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ORIGINAL RESEARCH ARTICLE

Systematic mutation screening and association study of the A_1 and A_{2a} adenosine receptor genes in panic disorder suggest a contribution of the A_{2a} gene to the development of disease

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Several lines of evidence suggest a contribution of adenosinergic neurotransmission to the development of panic disorder. We therefore hypothesized that variation in the A_1 and A_{2a} adenosine receptor (AR) genes modifies genetic susceptibility to panic disorder. To test this hypothesis, we screened 38 patients with panic disorder for mutations in the coding sequence of the A_1AR and A_{2a}AR genes. An association study between the identified DNA sequence variants and panic disorder was performed in an extended sample of 89 patients and matched controls. One silent mutation (716T/G) in the A_1AR gene and two silent mutations (432C/T and 1083C/T) in the A_{2a}AR gene were detected. The association sample shows a significant association between the 1083T allele ($P = 0.01$) and 1083T/T genotype ($P = 0.024$) of the A_{2A}R gene and panic disorder. Our findings thus lend further support to the hypothesis that the A_{2a}AR gene, or a locus in linkage disequilibrium with it, confers susceptibility to panic disorder. Replication studies in independent samples with nuclear families applying the transmission disequilibrium test (TDT) are warranted.

Abnormalities of adenosinergic neuromodulation have been associated with panic disorder. The adenosine receptor (AR) antagonist caffeine induces panic attacks in patients with the disorder[1] and caffeine intoxication (DSM-III-R)[2] resembles anxiety disorders. Adenosine analogues have depressant effects on respiratory function in the brainstem,[3] an impairment of depressant brainstem respiratory mechanisms being considered a central feature in panic disorder.[4] Adenosine levels have been found to be unchanged in patients with the disorder[5] and treatment with an uptake inhibitor has been shown to be ineffective.[6] These observations are consistent with the hypothesis that the effectiveness of adenosinergic neuromodulation in patients with panic disorder may be impaired due to changes in receptor function. Four different human AR subtypes have thus far been identified.[7] The A_1 and the A_{2a} adenosine receptors mediate the central nervous system effects of adenosine.

Family and twin studies propose that genetic factors contribute to the pathogenesis of panic disorder.[8–11] In the present study, we therefore systematically searched the A_1 and A_{2a}AR genes for mutations in patients with panic disorder. We detected one silent mutation (716T/G) in the A_1AR gene and two silent mutations (432C/T and 1083C/T) in the A_{2a}AR gene. A significant association between the A_{2a} AR polymorphism (1083C/T) and panic disorder was found, suggesting a contribution of the A_{2a}AR gene to the development of disease.

Screening the A_1 and A_{2a}AR genes of 38 patients with panic disorder for mutations by PCR-based single-strand conformation analysis (SSCA), three bandshifts indicating DNA sequence variation were observed for different PCR fragments (Table 1 and Figure 1). Sequence analysis revealed the presence of three silent mutations. One sequence variant was located in the A_1AR gene and two in the A_{2a}AR gene. While the C→T substitution at position 432 of the A_{2A}R gene was observed in a single individual, the C→T substitution in position 1083 of the A_{2a}AR gene and the T→G substitution in position 716 of the A_1AR gene were found to occur frequently among the patients. All variants can easily be typed using PCR-based restriction fragment length polymorphism (RFLP) assays (Table 2 and Figure 1).

Using these assays we determined the frequency of the variants in 51 additional patients and 89 matched controls. While the rare A_{2a}AR 432T variant was not observed among these individuals, the frequent A_{2a}AR 1083C/T and A_1AR 716T/G polymorphisms were found in both groups. Comparing patients and controls we observed a significant overrepresentation of the 1083T allele ($\chi^2 = 6.69$, d.f. = 1, $P = 0.010$) and the 1083T/T genotype ($\chi^2 = 7.48$, d.f. = 2, $P = 0.024$) among patients. No significant differences between patients and controls were observed for the A_1AR 716T/G polymorphism (Table 3).

Our results thus failed to provide evidence for an involvement of the A_1AR gene in the pathogenesis of panic disorder. However, they suggest that allelic variation at the adenosine A_{2a}AR gene may contribute to the development of the disease.

Positive association findings have to be interpreted with caution. It has been stressed that the candidate gene approach in association studies as applied in this study will provide for numerous false positive results by chance with the commonly accepted significance level of $P < 0.05$ given the numerous possible candi-

Table 1 PCR primers for amplification of overlapping fragments covering the coding region and the exon-intron boundaries of the human adenosine A_1 and A_{2a} receptor genes

Primer	*Primer sequences*	*Nucleotide position (5′–3′)*[a]	*PCR product (bp)*
A_1AR			
Exon 5			
D1	5′-CTGATGTGCCCAGCCTGT-3′	386→403	245
D2	5′-GTGGCCCAATGTTGATGAG-3′	630→612	
D112	5′-GTGGGTGCCCTGGTCATC-3′	582→599	206
D12	5′-TGTGGTGCTGCGAGTACTTC-3′	751+36→751+17	
Exon 6			
D9	5′-GAGGCAGATCCTCACACTCG-3′	752–37→752–17	223
D10	5′-CTGATGACCTTCTCGAACTCG-3′	937→917	
D7	5′-ATCCTCTCCTTCGTGGTGG-3′	807→825	235
D8	5′-GCTGCTTGCGGATTAGGTAG-3′	1041→1022	
D5	5′-CTCCTCATGGTCCTCATCTACC-3′	990→1011	241
D6	5′-TGGCAATGTAGGTAAGGATGC-3′	1230→1210	
D3	5′-TCACCCTCTTCTGCCCGT-3′	1177→1194	250
D4	5′-ACTGGATGTGGGCTGGTG-3′	1426→1409	
A_{2a}AR			
Exon 1			
E112	5′-TGAGCCTGCCTGTCGTCT-3′	1–24→1–7	224
E21	5′-CTGATGGTGATGGCAAAGG-3′	200→182	
E31	5′-TTTGTGGTGTCACTGGCG-3′	130→147	230
E4	5′-CACAGATGTAACCCCGGC-3′	332+27→332+10	
Exon 2			
E5	5′-GGCAAGATGCTGGTCTCTTC-3′	333–27→333–8	284
E6	5′AGACACCCAGCATGAGCAG-3′	589→571	
E7	5′-TCTTTGCCTGTGTGCTGGT-3′	545→563	279
E8	5′-CGATGGCCAGGTACATGAG-3′	823→805	
E9	5′-AACTGCTTCACTTTCTTCTGCC-3′	757→778	241
E10	5′-GCTCTCCGTCACTGCCAT-3′	997→980	
E11	5′-AGGCAGCAAGAACCTTTCAA-3′	925→944	236
E12	5′-CTAAGGAGCTCCACGTCTGG-3′	1160→1141	
E13	5′-GAGGGAGTGCCCAAGAGTC-3′	1103→1121	212
E14	5′-CCCAACGTGACTGGTCAAG-3′	1314→1296	

[a]The numbering of A_1AR exon 5 and 6 nucleotides is according to Ren and Stiles[20] and of A_{2a}AR exon 1 and 2 nucleotides according to Le *et al*.[21] Intron nucleotides were numbered – or + depending on their 5′- or 3′-localization in relation to the exon as the complete sequence of the introns has not yet been established.

date genes.[12] Also, artifacts such as population stratification effects can easily lead to false positive findings in case-control association studies.[13] Replication, therefore is necessary and should be attempted with family-based linkage studies using the transmission disequilibrium test.[14]

The 1083C/T polymorphism of the A_{2a}AR gene does not change the amino acid sequence of the protein. Most likely, the 1083C/T polymorphism is not involved directly in the etiology of panic disorder but is in linkage disequilibrium with the true functional variant either within the A_{2a}AR gene itself or within an adjacent gene. In the present study we found no evidence for a functional variant in the coding region of the A_{2a}AR gene. As the sensitivity of SSCA is not 100%,[15] the presence of such a coding variant cannot be completely excluded. An alternative possibility is that a functional variant resides in a regulatory region of the A_{2a}AR gene leading to altered levels of receptor expression. In fact, mice lacking the A_{2a}AR were recently shown to be markedly more anxious than wild-type mice.[16] The regulatory regions of the human A_{2a}AR gene have yet to be defined at the molecular level.[17] A systematic mutation screening of these regions will therefore be a matter of future studies.

In conclusion, the observed association between a silent polymorphism in the coding region of the A_{2a}AR gene and panic disorder is suggestive for an involvement of the A_{2a}AR in the pathogenesis of panic disorder. If the finding can be independently replicated,

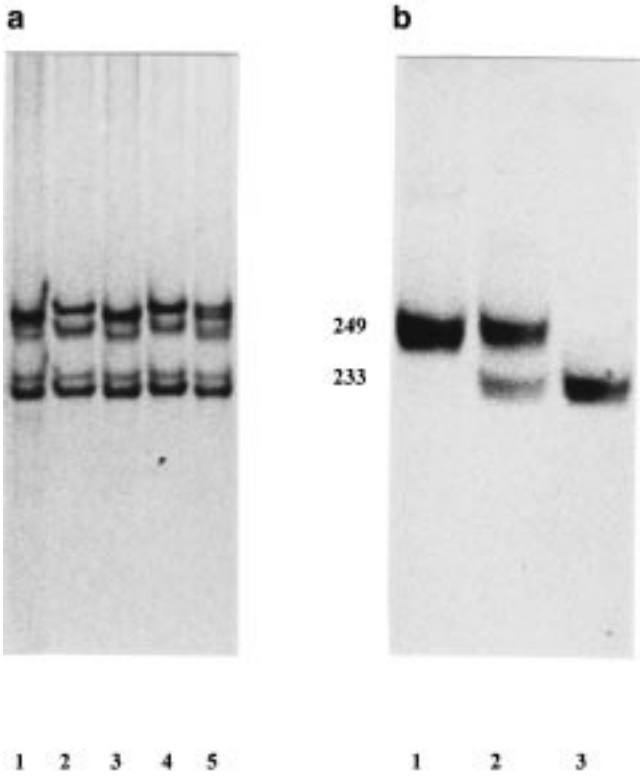

Figure 1 (a) Single-strand conformation analysis (SSCA) of A_{2a}AR PCR product E11/E12. SSCA was performed with 7 V cm^{-1} at 4°C. Lanes 1 and 3 show individuals homozygous for 1083T, lanes 2 and 4 individuals homozygous for 1083C and lane 5 a heterozygous individual. (b) Restriction fragment length polymorphism (RFLP) analysis of polymorphism 1083C/T. A heterozygous individual (lane 2) is surrounded by two homozygous individuals (lane 1: 1083T, lane 3: 1083C). Size of fragments shown is indicated on the left (249 bp and 233 bp respectively).

future studies will aim on the identification of the true functional variant in the A_{2a}AR gene.

Methods

Subjects

The mutation screening sample consisted of 38 patients with panic disorder. The association sample included an additional 51 patients. Eighty-nine anonymous blood donors, who were matched according to age, sex and ethnicity, served as controls. The patients were recruited consecutively from in- and outpatient facilities. Lifetime 'best estimate' diagnoses (DSM-III-R)[2] were assessed by an experienced psychiatrist based on multiple sources of information, including personal structured interviews [1. CIDI[18] or 2. SADS-LA[19]], family history reports and medical records. The inclusion criterion was panic disorder with or without agoraphobia: (a) occurring primary to other major psychiatric disorders (exclusion: simple and social phobia); and (b) representing the predominant syndrome during lifetime. Patients with organic mental or psychotic disorders were excluded.

All probands were unrelated and of German origin. After complete description of the study to the patients, written informed consent was obtained.

Mutation screening, sequence analysis and genotyping

EDTA anticoagulated venous blood samples were drawn from all probands and DNA was extracted by NaCl precipitation. Six (seven) sets of primers (Table 1) were chosen to produce six (seven) overlapping fragments encompassing the whole coding region and the exon-intron boundaries of the human A_1AR (A_{2a}AR) gene.[20,21] PCR was carried out in a 25-μl total volume, containing 80 ng genomic DNA, 10 pmol of each primer, 200 μM dNTPs, 1 U Taq polymerase (Life Technologies, Eggenstein, Germany), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, and 10 mM TRIS-HCl

Table 2 Detection of DNA sequence variants in the human adenosine A_1 and A_{2a} receptor genes

Variant	*Primerpair*	*Annealing temperature*	*PCR product (bp)*	*Restriction enzyme*	*Allele*	*Fragment sizes (bp)*
A_1AR						
716T/G	5′ATCGCCCTGGTCTCTGTG-3′	57°C	273	*Aci*I	716T	173+43+34+23
	5′-GACCCGGAGGTAGAGGTCC-3′[a]				716G	150+43+34+23
A_{2a}AR						
432C/T	5′-GACTCCCATGCTAGGTTGGTA-3′[b]	57°C	179	*Rsa*I	432C	105+54+20
	5′AGACACCCAGCATGAGCAG-3′				432T	125+54
1083C/T	5′-CGGAGGCCCAATGGGTA-3′[b]	59°C	249	*Rsa*I	1083C	233+16
	5′CCCAACGTGACTGGTCAAG-3′				1083T	249

[a]Mutagenic primer to abolish a constant restriction site (nucleotide substitution is underlined).
[b]Mutagenic primer to introduce a polymorphic restriction site (nucleotide substitution is underlined).

Table 3 Allele and genotype distribution of the adenosine receptor gene variants in patients with panic disorder and controls

	Allele		*P*	*Genotype*			*P*
A_1AR							
716T/G	716T	716G		716T/716T	716T/716G	716G/716G	
Panic disorder (n = 89)	127	51	0.113	47	33	9	0.253
Control (n = 89)	113	65		36	41	12	
A_{2a}AR							
1083C/T	1083C	1083T		1083C/1083C	1083C/1083T	1083T/1083T	
Panic disorder (n = 89)	93	85	0.010	28	37	24	0.024
Control (n = 89)	117	61		38	41	10	

Statistical results are given for comparison of allele and genotype distribution between patients and controls using a standard χ^2-test (1 degree of freedom for comparison of allele distribution, 2 degrees of freedom for comparison of genotype distribution).

(pH 8.3). After an initial 5-min denaturation at 95°C, 35 cycles were carried out consisting of 20 s at 94°C, 20 s at 55–59°C,[22,23] and 20 s at 72°C, followed by a final extension step of 5 min at 72°C. For SSCA, 4 μl of PCR-product were mixed with 6 μl denaturing solution containing 85% formamide, 0.001% bromophenol blue, 1.1% Ficoll 400, 0.1 × TBE and denatured for 5 min at 95°C. Samples were subsequently chilled on ice and then loaded on a 10% polyacrylamide gel (acrylamide : bisacrylamide = 49 : 1;, 110 mm × 120 mm × 1 mm, Multigel-Long/Biometra, Gottingen, Germany) containing 0.5× TBE. Gels were allowed to run for 16–18 h under two conditions (6 V cm^{-1} at room temperature and 7 V cm^{-1} at 4°C) to increase sensitivity. Bands were visualized by silver staining.

For sequence analysis PCR products were cloned into pUC18 *Sma*I/BAP vector and DH5α *Escherichia coli* transfected. Single colonies were lysated in 10 μl H_2O by boiling for 10 min. The lysates were used as template for PCR with insert specific primer pairs. SSCA of PCR products allowed the identification of clones containing different PCR alleles. From selected colonies a hemibiotinylated PCR product was generated using one biotinylated vector primer and one normal vector primer. The PCR product was incubated with streptavidine Dynabeads (Dynal, Hamburg, Germany) and magnetic beads were collected with a magnetic concentrator. After washing and denaturing, both strands of DNA were sequenced by the dideoxynucleotide chain termination method using Sequenase Version 2.0 Kit (US Biochemicals, Cleveland, OH, USA).

Genotyping of the DNA sequence variants was performed by means of PCR-based RFLP assays (Table 2). An 8-μl aliquot of PCR product was incubated with 8 U *Aci*I or 5 U *Rsa*I according to the manufacturer's recommendations (New England BioLabs, Schwalbach, Germany). Fragments were separated in a 15% polyacrylamide gel (acrylamide : bisacrylamide 49 : 1 containing 1× TBE) and visualized by silver staining.

Statistical analysis

Control and patient group were compared using a standard χ^2 test.

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References

1 Boulenger JP, Uhde TW, Wolff EA, Post RM. Increased sensitivity to caffeine in patients with panic disorder: preliminary evidence. *Arch Gen Psychiatry* 1984; **40**: 1067–1071.
2 American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders*, 3rd edn, revised. American Psychiatric Association: Washington, DC, 1987.
3 Barraco RA, Janusz CA, Schoener EP, Simpson LL. Cardiorespiratory function is altered by picomole injections of 5′-N-ethylcarboxamidoadenosine into the nucleus tractus solitarius of rats. *Brain Res* 1990; **507**: 234–246.
4 Gorman JM, Fyer MR, Goetz R, Askanazi J, Liebowitz MR, Fyer AJ *et al*. Ventilatory physiology of patients with panic disorder. *Arch Gen Psychiatry* 1988; **45**: 31–39.
5 Boulenger JP, Salem N, Marangos PJ, Uhde TW. Plasma adenosine levels: measurement in humans and relationship to the anxiogenic effects of caffeine. *Psychiatry Res* 1987; **21**: 247–255.
6 Stein M, Black B, Brown TM, Uhde TW. Lack of efficacy of the adenosine reuptake inhibitor dipyridamole in the treatment of anxiety disorders. *Biol Psychiatry* 1993; **33**: 647–650.
7 Williams M. Purinoceptors in central nervous system function. In: Bloom FE, Kupfer DJ (eds). *Psychopharmacology: The Fourth Generation of Progress*. Raven Press: New York, NY, 1995, pp 643–655.
8 Noyes R, Crowe RR, Harris EL, Hamra BJ, McChesney CM, Chaudry DR. Relationship between panic disorder and agoraphobia. *Arch Gen Psychiatry* 1986; **43**: 227–232.
9 Skre I, Onstad S, Torgersen S, Lygren S, Kringlen E. A twin study of DSM-III-R anxiety disorders. *Acta Psychiatr Scand* 1993; **88**: 85–92.
10 Maier W, Lichtermann D, Minges J, Ohrlein A, Franke P. A controlled family study in panic disorder. *J Psychiatr Res* 1993; **27** (Suppl 1): 79–87.
11 Kendler KS, Neale MC, Kessler RC, Heath AC, Eaves LJ. Panic disorder in women: a population-based twin study. *Psychol Med* 1993; **23**: 397–406.
12 Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science* 1996; **273**: 1516–1517.
13 Lander ES, Schork NJ. Genetic dissection of complex traits. *Science* 1994; **265**: 2037–2048.
14 Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993; **52**: 506–513.
15 Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 1989; **5**: 874–879.
16 Ledent C, Vaugeois J-M, Schiffmann SN, Pedrazzini T, Yacoubi ME,

Vanderhaegen J-J *et al*. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the A_{2a} receptor. *Nature* 1997; **388**: 674–678.

17 Peterfreund RA, MacCollin M, Gusella J, Fink S. Characterization and expression of the human A_{2a} adenosine receptor gene. *J Neurochem* 1996; **66**: 363–368.

18 Robins LN, Wing J, Wittchen HU, Helzer JE, Babor TF, Burke J *et al*. The composite international diagnostic interview: an epidemiologic instrument suitable for use in conjunction with different diagnostic systems and in different cultures. *Arch Gen Psychiatry* 1988; **45**: 1069–1077.

19 Manuzza S, Fyer AJ, Klein DF, Endicott J. Schedule for Affective Disorders and Schizophrenia — Lifetime version (modified for the study of anxiety disorders): rationale and conceptual development. *J Psychiatr Res* 1986; **20**: 317–325.

20 Ren H, Stiles GL. Characterization of the human A1 adenosine receptor gene. *J Biol Chem* 1994; **269**: 3104–3110.

21 Le F, Townsend-Nicholson A, Baker E, Sutherland GR, Schofield PR. Characterization and chromosomal localization of the human A2a adenosine receptor gene: ADORA2A. *Biochem Biophys Res Commun* 1996; **223**: 461–467.

22 Deckert J, Nothen MM, Bryant S, Ren H, Wolf HK, Stiles GL *et al*. Human adenosine A_1 receptor gene. Systematic screening for DNA sequence variation and linkage mapping on chromosome 1q31–32.1 using a silent polymorphism in the coding region. *Biochem Biophys Res Commun* 1995; **214**: 614–621.

23 Deckert J, Nothen MM, Rietschel M, Wildenauer D, Bondy B, Ertl MA *et al*. Human adenosine A_{2a} receptor (A_{2a}AR) gene: systematic mutation screening in patients with schizophrenia. *J Neural Transm* 1996; **103**: 1447–1455.

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