-25. Further studies demonstrate that the SNAP-25 deficiency in mouse mutant coloboma results in regional and neurotransmitter-specific deficits in the brain.[21] These studies showed a significant reduction in KCl-induced release of dopamine from the dorsal striatum region of the coloboma (Cm/+) mice when compared to wild-type mice. However, no significant difference was observed between the level of KCl-induced release of dopamine in the ventral striatum of the Cm/+ and the wild-type mice. Also observed was a 50% reduction in serotonin release in the dorsal striatum of Cm/+ mice when compared with wild-type mice. This region-specific effect of the SNAP-25 deficiency may imply a specific role for SNAP in the aetiology of ADHD. Deficits in the executive functions performed in the prefrontal cortex have been proposed as the primary deficit in ADHD.[22] This region of the brain receives modulatory input from the striatum and this is suggestive of an association between ADHD and a deficit in SNAP-25 expression.

In a recent study, Barr *et al*[1] reported an increased transmission of allele 2 of the *Dde*I polymorphism, though not statistically significant at the level of $P = 0.05$. However, haplotype analysis showed a marginally significant association between a haplotype consisting of allele 1 of the *Mnl*I polymorphism and allele 2 of the *Dde*I polymorphism and ADHD. We have also conducted a similar analysis using the program TRANSMIT and found no evidence of preferential transmission of any particular haplotype to the ADHD cases. It is difficult to reconcile these differences in the absence of literature investigating the relation between the SNAP-25 polymorphisms and ADHD. Both of the examined polymorphisms are mapped to the 3′ untranslated region of the gene.[1] It is unlikely therefore that they will have any direct effect on the three-dimensional structure or the expression of SNAP-25. However, these polymorphisms may be in linkage disequilibrium with other unidentified polymorphism(s) in the coding or the promoter regions of the gene that might alter the function or the level of gene expression. This postulated linkage disequilibrium may differ between the Irish and Canadian populations. A systematic search of the coding and regulatory regions (within introns or upstream and downstream of coding regions) of SNAP-25 for DNA variants is required to substantiate these speculations.

Analyses by sex indicated that the association of SNAP-25 with ADHD is largely due to the transmission of the paternal allele to the cases (Table 3). Similar findings of preferential paternal allele transmission have been observed in polymorphisms at the dopamine transporter (DAT1), tyrosine hydroxylase (TH), dopamine receptor 2 (DRD2) genes.[23] This may suggest that genomic imprinting is acting in or near this locus. Evidence of imprinting has been reported on chromosome 20q13.11. This region is known to contain the GNAS1 gene which has been implicated in pseudohypoparathyroidism type Ia and has a highly complex imprinted expression.[24] However, SNAP-25 was isolated and mapped to chromosome 20p11.2,[19,25] thus the imprinting acting at the 20q13.11 locus is unlikely to have any effect on the development of ADHD. Currently, there is no independent evidence to suggest that the SNAP-25 associated allele is imprinted, and further work is required.

Finally, given the complex nature of a multifactorial neuropsychiatric disorder such as ADHD and the relatively small size of the studied sample, it is important to investigate these findings in other samples preferably from different ethnic groups to confirm or refute the association of SNAP-25 with ADHD.

Materials and methods

Sample

ADHD cases were recruited from child psychiatric clinics and schools in West County Dublin and from the Hyperactive and Attention Deficit Children's Support Group of Ireland. Ninety-three children were assessed from 93 families. Twenty-four families consisted of parent probands and 69 trios consisted of mother, father and affected child. The age range of the probands was between 4 and 14 years, with males accounting for 87%. The families were ethnically Irish with the exception of one family in which the father was Croatian. Details of diagnosis and clinical criteria can be found in Daly.[14] Briefly, consensus diagnoses were made according to DSM-IV ADHD or UADD either with or without comorbidity. These diagnoses were based on all available clinical information and the rating scales described below. The rating scales used were: (1) the Child Behavioral Checklist (CBCL), a widely based behavioral symptom measure and the records of child behavior problems and social competencies as reported by parents; (2) the Connors Parents and Teachers Rating Scales, 48-item parent and 39-item teacher rating

scales; (3) the Comprehensive Teachers Rating Scale (ACTeRS) which includes 24 items relevant to classroom behavior. The 25-item Wender-Utah rating scale (WURS) children[26] was applied to all parents. This rating scale seeks to retrospectively make a diagnosis of ADHD during childhood. A cut-off score of 36 or higher is 96% sensitive and 96% specific for adults with ADHD as children.[26] Familiality, for the purposes of the present study, was defined as the presence of one or more parents with a score on the Wender of >36.

DNA amplification

The PCR primer sequences and conditions used to amplify the polymorphisms at the 3′ untranslated region of the SNAP-25 gene were those used by Barr *et al*.[1] Standard PCR reactions were carried out in reaction volumes of 25 μl containing 60 ng of DNA, 10 picomoles of each primer, 200 μM of each dNTP, 50 mM of KCl, 10 mM of Tris-HCl (pH 9), 1.5 mM of $MgCl_2$, 0.01% of gelatin and 1 U of Taq polymerase. The PCR conditions consisted of an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 30 s, A final extension step of 72°C for 10 min was also carried out. Genotyping was achieved by restriction fragment length polymorphism (RFLP) analysis. Ten μl of PCR product were digested with either 4 units of *Mnl*I which recognize the sequence 5′..CCTC$(N)_7$.3′ or *Dde*I which recognize the sequence 5′..CTNAG..3′. The digests were separated on an 8% polyacrylamide gel followed by ethidium bromide staining. For allele 1 of the *Mnl*I polymorphism, the 261 bp was cut into two fragments of 256 bp and 5 bp while for allele 2, the 261 bp was digested into three fragments of 210 bp, 46 bp and 5 bp. For the *Dde*I polymorphism, allele 1 (261 bp) was not digested and for allele 2, the 261 bp fragment was cut into two fragments of 228 bp and 33 bp.

Statistics

We used the haplotype based haplotype relative risk[27] and transmission disequilibrium test[28] design to avoid any potential population stratification. The Chi-square test and Fisher's exact *P* were used to assess the significance of the transmission of alleles from parents to their affected children. We also used the programme TRANSMIT to examine haplotypes that might be transmitted more frequently to the ADHD cases and the program EH[29] to assess linkage disequilibrium between the studied markers. We tested the significance of the comparison between preferential maternal and paternal transmissions by plotting 95% confidence intervals and examining for overlap.

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