

Allelic variants of *SNAP25* in a family-based sample of ADHD

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Summary. Altered neurotransmission has been suggested to be a crucial factor in the pathophysiology of attention-deficit/hyperactivity disorder ADHD. Subsequently genes encoding for synaptic proteins have been investigated in candidate gene studies. These proteins mediate the release of neurotransmitters into the synaptic cleft in the process of signal transduction by forming a transient complex, enabling the junction of vesicle and synaptic membrane. One of the core proteins of this complex is the synaptosomal-associated protein 25 (*SNAP25*). It is one of the most validated candidate genes in ADHD according to meta-analyses. However, differing results were observed in previous studies, some of which were not able to observe association with ADHD. In this study we aimed to investigate association of genetic variants of *SNAP25* located in the putative promoter region of *SNAP25* and a SNP in intron 8, previously reported to associated with ADHD. A family based design was applied to detect preferential transmission of genetic variants. In our German ADHD sample no preferential transmission of either variant could be observed. Further investigation considering sub-sample analysis regarding response to D-amphetamine could enlighten the role of *SNAP25* in ADHD.

Keywords: Synapse; gene; neurotransmission; association; monoamines

Introduction

Investigations of the pathophysiology of the highly heritable attention-deficit/hyperactivity disorder (ADHD) have suggested a dysbalance of interacting neurotransmitters (Mehler-Wex et al. 2006; Russell 2007). Especially effectors of monoaminergic neurotransmission have been in the focus of previous studies (Pliszka 2005). Due to the high

heritability of up to 80 percent (APA 2000; Faraone and Doyle 2001), molecular genetic studies have been performed on candidate genes, based on clinical and pathophysiological considerations, as well as on evidence from animal models (Heiser et al. 2004; Faraone et al. 2005).

Genes encoding synaptic proteins involved in the release of neurotransmitters into the synaptic cleft are particularly interesting candidates (Brookes et al. 2005). One of these candidates is the gene for the synaptosomal associated protein 25 (*SNAP25*) which is located presynaptically and is a core member of the soluble NSF attachment receptor (SNARE) complex. This complex is necessary to mediate neurotransmission, stabilizing the junction of the vesicle and the synaptic membrane and the formation of the porous releasing the neurotransmitters (Chen et al. 1999; Sudhof 2004). Playing a crucial role in this process *SNAP25* is to be considered of being capable to influence the balance of several neurotransmitter systems (Jones et al. 2001; Tafoya et al. 2006).

This notion is supported by an animal model, the coloboma mouse. In these mice a chromosomal deletion includes *SNAP25* and besides the occurrence of colobomas, a dysbalance between norepinephrine (NE) and dopamine (DA) in favor of NA is assumed (Jones et al. 2001). These mice feature behaviour resembling ADHD-like symptoms, including spontaneous hyperactivity, increased impulsivity, and signs of inattention (Wilson 2000; Bruno et al. 2007). Notably, coloboma mice respond to prototypical pharmacological treatment of ADHD with D-amphetamine, but not to methylphenidate (Hess et al. 1996). Furthermore,

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(Steffensen et al. 1999) were able to show that a knock-in of *SNAP25* rescues the mice of hyperlocomotion and normalizes the response to D-amphetamine. This indicates a link to pharmacological D-amphetamine effects, further supporting the relevance of *SNAP25* as a candidate gene in ADHD.

SNAP25 which is located on chromosome 20p11.2 has previously been tested for association in different ADHD samples. For investigated variants in the 3'UTR findings were unanimous (Barr et al. 2000; Brophy et al. 2002; Kustanovich et al. 2003; Mill et al. 2004). Mill et al. (2004) reported association of the promoter single nucleotide polymorphism (SNP) rs6077690 and SNP rs363006 located in intron 7. The findings on the intron 7 SNP could not be replicated by Feng et al. (2005) in a combined study on a Canadian and US sample, though their results further supported *SNAP25* as a candidate gene. A study by Brookes et al. (2005) on 51 candidate genes confirmed effectors of synaptic neurotransmission as likely candidates and identified *SNAP25* as positively associated with ADHD, though the previously described variants were not investigated due to technical reasons. Furthermore, recent meta-analyses on association studies named *SNAP25* as one only few genes that remain associated on the basis of studies, further supporting the notion of role in the pathophysiology of ADHD (Faraone and Khan 2006; Schimmelmann et al. 2006).

To reevaluate the observed relevance of *SNAP25* in a German ADHD sample, we investigated two common polymorphic variants in the putative promoter region of *SNAP25*. Disease risk is frequently modulated by functional variants in regions which control expression or stability of gene transcripts (Lesch et al. 1996). Since up to now no biochemical verification of the actual functional impact on gene expression of a polymorphism or haplotype in *SNAP25* is available, selection of the investigated SNPs was based on previous results of association studies, including SNP rs363006 in intron 7. The study used a family-based design, investigating preferential transmission of risk alleles.

Material and methods

Sample

One hundred and sixty-one children with ADHD from 111 families were recruited and phenotypically characterized by a team of experienced psychiatrists in the outpatient unit of the Department of Child and Adolescent Psychiatry and Psychotherapy, University of Würzburg according DSM-IV criteria (Apa 2000). All patients agreed to participate in the study and written informed consent was obtained for all participants. The study was approved by the local Ethics Committee of the University of Würzburg.

Families were included if they had one or more children affected with ADHD to perform family-based association and genome-wide linkage studies. The index patient was required to be older than 8 years and to fulfil DSM-IV criteria for the combined subtype, other affected siblings in a family had to be older than 6 years. The lower limit was chosen in order to ensure relative persistence of ADHD symptoms and to exclude children who may show phenocopies of the disorder during preschool age but do not fulfil diagnostic criteria for ADHD during subsequent developmental stages (Shelton et al. 2000; Barkley et al. 2002). The mean age of the affected children was 11.9 years (SD: 3.6 years). The sample includes 129 males (78.2%) and 36 females (21.8%). In 72 families one child, in 26 families two, in 11 families three and in 2 families four affected children were recruited.

Exclusion criteria were: (a) general IQ ≤ 75 , (b) potentially confounding psychiatric diagnoses such as schizophrenia, any pervasive developmental disorder, Tourette's disorder, and primary mood or anxiety disorder, (c) neurological disorders such as epilepsy, (d) history of any acquired brain damage or evidence of the fetal alcohol syndrome, (e) premature deliveries, and/or (f) maternal reports of severe prenatal, perinatal or postnatal complications. Psychiatric classification was based on the Schedule for Affective Disorders and Schizophrenia for School-age Children Present and Lifetime version (K-SADS-PL). Mothers received: (1) the unstructured Introductory Interview, (2) the Diagnostic Screening Interview, and (3) the Supplement Completion Checklist and upon fulfilment of screening criteria the appropriate Diagnostic Supplements. The child was interviewed with the screening interview of the K-SADS and in case of positive screening for mood or anxiety disorders with the respective supplements of the K-SADS-PL. Additionally, we employed the Child Behavior Checklist and a German Teachers' Report on ADHD symptoms according to DSM-IV. We consider our index patients as representative of ADHD patients of child and adolescent psychiatric units in Germany. See Table 1 for clinical characteristics.

Table 1. Clinical characteristics of the ADHD sample

	N	%
ADHD subtype ^a		
Combined	139	84.3
Predominantly inattentive	23	13.9
Predominantly hyperactive	3	1.8
Co-morbidities ^{a,b}		
Conduct disorder	21	12.7
Oppositional defiant disorder	57	34.5
Mood disorder ^c	20	12.1
Anxiety disorder ^d	9	5.5
Tic disorder	9	5.5

^a Current DSM-IV diagnosis according to K-SADS.

^b Multiple scoring possible.

^c Diagnoses: major depression and dysthymic disorders.

^d Diagnoses: separation anxiety disorder, social phobia, specific phobia.

Table 2. Oligo sequences and restriction enzymes used for genotyping

Marker	Position	Primers	Enzyme
rs6077690	-2015	forward: CCTCTCCATTCCTTCACAA reverse: GAATAGGGGGAAAGGGGTTT	<i>ApoI</i>
rs6039769	-523	forward: CGCTGCTTCGATAACATGAA reverse: AAGGCAGAGGAGGGGTAATA	<i>MspI</i>
rs363006	80609	forward: ATGCCCGAGAAAATGAAATG reverse: CCTGAATCCAAGGTCGTGTT	<i>BsmFI</i>

Table 3. Single marker family-based association analysis of *SNAP25* markers (numbers of transmissions from heterozygous parents only)

Marker	Risk allele	Transmitted	Non-transmitted	Transmission ratio (%)	<i>p</i> -value (FAMHAP)
rs6077690	A	77	74	51	0.86
rs6039769	A	71	63	53	0.54
rs363006	A	38	37	51	1

Genotyping

DNA was extracted out of whole blood samples according to standard protocols. SNPs in the regulatory region of *SNAP25*, respectively previously published to be associated with ADHD were chosen. SNP rs6077690, located –2015 base pairs upstream of the transcriptional start site (TSS), and SNP rs363006 in intron 7 were reported to be associated. Additionally SNP rs6039769 located in the putative core promoter region –523 base pairs to the TSS was investigated. Genotyping for was performed via PCR, enzyme digestion and subsequent gel electrophoresis. Primers were designed by using FastPCR (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) and Autodimer (www.cstl.nist.gov/biotech/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm), respectively. Oligo sequences and used restriction enzymes are given in Table 2. Further information on protocols is given upon request.

Statistical analysis

All markers were checked for Mendelian inconsistencies using PedCheck (O'Connell and Weeks 1998) and in case of inconsistencies the genotypes of all family members for the respective marker and family were set on missing. Hardy-Weinberg equilibrium was checked for all genetic markers on parental genotypes by chi-square tests as implemented in Pedstats (Wigginton and Abecasis 2005). Of the recruited sample 102 families with 151 affected children were finally included in the statistical analysis, 9 families dropped out due technical problems in genotyping. A family-based association test which is valid for families with an arbitrary number of affected children (basically a generalisation of the TDT) was performed for three SNPs in *SNAP25* by using a permutation test (Zhao et al. 2000) as implemented in FAMHAP (Knapp and Becker 2003). Transmitted and non-transmitted alleles are permuted jointly for the affected children in one family. To consider all possible haplotype configurations, this test was performed for all 7 combinations of one to three markers, nominal *p*-values for the single marker tests and a *p*-value that is corrected for multiple testing (of 7 combinations) is given (Becker and Knapp 2004). Analysis of linkage disequilibrium (LD) between the investigated *SNAP25* polymorphisms was also done with the program FAMHAP. This gives *D'* as a measure of LD between pairs of markers and estimates haplotype frequencies from the sample.

Results

A family-based association study was performed for three common *SNAP25* variants in a sample of 102 families including 151 affected children. The association analysis was both single marker- and haplotype-based. All three SNPs were found to be in significant linkage disequilibrium with pair-wise *D'* values of 0.88 (for rs6077690 and rs6039769), 0.32 (for rs6077690 and rs363006) and 0.77 (for rs6039769 and rs363006). Genotype distributions in the parental generation did not deviate from Hardy-Weinberg equilibrium (all *p* > 0.1).

No significant preferential transmissions to affected children with ADHD were detected at any of the three SNPs (Table 2). Haplotype analyses also did not show any significant transmission disequilibrium (all *p* > 0.7). The global *p*-value corrected for multiple testing for the haplotype analysis of all marker combinations in *SNAP25* was *p* = 0.89.

Discussion

We investigated association between genetic variants of the candidate gene *SNAP25* and ADHD in a German family-based sample. No transmission disequilibrium in single nor haplotype analysis was observed, neither of two previously published SNPs in the promoter region and in intron 7, nor of a SNP located –523 base pairs upstream of the putative TSS of *SNAP25*. Our results do not confirm association of *SNAP25* SNPs suggested being of functional importance, either by themselves or by being in linkage disequilibrium with a unknown functional variant.

Research on genetic factors in the development of ADHD has been complicated by a discrepancy between high heritability estimates and a scarcity of replicable gene-disorder associations. Generally, criteria for ADHD according to DSM-IV were applied in the phenotypical classification during the ascertainment of the various patient samples. The distribution of the clinical subtypes of ADHD according to DSM-IV might have a potential impact on the results. Feng et al. (2005) found differing results regarding *SNAP25* in two independent ADHD samples. In the "Irvine" sample, in which only patients with combined type of ADHD were included, no association with *SNAP25* was observed. Positive results were observed in the "Toronto" sample, which included all subtypes of ADHD. But the different results were not attributable to ADHD subtypes and quantitative analysis of the dimensions of hyperactivity/impulsivity and inattention in this second sample revealed that both behavioural traits were associated with *SNAP25*.

Since our sample includes nearly 85 percent of children with the combined type of ADHD, the result supports the suggestion that in patients affected by full-scale ADHD *SNAP25* plays a minor role. On the other hand, subtypes

of ADHD (according to DSM-IV) could still have an impact on association with *SNAP25* in our sample. Differences in ethnicity, differential medication response, and other clinical characteristics of the samples should be considered in an appraisal of the present results. Given the limitations of clinical phenotyping, the diagnostic criteria of DSM-IV that are based on a categorical system may not be applicable for the assessment of ADHD that follows a dimensional approach. Neurobiological heterogeneity of the investigated ADHD samples has been proposed to be responsible for the differing results (Casey et al. 2007). Some candidate genes might be of importance in specific subgroups with distinct phenotypes, but not on basic neuropathology of ADHD. Thus, meta-analyses may be helpful to evaluate the relevance of candidate genes in the general pathophysiology of ADHD by considering positive, as well as negative results.

The study of Hess et al. (1996) on the specific effectiveness of D-amphetamine in coloboma mice indicates a role of *SNAP25* in the pathophysiology of ADHD. In a systematic review by King et al. (2006) D-amphetamine has been shown as very effective, comparably with methylphenidate, in the therapy of patients with ADHD. However, some patients do not respond to methylphenidate but to D-amphetamine (Elia et al. 1991). Feng et al. (2005) discussed that alteration of *SNAP25* expression could be relevant for such a subpopulation, which may not be represented by our sample. To elucidate this potential effect, further studies on *SNAP25* should include a design to investigate response to stimulants, especially to D-amphetamine.

In conclusion, in the present study we were not able to confirm association of variants of *SNAP25* with ADHD. However, *SNAP25* remains an interesting candidate due to its capacity to modulate synaptic transmission by its close interaction with other synaptic proteins. Further studies should consider whether specific gene effects are conditional on environmental factors or gene-gene interaction as well as potential effects in a subgroup responding to administration of D-amphetamine.

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