Neuropsychopharmacology (2003) 28, 1694–1702

www.neuropsychopharmacology.org

Association Between A_{2a} Receptor Gene Polymorphisms and Caffeine-Induced Anxiety

Karen Alsene[1,4], Jürgen Deckert[2], Philipp Sand[3] and Harriet de Wit*[,1]

[1]Department of Psychiatry, The University of Chicago, Chicago, IL, USA; [2]Department of Psychiatry, University of Munster Albert-Schweitzer-Straße, Münster, Germany; [3]Department of Psychiatry, University of Würzburg Füchsleinstraße, Würzburg, Germany

The adenosine receptor system, which mediates the psychoactive effects of caffeine, is also thought to be involved in the regulation of anxiety. In this study, we examined the association between variations in anxiogenic responses to caffeine and polymorphisms in the A_1 and A_{2a} adenosine receptor genes. Healthy, infrequent caffeine users ($N = 94$) recorded their subjective mood states following a 150 mg oral dose of caffeine freebase or placebo in a double-blind study. We found a significant association between self-reported anxiety after caffeine administration and two linked polymorphisms on the A_{2a} receptor gene, the 1976C > T and 2592C > Tins polymorphisms. Individuals with the 1976T/T and the 2592Tins/Tins genotypes reported greater increases in anxiety after caffeine administration than the other genotypic groups. The study shows that an adenosine receptor gene polymorphism that has been associated with Panic Disorder is also associated with anxiogenic responses to an acute dose of caffeine.
Neuropsychopharmacology (2003) **28,** 1694–1702, advance online publication, 25 June 2003; doi:10.1038/sj.npp.1300232

Keywords: caffeine; adenosine A_{2a} receptor; adenosine A_1 receptor; polymorphism; anxiety; panic disorder

INTRODUCTION

Caffeine is the most widely used psychoactive drug in the world: 82–92% of the adults in North America report regular consumption of caffeine-containing beverages (Graham, 1978; Gilbert, 1976). The popularity of caffeine is thought to be related to its subjective effects, which include increased alertness and stimulation (eg Griffiths and Mumford, 1996; Lieberman *et al*, 1987). However, large interindividual variations in acute responses to caffeine have been reported, especially in anxiety-inducing effects of the drug. Controlled studies have confirmed that while caffeine produces positive effects such as increased stimulation in many, others experience negative effects such as increased anxiety (Chait, 1992; Evans and Griffiths, 1991).

While the reason for the inter-individual variability in responses to caffeine is not clear (eg Lieberman *et al*, 1987; Svensson *et al*, 1980; Loke, 1988; Liguori *et al*, 1999), there is evidence that some of the variability in acute responses to caffeine may have a genetic basis. A recent study comparing habitual caffeine use in monozygotic *vs* dizygotic female twin pairs reported a high heritability ratio in caffeine use, toxicity, tolerance, and dependence (Kendler and Prescott, 1999). One source of this inherited variability in responses to caffeine may be variation in genes coding for receptors where caffeine acts, in particular, the adenosine receptor. Caffeine is thought to produce its central effects through its action as an adenosine receptor antagonist (Snyder and Sklar, 1984). It binds with high affinity at both the A_1 and A_{2a} adenosine receptors, the two subtypes thought to be responsible for many of caffeine's behavioral effects (Svenningsson *et al*, 1997). Both of these adenosine receptor subtypes are expressed in the human brain, with A_1 receptors being widely distributed throughout the brain and A_{2a} receptors concentrated mainly in the dopamine-rich basal ganglia areas of the brain (Fredholm *et al*, 2000).

Polymorphisms in the A_1 and A_{2a} adenosine receptor genes may account for variations in subjective responses to caffeine. Two noncoding adenosine receptor gene polymorphisms have been reported in the coding regions of the A_1 receptor gene (Deckert *et al*, 1998a) and the A_{2a} receptor gene (Deckert *et al*, 1996). Polymorphisms are commonly referred to by the nucleotide base site at which they occur on the gene (eg 1976) followed by the nucleotide base substitution comprising the polymorphism (eg C > T indicates that a T is substituted for a C; den Dunnen and Antonorakis, 2000). The adenosine A_{2a} receptor gene polymorphism 1976T > C, formerly 1083T > C, but not the adenosine A_1 receptor gene polymorphism 716T > G, was

*Correspondence: Dr H de Wit, Department of Psychiatry, The University of Chicago, MC3077, 5841 S. Maryland Avenue, Chicago, IL 60637, USA, Tel: +1 773 702 1537; Fax: +1 773 834 7698, E-mail: hdew@midway.uchicago.edu

[4]Neuroscience Training Program, University of Wisconsin Madison, USA.

Received 13 November 2002; revised 30 April 2003; accepted 05 May 2003

Online publication: 14 May 2003 at http://www.acnp.org/citations/Npp05140341O/default.pdf

found to be associated with Panic Disorder (PD) (Deckert *et al*, 1998b). PD is characterized by recurrent and unexpected attacks of anxiety or fear (APA, 2000). The T allele of the noncoding adenosine A_{2a} receptor gene polymorphism was more prevalent in a population of PD patients compared to a control population, indicating that genetic variation in the A_{2a} receptor gene may be related to susceptibility to anxiety. As this noncoding polymorphism may not be functionally or clinically significant, a systematic mutation screening for further, potentially functional and clinical relevant polymorphisms in 5′- and 3′-regulatory cDNA regions of the A_{2a} adenosine receptor gene had been performed in patients with PD, which resulted in two additional adenosine A_{2a} receptor gene polymorphisms (Deckert *et al*, 2001). Both the A_{2a} and the A_1 receptor have been linked to anxiety in animal studies, showing that knockout mice lacking the A_{2a} or the A_1 receptor score higher on behavioral measures of anxiety (Ledent *et al*, 1997, Johansson *et al*, 2001). Thus, we reasoned that polymorphisms in the A_{2a} adenosine receptor gene, and also possibly the A_1 receptor gene, might be responsible for the inter-subject variability in anxiogenic responses to caffeine.

This study was designed to assess responses to an acute dose of caffeine in relation to polymorphisms in the A_1 and A_{2a} receptor genes in infrequent caffeine users. Infrequent caffeine users (ie individuals who consume less than 300 mg of caffeine per week) were chosen to minimize the confounding effects of tolerance and withdrawal. The volunteers participated in two sessions, in which they ingested capsules containing placebo or 150 mg of caffeine (equivalent to the caffeine in about two cups of coffee). It was hypothesized that variations in anxiogenic responses to caffeine would be related to polymorphisms in the A_1 and A_{2a} adenosine receptor genes.

MATERIALS AND METHODS

Volunteers

A total of 100 healthy, infrequent caffeine users, 54 men and 46 women, completed the behavioral portion of the study. Only individuals who reported a weekly caffeine consumption of less than 300 mg, including the intake of coffee, tea, caffeinated sodas, and other dietary sources of caffeine, were accepted. Caffeine consumption was based on estimates of 50 mg per 12 oz serving of caffeinated soft drinks, 60 mg per 8 oz serving of tea, 100 mg per 8 oz serving of coffee, and 10 mg per bar of chocolate (Durrant, 2002).

Eligibility was determined via a telephone interview and a brief in-person psychiatric interview. The psychiatric interview consisted of questions relating to current anxiety, depression, and psychosis, and it was conducted by an individual knowledgeable in DSM-IV diagnosis (APA, 2000). Subjects were excluded if they had any history of substance abuse or dependence, if they reported high levels of anxiety or depression, any history of psychosis, or use of more than 300 mg of caffeine per week. Cigarette smokers were not accepted because smoking has been shown to reduce the half-life of caffeine (Hart *et al*, 1976). Subjects were also excluded if they had high blood pressure or used any medication on a daily basis.

Drugs

Subjects participated in two sessions, separated by at least 2 days, in which they received either placebo or caffeine (150 mg freebase). Caffeine and placebo were administered in random order and under double-blind conditions. Caffeine was administered in the form of caffeine citrate because it is more bioavailable than anhydrous caffeine. The caffeine citrate (300 mg) is equivalent to about 150 mg caffeine freebase, and this dose has been shown to produce subjective, physiological, and behavioral effects (Lader, 1969; Gupta, 1993; Gupta *et al*, 1994; White *et al*, 1980). The caffeine capsules were white and contained caffeine citrate (Mallinckrodt Baker, Inc., Paris, KY) with dextrose filler. Placebo capsules contained dextrose alone. For blinding purposes, subjects were informed that they would receive a commonly used drug that could be a stimulant, sedative, or placebo.

Sessions

Before the study began, volunteers attended a short orientation session in which they read and signed the consent form. The study was approved by the University of Chicago's Institutional Review Board. Subjects were instructed to abstain from taking any recreational drugs, including alcohol, nicotine, and caffeine, for 24 h before the sessions. They were also instructed to abstain from eating after midnight the night before the sessions.

Sessions were conducted from 08.30 to 12.00 h noon with a minimum of 2 days between the sessions. Sessions for women were scheduled without regard to menstrual cycle phase as it has been shown that the pharmacokinetics of caffeine are not significantly altered during the menstrual cycle (Kamimori *et al*, 1999). Upon arrival in the laboratory, subjects provided breath and urine samples to confirm their compliance with abstinence instructions. They were given a light meal (bagel with cream cheese) to reduce stomach irritation from the caffeine capsule. Then they completed baseline (precapsule) mood questionnaires and measures. At 09.10 h, 30 min after consuming the bagel, they ingested a capsule with 150 ml of orange juice. Physiological, subjective, and behavioral measures were obtained 20, 40, 60, and 120 min after capsule administration. Physiological measurements included heart rate, blood pressure, and temperature. Subjective measurements included ratings of drug effects and mood (see below). Behavioral measurements consisted of two measures of psychomotor performance (see below). Subjective and behavioral tasks were administered via computer. Volunteers were allowed to watch emotionally neutral movies and read during the sessions when measurements were not being taken. On a separate visit, subjects provided a blood sample for genotyping and completed personality questionnaires.

Behavioral Measurements

To assess the volunteers' baseline mood states and the effect of caffeine on mood, they completed several standardized questionnaires. The primary mood measures assessed the state of anxiety as measured by Anxiety scale on the shortened version of the Profile of Mood States (POMS)

(Shacham, 1983), which is highly correlated with the longer 72-item version (McNair *et al*, 1971), and ratings of Anxiety on a visual-analog scale (VAS). Secondary mood measures included POMS scales assessing anger, confusion, depression, elation, fatigue, and vigor and VAS items indicating if subjects felt stimulated, interested, content, confused, drowsy, hungry, elated, sedated, and nauseous. In addition, subjects completed the Addiction Research Center Inventory (ARCI) (Martin *et al*, 1971) and a locally developed visual analog questionnaire (Drug Effects Questionnaire, or DEQ) that assesses the extent to which subjects experience four subjective states: 'Feel Drug,' 'Feel High,' 'Like Drug,' and 'Want More.' Finally, at the end of both sessions subjects were asked to identify the class of drug they had received from a list of possible classes (stimulant, sedative, or placebo).

Behavioral and physiological measures provided objective indicators of the drug's effects. The behavioral measures included the Digit Symbol Substitution Task (DSST) (Wechsler, 1958) and a digit tapping speed task. The DSST is a measure of psychomotor performance on which subjects match symbols with numbers for 90 s. The digit tapping task is a motor task which measures the time required for the volunteer to tap on a computer key 150 times with their index finger. Both measures have been found to be sensitive to acute caffeine administration in subjects (Kaplan *et al*, 1997). Physiological measures included heart rate, blood pressure, and body temperature. Blood pressure and heart rate were measured using a Digitronic monitor (New Brunswick, NJ) and temperature was measured using an IVAC thermometer (San Diego, CA).

Subjects also completed two personality questionnaires to investigate correlations between responses to caffeine, genotype, and the personality trait of impulsivity. These included the Barratt Impulsiveness Scale-11 (BIS-11) (Patton *et al*, 1995) and the Eysenck I7 (Eysenck, 1993), which includes measures of impulsivity, venturesomeness, and empathy.

Genotyping

EDTA anticoagulated venous blood samples and DNA was extracted using the Genepure kit. All presently confirmed adenosine A_1 and A_{2a} receptor gene cDNA polymorphisms were investigated. The adenosine A_1 receptor gene 716T > G polymorphism and the adenosine A_{2a} 1976T > C (previously 1083T > C) polymorphism were genotyped by means of PCR-based restriction fragment length polymorphism (RFLP)-assays as previously described (Deckert *et al*, 1998a, b). The 263C > T polymorphism was genotyped by means of PCR-based RFLP-assay using 5U Bse NI (MBI Fermentas) as a restriction enzyme . The 2592C > Tins polymorphism was genotyped both by a PCR-based RFLP-assay using Mbo II (MBI Fermentas) as restriction enzyme and by a PCR-based SSCP-analysis using 5′-GGG CCC AGA GGT GAC ATT-3′ (forward) and 5′-CCT GGG ACT GAG AAG TGG AT-3′ (reverse) as primer pair at an annealing temperature of 58°C. Conditions of the RFLP-assays and fragment lengths are summarized in Table 1. Each PCR-assay contained 1.5 mM MgCl2, 200 μM dNTP-mix (Eppendorf), 10 pmol primer, 1 U Hot Star Taq plus buffer (Quiagen), and 80 ng genomic DNA in a total volume of 25 μl. For the PCR-assay prior to Mbo II digestion Q-solution was added. In all, 35 cycles were performed with cycle lengths varying between 30 and 60 min preceded by 5′ denaturation at 94°C and followed by 5′ elongation at 72°C. Digestion was performed overnight for 20 h according to the manufacturer's directions and digestion products were separated on 15%-polyacrylamide gels (acrylamide:bisacrylamide = 49:1) in 1 × TBE buffer at 200 V for 3 h (Multigel-Long, Biometra), SSCP-analysis was performed on 10%-polyacrylamide gels (acrylamide:bisacrylamide = 49:1) in 0.5 × TBE at 70 V and 4°C overnight for 20 h. Bands were visualized by silver staining. Genotyping was performed blind, that is, the analyzers were unaware of the clinical, physiological, and behavioral characteristics of the subjects.

In Silico Sequence Analysis

In order to examine whether the polymorphisms may be functionally relevant, ortholog mRNA sequences from the mouse (U05672), rat (M91214), guinea pig (U04201), and dog (X14052) were investigated for conserved features using the multiple alignment construction and analysis workbench (MACAW) V.2.0.5 (Schuler *et al*, 1991) by pairwise

Table 1 DNA Sequence Polymorphisms in the Human A_1 and A_{2a} Adenosine Receptor Genes

	Primer pair	Annealing temperature (°C)	PCR product (bp)	Restriction enzyme	Allele	Fragment size (bp)
A_1AR						
716T > G	5′-ATCGCCCTGGTCTCTGTG-3′	57	273	AciI	716T	173+43+34+23
	5′-GACCCGGAGGTAGAGGTCC-3′				716G	150+43+34+23
A_{2A}AR						
263C > T	5′-TACCCAGAGGCAACCAGATAAA-3′	56	224	BseNI	263C	224
	5′-CGAAAAGCCCATTTCTACCAAA-3′				263T	167+57
1976T > C	5′-CGGAGGCCCAATGGGTA-3′	63	249	RsaI	1976C	233+16
	5′-CCCAACGTGACTGGTCAAG-3′				1976T	249
2592C > Tins	5′-CAGAGGTGACATTTGACTTTCTT-3′	54	194	MboII	C	182+12
	5′-CCTGGGACTGAGAAGTGGAT-3′				Tins	195

Substitutions in mutagenic primers are underlined. Nomenclature of the A_1AR polymorphism is according to cDNA sequence GenBank Acc. No. NM_000674, the nomenclatures of the A_{2a}R polymorphisms are according to cDNA sequence GenBank Acc. No. X68486 following the recommendations of den Dunnen and Antonarakis (2000). NCBI SNP cluster IDs were not given because none of the investigated SNPs is listed.

segment overlap at a pairwise score cutoff of 60. Editing of multiple alignments was performed manually. For the identification of specific signals, we applied a succession of motif search tools, UTR scan (http://bighost.area.ba.cnr.it/BIG/UTRScan/) against the current release (15.0) of the UTRdb (http://bighost.area.ba.cnr.it/BIG/UTRHome/, 154393 nonredundant entries), and Gene Tool Motif Search against the GeneToolLite V.1.0 custom database of eukaryotic motifs, respectively, to the entire A_{2a} 3′-UTR. Curvature propensity and GC-content plots of the A_{2a} terminal exon were obtained from the bend.it server (http://www2.icgeb.trieste.it/~dna/bend_it.html). Curvature propensity was calculated using the Dnase I-based bendability parameters of Brukner *et al* (1991) and the consensus bendability scale (Gabrielian and Pongor, 1996).

Statistical Analysis

To determine the overall effects of caffeine, data from caffeine and placebo sessions were examined using separate two-way repeated measures ANOVA (drug (two levels) × time (five levels)) for each dependent measure. To determine whether genetic variations in the A_1 and A_{2a} receptor were related to subjective or behavioral measurements, individuals were assigned to one of three genotypic groups (eg TT, CT, or CC) at each of the four loci (716T > G, 263C > T, 1976T > C, and 2592C > Tins). The direction and magnitude of response to caffeine was calculated for each subject using peak change scores on the caffeine and placebo sessions. Peak change scores were calculated by subtracting the precapsule baseline score from either the maximum or minimum response reached within 1 h after capsule administration. Only peak scores reached within the first hour were used because studies have shown peak subjective effects of caffeine to occur within 20–40 min after oral administration (Mumford *et al*, 1994). Separate two-way ANOVAs ((drug (two levels) × group (three levels)) were used to analyze each dependent measure. The Hardy–Weinberg equilibrium was examined using an online resource provided by Professor Christensen (http://www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm). Linkage disequilibrium analysis was performed at the allelic level using the Genetix program, version 4.02 (Belkhir *et al*, 2001). The significance level for all statistical tests was set at $P < 0.05$.

RESULTS

Demographic Data

Genetic data could not be analyzed for six subjects because of problems in obtaining the blood sample or extracting the DNA. Therefore data are reported for 94 volunteers (51 male and 43 female subjects). Most subjects were Caucasian college students in their early 20s. They reported consuming an average of one to two caffeine-containing beverages per week and an average of one to two alcoholic beverages per week. When total weekly caffeine consumption (including sodas and chocolate) was converted to cups of coffee per week, 26 participants reported no caffeine use, 43 reported less than one cup per week, 18 reported consuming between one and two cups of coffee, and seven reported consuming the equivalent of two to three cups of coffee per week. There was no relationship between demographic variables such as gender, age, or race and subjective responses to caffeine (data not shown).

Overall Caffeine Effects

When the data from all 94 volunteers were examined together, caffeine produced its prototypic effects. On the subjective measurements, caffeine significantly ($P < 0.05$) increased scores on Stimulation (VAS), Anxiety (VAS and POMS), and the ARCI BG and A (Stimulant-like) scales, and decreased scores on Depression (POMS) and Fatigue (POMS). On the behavioral tasks, caffeine significantly increased the number of symbols completed on the DSST but had no effect on the digit tapping task. Caffeine significantly increased the heart rate, systolic and diastolic blood pressure, but had no effect on body temperature. The effects of caffeine analyzed using the peak change scores (used for association analyses) were similar to the effects obtained in the analysis by time (data not shown). Approximately half of the subjects correctly identified caffeine as a stimulant (41 were correct and 53 were incorrect), a level that was not significantly greater than chance.

Adenosine A_1 And A_{2a} Receptor Gene Polymorphisms

The frequencies of the alleles and genotypes for each of the four polymorphisms examined in the study sample are shown in Table 2. The 1976T > C on the A_{2a} receptor gene and the 716T > G on the A_1 receptor gene are the only polymorphisms which have previously been reported and their representation in our study sample is similar to previous studies (Deckert *et al*, 1998b; Yamada *et al*, 2001). All polymorphisms were in Hardy–Weinberg equilibrium. The 1976T > C and 2592C > Tins polymorphisms were in nearly complete linkage disequilibrium ($P < 0.0001$, $\chi^2 = 106.35$, df = 1). Therefore, these two linked polymorphisms formed only three genotypic groups: the 1976C/C and

Table 2 Allele and Genotype Distribution of the A_1 and A_{2a} Receptor Polymorphisms

Variant	Allele		Genotype		
A_1 receptor gene	T	G	T/T	T/G	G/G
716T > G	126	56	42	42	7
A_{2a} receptor gene	C	T	C/C	C/T	T/T
263C > T	161	27	69	23	2
1976T > C	92	96	25	42	27
	C	Tins	C/C	C/Tins	Tins/Tins
2592C > Tins	91	97	25	41	28

Due to low DNA yields for several subjects, the A1 receptor gene was only typed for 91 subjects. The distributions from this study are comparable to previous studies. In a German sample ($N = 281$), genotype frequencies were CC = 107, CT = 135 and TT = 37 with allele frequencies of C = 349 and T = 209 (Deckert *et al*, 1998b). A Japanese study (N = 99) reported CC = 26, CT = 54, and TT = 19, with C = 106 and T = 92 (Yamada *et al*, 2001).

2592C/C group, the 1976C/T and 2592C/Tins group, and the 1976T/T and 2592Tins/Tins group.

A_{2a} 1976T > C and 2592C > Tins Polymorphism

Responses to caffeine were compared in the three groups based on their genotype at the 1976T > C and 2592C/Tins polymorphisms. Both these polymorphisms were significantly related to increases in anxiety after caffeine. Individuals with the 1976T/T and 2592Tins/Tins genotypes reported a greater increase in anxiety after caffeine than did individuals from either of the other two groups on both the POMS anxiety measure (Figure 1 ; $P<0.05$) and the VAS anxiety scale (Figure 2 ; $P<0.05$). Figure 3 shows the time course of the anxiogenic effect in the three genotyped groups. The association between genotype and increased anxiety after caffeine was apparent in both men and women. The groups did not differ on measures of anxiety either at baseline (before capsule administration) or after placebo. The groups did not differ in their responses to caffeine on other measures of mood, behavior, or in the identification of the class of drug they had received. Figure 4 shows the peak change scores for the genotypic groups on a representative measure (for Vigor (POMS) that did not differ across the groups ($P<0.7$)). The time to peak increases in subjective ratings of Anxiety and Vigor did not differ across the genotypic groups. The groups also did not differ on any demographic measures such as sex, race, age, or drug use (Table 3). Interestingly, however, the groups did differ on a personality measure of 'venturesomeness', a form of extraversion (Eysenck, 1993). The subjects in the 1976T/T and 2592Tins/Tins groups scored lower on this trait measure than subjects from the other two genotypic groups ($P<0.05$).

Other Polymorphisms

The volunteers were also divided into three groups based on their genotype at the A_{2a} 263C > T and the A_1 716T > G loci and their responses to caffeine were compared. There were no significant differences between these groups on any measure (data not shown).

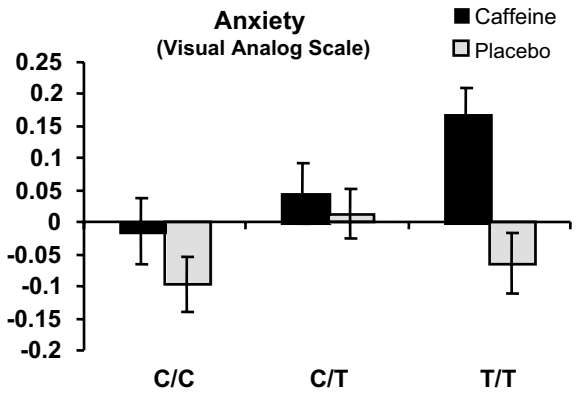

Figure 1 Mean (SEM) peak change scores on anxiety (VAS) between the three genotypic groups at the 1976T > C and 2592C > Tins polymorphism locus after placebo and caffeine (150 mg). Only the 1976T/T group reported a significant increase in anxiety.

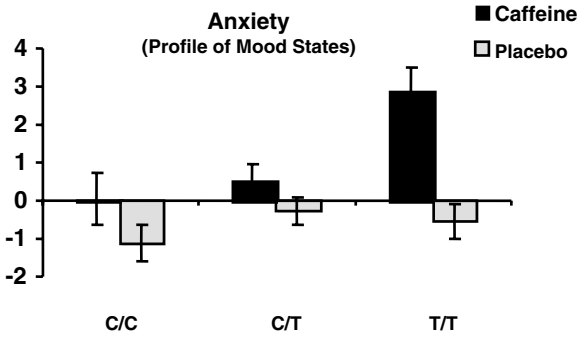

Figure 2 Mean (SEM) peak change scores on anxiety (POMS) between the three genotypic groups at the 1976T > C and 2592C > Tins polymorphism locus after placebo and caffeine (150 mg). Only the 1976T/T group reported a significant increase in anxiety.

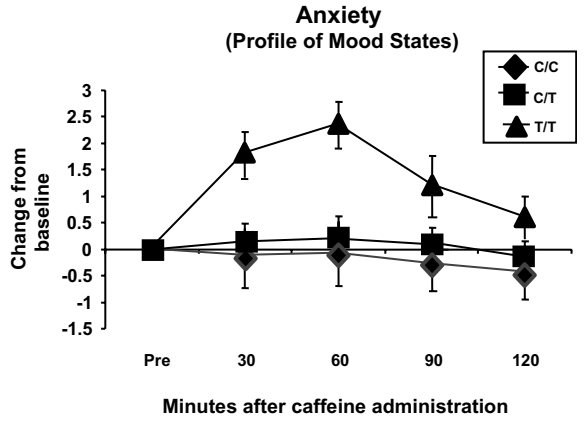

Figure 3 Time course of self-reported anxiety (POMS) after caffeine administration (150 mg) for the three genoptyed groups. Data shown are mean (SEM) ratings of anxiety (POMS) as change from predrug baseline. Baseline scores did not differ among the groups. The 1976T/T group reported higher levels of anxiety after caffeine than either of the other two groups.

Functional Elements in the 3′-Utr Adenosine A_{2a} Receptor Gene Sequence

It is not known whether the 2592C > Tins polymorphism is functionally relevant. However, as a first step to investigating this important question, mRNA sequences were compared from several species, including the mouse, rat, guinea pig, and dog. Comparative sequence analysis revealed a strikingly similar architecture of conserved cis-acting elements across species that coincided with regions of proposed curvature (Figure 5). Curvature propensity plots of the entire exon (data not shown) peaked at the termination codon, at the polymorphic (UUUUUU)-motif, and at the terminal 88 bp of the mRNA sequence. Interestingly, the U-rich motif displays some variability in ortholog DNA, reflecting a mononucleotide run prone to slippage during replication. In addition to the nucleotide identities and an elevated curvature propensity at the (UUUUUU)-motif, two equally conserved, flanking class I A + U-rich elements (AREs) suggest a region of functional importance. With regard to the terminal 88-bp stretch, the pattern-sensitive search disclosed a highly conserved, terminal transcription enhancer, internal ribosome entry

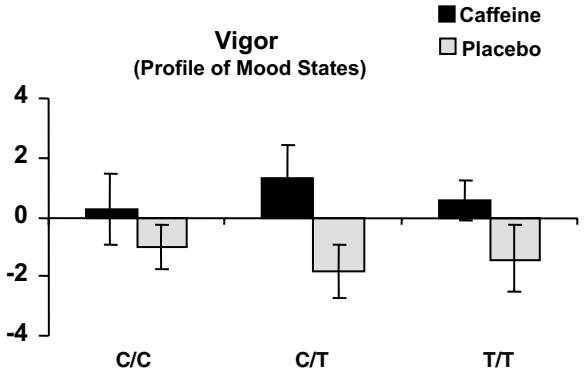

Figure 4 Mean (SEM) peak change scores on vigor (POMS) between the three genotypic groups at the 1976T>C and 2592C>Tins polymorphism locus after placebo and caffeine (150 mg). Caffeine increased self-reports of vigor in all three genotyped groups and there were no differences between the groups on this measure.

Table 3 Demographic Variables between the Genotypic Groups at the 1976T>C Polymorphism

Variable	1976C/C group (*N* = 25)	1976C/T group (*N* = 42)	1976T/T group (*N* = 27)
Age, years (mean ± SD)	21.3 ± 2.7	21.8 ± 3.8	21.2 ± 3.7
BMI	22.7 ± 2.1	22.7 ± 2.9	22.6 ± 2.4
Sex, *n* (male/female)	16/9	19/23	16/11
Race/ethnicity			
White	17	23	14
Black	1	7	7
Asian	6	9	5
Hispanic	1	3	1
Education			
High school	0	2	0
Current college student	18	30	23
Partial college	1	2	1
College degree	3	3	1
Current drug use			
Caffeine, mean ± SD (drinks/week)	1.44 ± 0.73	1.32 ± 0.65	1.13 ± 0.5
Alcohol, mean ± SD (drinks/week)	1.6 ± 1.8	1.5 ± 1.8	1.2 ± 1.4
Marijuana, mean number used	6	11	7

site (IRES), corresponding to the DNA region of proposed curvature. Thus, although definitive functional studies have yet to be performed, these observations support the idea that the polymorphism is functionally relevant.

DISCUSSION

The primary finding of this study was that acute anxiogenic responses to caffeine were associated with two linked polymorphisms in the A_{2a} adenosine receptor gene. Healthy volunteers received an oral dose of caffeine (150 mg), and were genotyped at three loci on the A_{2a} adenosine receptor gene and one on the A_1 receptor gene. Based on previous reports indicating variability in anxiogenic responses to caffeine (Chait, 1992; Evans and Griffiths, 1991) and studies indicating that the adenosine receptor regulates anxiety, we hypothesized that polymorphisms in the A_1 and A_{2a} adenosine receptor gene may account for inter-individual variations in anxiety after administration of caffeine. Consistent with the hypothesis, we found that individuals with the linked 1976T/T and the 2592 Tins/Tins variants in the A_{2a} adenosine receptor gene reported greater increases in anxiety after caffeine intake than did individuals in either of the other two genotypic groups. The three genotypic groups did not differ on other subjective measures of caffeine effects, including self-report measures of feeling stimulated or global ratings of drug effects. They also did not differ in physiological responses to caffeine (eg heart rate) or in the behavioral effects as measured by the psychomotor tasks. Thus, the genotypes were specifically related to the subjective experience of anxiety after caffeine, and not to other, more global measures of caffeine's effects. The genotypes were not associated with variability in baseline ratings of anxiety, or ratings of anxiety after placebo, suggesting that the observed relations were related to the pharmacological effects of caffeine. We did not find any evidence for an association between A_1 adenosine receptor gene polymorphisms and caffeine-induced anxiety. However, we cannot rule out the possibility that an association would be detected with a larger sample of subjects.

The main finding from this study, that genetic variation in the adenosine A_{2a} receptor gene resulted in differences in caffeine-induced anxiety levels, can be related to previous genetic and pharmacological research. The 1976T>C A_{2a} receptor gene polymorphism, which we found to be related to caffeine-induced anxiety, has also been found to be associated with PD (Deckert *et al*, 1998b), a condition characterized by recurrent and unexpected attacks of anxiety or fear (APA, 2000). Caffeine is known to produce anxiety in some individuals (Chait, 1992; Scott *et al*, 2002), and the anxiogenic effects of caffeine have been found to be greater in PD patients (Lee *et al*, 1988; Charney *et al*, 1985). Perhaps because of this, PD patients consume less caffeine (Boulenger *et al*, 1984). Further, caffeine administration more readily induces panic attacks in individuals with PD than in unaffected individuals (Charney *et al*, 1985; Uhde *et al*, 1984). Thus, if individuals with the 1976T/T genotype are more susceptible to PD and PD patients report higher anxiety after caffeine, it is plausible that 1976T/T individuals would report higher anxiety after caffeine.

An association finding with a DNA variant or polymorphism, however, can only be considered clinically relevant if the associated variant or polymorphism causes either a difference in function or expression level of the protein encoded for by the gene.

While at present there is no evidence for a functional relevance of the synonymous 1976T>C polymorphism in the coding region, in silico sequence analysis provided evidence that the 2592C>Tins polymorphism in the 3′-UTR region of the gene may be functionally relevant. U-rich motifs which are conserved across species and provide active sites for complex and dynamic interactions with RNA-binding proteins have been identified in the 3′-UTR of

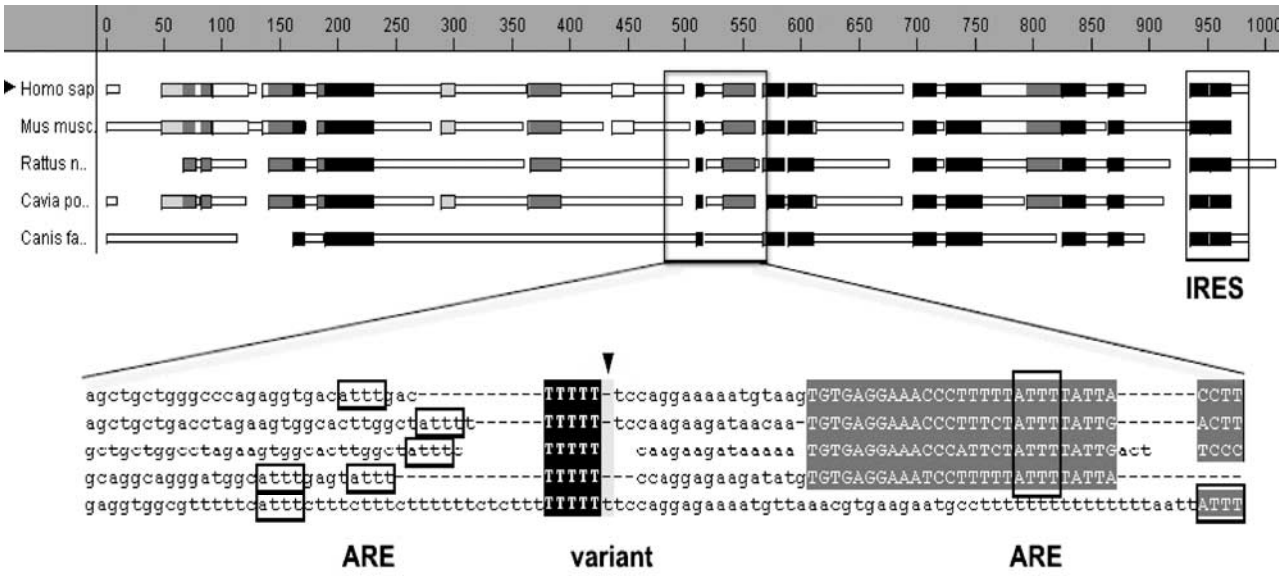

Figure 5 Conservation of tandem AREs (ATTT), a nested (TTTTTT)-motif, and an IRES in the human, rodent, and canine lineages in the 3′-UTR region of the adenosine A_{2a} receptor gene. The human 2592C > Tins polymorphism is indicated by an arrow. Abbreviations are Homo sap. = Homo sapiens (man), Mus mus. = Mus musculus (mouse), Rattus n. = Rattus norvegicus (rat), Cavia po. = Cavia porcellus (guinea pig) and Canis fa. = Canis familiaris (dog).

several mammalian genes (eg Song and Singh, 2001). In humans, U-rich motifs have been shown to mediate translational repression of oncogenes (eg Fu *et al*, 1999). The 2592C > Tins polymorphism may therefore be associated with variation in A_{2a} receptor expression. Although the *in silico* sequence analysis is suggestive, experimental evidence, for example, from bandshift-assays is still needed to prove a functional relevance of the 2592C > Tins polymorphism with confidence. If the 2592Tins/Tins polymorphism changes A_{2a} receptor expression, this may explain why individuals with the 2592Tins/Tins genotype are more sensitive to caffeine. Studies have shown that only high doses of caffeine increase anxiety and that there are differences between individuals in what constitutes a high, anxiogenic dose of caffeine (Evans and Griffiths, 1991; Griffiths and Woodson, 1988). Individuals with the 2592Tins/Tins genotype may report the greatest increases in anxiety because they require a lower dose to feel caffeine's anxiogenic effects. An alternate explanation may be that both polymorphisms are linked to still another, at present unknown, but functional polymorphism in the A_{2a} receptor gene.

Interestingly, we also found that the 1976T/T and the 2592Tins/Tins group differed from the other two genetic groups on the venturesomeness scale of the Eysenck Personality Inventory (Eysenck, 1993). The 1976T/T and 2592Tins/Tins group had significantly lower venturesomeness scores, suggesting that they are lower in sensation seeking and sociability than the other genotypic groups. It can be hypothesized that these individuals are less 'venturesome' because of their disposition to anxiety. However, we found no correlation between the personality measure of venturesomeness scores and the peak increase in anxiety ratings after caffeine, indicating that the lower scores on the personality measure of venturesomeness did not account for the increased anxiety in the 1976T/T and 2592Tins/Tins groups. Therefore, it appears that the relationship between the personality trait and the acute pharmacological response to caffeine are independent variables.

It is important to point out several limitations of the study. First, we recruited light or noncaffeine users (less than 300 mg per week self-reported caffeine use) in order to avoid subjects with tolerance or withdrawal. However, by limiting the subject pool to nonchronic users, we only examined those individuals who had self-selected to abstain from caffeine. Thus, they may not be representative of the general population. Goldstein *et al* (1969) found that heavy caffeine users, who consumed five to six cups of coffee a day, reported more 'positive' effects from caffeine (such as euphoria) whereas light users, who consumed one or less cups of coffee per day, reported more 'negative' effects such as anxiety. By limiting the subjects in our study to light users, we may have preferentially recruited negative responders to caffeine. This might have resulted in a lack of sensitivity to the positive mood effects of caffeine, such as 'liking' of drug effects. It would be of interest to study the same polymorphisms in a more general population of caffeine users, including moderate users. Second, we tested only a single dose of caffeine. The effects of caffeine are dose-dependent and caffeine typically produces more positive effects at lower doses and more adverse effects at higher doses (Griffiths and Woodson, 1988). Even though the dose given in this study was equivalent to only 150 mg caffeine, it is possible that this dose was too high for these subjects, and limited the occurrence of positive responses to the drug. Third, the present study involved a relatively small number of subjects for a genotypic analysis, raising the possibility that the findings occurred by chance. Although human genetic studies typically involve hundreds, or even thousands, of subjects, it could be argued that fewer subjects may be needed in this type of study, involving response to a specific, pharmacologic challenge. In the present study, the phenotype was acute response to a drug

with a specific action on the adenosine receptor, in a relatively homogeneous population. As such, there may have been fewer sources of uncontrolled variability, or variability related to other factors. Nevertheless, the findings must be considered preliminary based on the relatively small sample size.

In summary, this study provided evidence that genetic variations in the adenosine A_{2a} receptor gene are related to anxiety induced by caffeine in light caffeine users. Finding the genetic basis for variations in the quality or magnitude of responses to addictive drugs may help researchers understand why some individuals are vulnerable to, or protected from, drug addiction.

ACKNOWLEDGEMENTS

This research was supported by DA02812 and Innovative Medizinische Forschung (IMF). We thank Jennelle Fleck RN and Francis McMahon MD for their assistance with the blood samples and Sommer Lindsey, Liz Young, and Kirsten Weiss for their technical assistance.

REFERENCES

American Psychiatric Association (2000). *Diagnostic and Statistical Manual of Mental Disorders, 4th Edition Text Revised*. American Psychiatric Association: Washington, DC.

Belkhir K, Borsa P, Chiki L, Raufaste N, Bonhomme F (2001). 1996–2001 GENETIX 4.02, logiciel sous Windows TM pour la genetique des populations. Laboratoire Genome, Populations, Interactions, CNRS UMR 5000. Universite de Montpellier II: Montpellier.

Boulenger JP, Uhde TW, Wolff EA, Post RM (1984). Increased sensitivity to caffeine in patients with panic disorders. *Arch Gen Psychiatry* **41**: 1067–1071.

Brukner I, Jurukovski V, Konstantinovic M, Savic A (1991). Curved DNA without AA/TT dinucleotide step. *Nucleic Acids Res* **19**: 3549–3551.

Chait LD (1992). Factors influencing the subjective response to caffeine. *Behav. Pharmacol* **3**: 219–228.

Charney DS, Heninger GR, Jallon PI (1985). Increased anxiogenic effects of caffeine in panic disorder. *Arch Gen Psychiatry* **42**: 233–243.

Deckert J, Kuhlenbäumer G, Weiß K, Schirmacher A, Ringelstein EB, Arolt V *et al* (2001). Chromosome 22q11.2 as a candidate gene locus: association of the more active COMT-allele with panic disorder. *Am J Med Genet* **105**: 570.

Deckert J, Nothen MM, Albus M, Franzek E, Rietschel M, Ren H *et al* (1998a). Adenosine A1 receptor and bipolar affective disorder: systematic screening of the gene and association studies. *Am J Med Genet* **81**: 18–23.

Deckert J, Nothen MM, Franke P, Delmo C, Fritze J, Knapp M *et al* (1998b). Systematic mutation screening and association study of the A1 and A_{2a} adenosine receptor genes in panic disorder suggest a contribution of the A_{2a} gene to the development of disease. *Mol Psychiatry* **3**: 81–85.

Deckert J, Nothen MM, Rietschel M, Wildenauer D, Bondy B, Ertl MA *et al* (1996). Human adenosine A2a receptor (A_{2a}AR) gene: systematic mutation screening in patients with schizophrenia. *J Neural Transm* **103**: 1447–1455.

den Dunnen JT, Antonorakis SE (2000). Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat.* **15**: 7–12.

Durrant KL (2002). Known and hidden sources of caffeine in drug, food, and natural products. *J Am Pharm Assoc (Wash.)* **42**: 625–637.

Evans SM, Griffiths RR (1991). Dose-related caffeine discrimination in normal volunteers: individual differences in subjective and self-reported cues. *Behav Pharmacol* **2**: 345–356.

Eysenck SBG (1993). The I7: development of a measure of impulsivity and its relationship to personality. In: McCown WG, Johnson JL, Shure MB (eds) *The Impulsive Client*. American Psychological Association: Washington, DC. pp 141–149.

Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W (2000). Structure and function of adenosine receptors and their genes. *Arch Pharmacol* **362**: 364–377.

Fu L, Ma WL, Benchimol S (1999). A translation repressor element resides in the 3′ untranslated region of human p53 mRNA. *Oncogene* **18**: 6419–6424.

Gabrielian A, Pongor S (1996). Correlation of intrinsic DNA curvature with DNA property periodicity. *FEBS Lett* **393**: 65–68.

Gilbert RM (1976). Caffeine as a drug of abuse. In: Gibbins RJ, Israel Y, Kalant H, Popham RE, Schmidt W, Smart RG (eds) *Research Advances in Alcohol and Drug Problems*, Vol. 3 Wiley: New York. pp 49–176.

Goldstein A, Kaizer S, Whitby O (1969). Psychotropic effects of caffeine in man. IV. Quantitative and Qualitative differences associated with habituation to coffee. *Clin Pharmacol Therap* **10**: 489–497.

Graham DM (1978). Caffeine—its identity, dietary sources, intake and biological effects. *Nutr Rev* **36**: 97–102.

Griffiths RR, Mumford GK (1996). Caffeine reinforcement, discrimination, tolerance and physical dependence in laboratory animals and humans. In: Schuster CR, Kuhar MJ (eds). *Pharmacological Aspects of Drug Dependence*, (Chapter 9) Springer Verlag: Berlin.

Griffiths RR, Woodson PP (1988). Reinforcing properties of caffeine: studies in human and laboratory animals. *Pharmacol Biochem Behav* **29**: 419–427.

Gupta U (1993). Effects of caffeine on recognition. *Pharmacol Biochem Behav* **44**: 393–396.

Gupta U, Dubey GP, Gupta BS (1994). Effects of caffeine on perceptual judgement. *Neuropsychobiology* **30**: 185–188.

Hart P, Farrell GC, Cooksley WGE, Powell L (1976). Enhanced drug metabolism in cigarette smokers. *Br Med J* **2**: 147–149.

Johansson B, Halldner L, Dunwiddie TV, Masino SA, Poelchen W, Gimenez-Llort L *et al* (2001). Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. *Proc Natl Acad Sci USA* **98**: 9407–9412.

Kamimori GH, Joubert A, Otterstetter R, Santaromana M, Eddington ND (1999). The effects of the menstrual cycle on the pharmacokinetics of caffeine in normal, healthy eumenorrheic females. *Eur J Clin Pharmacol* **55**: 445–449.

Kaplan GB, Greenblatt DJ, Ehrenberg BL, Goddard JE, Cotreau NM, Harmatz JS *et al* (1997). Dose-dependent pharmacokinetics and psychomotor effects of caffeine in humans. *J Clin Pharmacol* **37**: 693–703.

Kendler KS, Prescott CA (1999). Caffeine intake, tolerance, and withdrawal in women: A population-based twin study. *Am J Psychiatry* **156**: 223–228.

Lader M (1969). Comparison of amphetamine sulphate and caffeine citrate in man. *Psychopharmacologia* **14**: 83–94.

Ledent C, Vaugeois JM, Schiffmann SN Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ *et al* (1997). Aggressiveness, hypoalgesia, and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388**: 674–682.

Lee MA, Flegel P, Greden JF, Cameron OG (1988). Anxiogenic effects of caffeine on panic and depressed patients. *Am J Psychiatry* **145**: 632–635.

Lieberman HR, Wurtman RJ, Emde GG, Roberts C, Coviella ILG (1987). The effects of low doses of caffeine on human performance and mood. *Psychopharmacology* **92**: 308–312.

Liguori A, Grass JA, Hughes JR (1999). Subjective effects of caffeine among introverts and extraverts in the morning and evening. *Exp Clin Psychopharmacol* **7**: 244–249.

Loke WH (1988). Effects of caffeine on mood and memory. *Physiol Behav* **44**: 367–372.

Martin WR, Sloan JW, Sapira JD, Jasinski RM (1971). Physiological, subjective and behavioral tests of amphetamine, methamphetamine, ephedrine, phenmetrazine and methylphenidate in man. *Clin Pharmacol Ther* **12**: 245–258.

McNair D, Lorr M, Droppleman DL (1971). *Profile of Mood States*. Educational and Industrial Testing Service: San Diego.

Mumford GK, Evans SM, Kaminski BJ, Preston KL, Sannerud CA, Silverman K *et al* (1994). Discriminative stimulus and subjective effects of theobromine and caffeine in humans. *Psychopharmacology* **115**: 1–8.

Patton JH, Stanford MS, Barratt ES (1995). Factor structure of the Barratt Impulsiveness Scale. *J Clin Psychol* **51**: 768–774.

Schuler GD, Altschul SF, Lipman DJ (1991). A workbench for multiple alignment construction and analysis. *Proteins* **9**: 180–190.

Scott Jr WH, Coyne KM, Johnson MM, Lausted CG, Sahota M, Johnson AT (2002). Effects of caffeine on performance of low intensity tasks. *Percept Motor Skills* **94**: 521–532.

Shacham S (1983). A shortened version of the profile of mood states. *J Pers Assess* **47**: 305–307.

Snyder SH, Sklar P (1984). Behavioral and molecular actions of caffeine: Focus on adenosine. *J Psychiatry Res* **18**: 91–106.

Song X, Singh SM (2001). Distribution and molecular characterization of mRNA-bending proteins specific to the (U)15 region of 3′UTR of the mouse catalase (Cas-1). *DNA Cell Biol* **20**: 339–348.

Svenningsson P, Nomikos GG, Ongini E, Fredholm BB (1997). Antagonism of adenosine A2A receptors underlies the behavioral activating effect of caffeine and is associated with reduced expression of messenger RNA for NGFI-A and NGFI-B in caudate-putamen and nucleus accumbens. *Neuroscience* **79**: 753–764.

Svensson E, Persson LO, Sjoberg L (1980). Mood effects of diazepam and caffeine. *Psychopharmacology* **67**: 73–80.

Uhde TW, Boulanger JP, Vittone B, Jimerson DC, Post PM (1984). Caffeine: relationship to human anxiety plasma MHPG and cortisol. *Psychopharmacol Bull* **20**: 426–430.

Wechsler D (1958). *The Measure and Appraisal of Adult Intelligence*. Williams and Wilkins: Baltimore.

White BC, Lincoln CA, Pearce NW, Reeb R, Vaida C (1980). Anxiety and muscle tension as consequences of caffeine withdrawal. *Science* **209**: 1547–1548.

Yamada K, Hattori E, Shimizu M, Sugaya A, Shibuya H, Yoshikawa T (2001). Association studies of the cholecystokinin B receptor and A2a adenosine receptor genes in panic disorder. *J Neural Transm* **108**: 837–848.