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**Association of the anxiogenic and alerting effects of caffeine with *ADORA2A* and *ADORA1* polymorphisms and habitual level of caffeine consumption**

Abbreviated title: ADORA SNPs, caffeine tolerance and anxiety

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## ABSTRACT

Caffeine, a widely consumed adenosine A<sub>1</sub> and A<sub>2A</sub> receptor antagonist, is valued as a psychostimulant, but it is also anxiogenic. An association between a variant within the *ADORA2A* gene (rs5751876) and caffeine-induced anxiety has been reported for individuals who habitually consume little caffeine. The present study investigated whether this SNP might also affect habitual caffeine intake, and whether habitual intake might moderate the anxiogenic effect of caffeine. Participants were 162 non-/low (NL) and 217 medium/high (MH) caffeine consumers. In a randomized, double-blind, parallel groups design they rated anxiety, alertness and headache before and after 100 mg caffeine and again 90 min after another 150 mg caffeine, or after placebo on both occasions. Caffeine intake was prohibited for 16 h before the first dose of caffeine/placebo. Results showed greater susceptibility to caffeine-induced anxiety, but not lower habitual caffeine intake (indeed coffee intake was higher), in the rs5751876 TT genotype group, and a reduced anxiety response in MH versus NL participants irrespective of genotype. Apart from the almost completely linked *ADORA2A* SNP rs3761422, no other of eight *ADORA2A* and seven *ADORA1* SNPs studied were found to be clearly associated with effects of caffeine on anxiety, alertness or headache. Placebo administration in MH participants decreased alertness and increased headache. Caffeine did not increase alertness in NL participants. With frequent consumption, substantial tolerance develops to the anxiogenic effect of caffeine, even in genetically susceptible individuals, but no net benefit for alertness is gained, as caffeine abstinence reduces alertness and consumption merely returns it to baseline.

**Keywords:** caffeine; adenosine; polymorphism; anxiety; alertness; headache

## INTRODUCTION

Caffeine is prized for its alerting effect which may, at least in part, explain the worldwide popularity of tea, coffee and other caffeine-containing products. However, while frequent consumers feel alerted by caffeine, especially by their morning tea, coffee or other caffeine-containing drink, evidence suggests that this is actually merely the reversal of the fatiguing effects of acute caffeine withdrawal (James and Rogers, 2005; Sigmon *et al*, 2009). That is, little or perhaps no net benefit for alertness is gained. Additionally, caffeine has the undesirable effects of, for example, increasing anxiety and raising blood pressure (e.g., Alsene *et al*, 2003; Goldstein *et al*, 1969; James, 2004). These behavioural and physiological effects of caffeine occur primarily via antagonism by caffeine of the action of endogenous adenosine at adenosine A<sub>1</sub> and A<sub>2A</sub> receptors (Fredholm *et al*, 1999). Both receptors play a role in miscellaneous biological processes, particularly the cAMP-protein kinase A signalling cascade and the fine-tuning of glutamatergic information flow (Schiffmann *et al*, 2007; Calker and Biber, 2005). They are considered to be modulators of glial function, neuronal communication and neuronal activity, and to be involved in sleep and arousal, and cognition, as well as different psychiatric disorders, including anxiety and other mood disorders (Ribeiro *et al*, 2003; Cunha *et al*, 2008; Freitag *et al*, 2010). In mice, genetic knockout of adenosine A<sub>1</sub> or A<sub>2A</sub> receptors has been linked to increased anxiety (Ledent *et al*, 1997; Johansson *et al*, 2001), implicating the corresponding genes or, more precisely, polymorphisms within these genes, as promising candidates for increased anxiety reactions. Consistent with this, recent studies have discovered an association between caffeine-induced anxiety and a single-nucleotide polymorphism (SNP) in the gene coding for the adenosine A<sub>2A</sub> receptor (*ADORA2A*). Specifically, it was found that 150 mg of caffeine (equivalent to the amount of caffeine present in, e.g., 1½ cups of ground coffee) increased

anxiety in individuals carrying the TT genotype of the *ADORA2A* SNP rs5751876, but not in the CT and CC genotype groups (Alsene *et al*, 2003; Childs *et al*, 2008).

The significance of these findings on anxiety for assessing the balance of benefit and harm of everyday caffeine consumption is, however, uncertain, because all the participants in these two genetic studies were infrequent consumers of caffeine – no individual reported consuming more than 3 cups of coffee or equivalent per week, and many were recorded as consuming no caffeine. It is possible, for example, that the experience of increased anxiety after caffeine may cause vulnerable individuals to avoid caffeine subsequently (cf Evans and Griffiths, 1992; Stern *et al*, 1989), so that actually there are rather few such individuals among populations of frequent caffeine consumers. Indeed, even among infrequent consumers of caffeine a minority (19-29%) carry the rs5751876 TT genotype (Alsene *et al*, 2003; Childs *et al*, 2008). Furthermore, anxiety effects of caffeine might be diminished with frequent consumption due to tolerance.

Accordingly, the present study investigated the effects of caffeine on anxiety in both frequent and infrequent consumers. It also measured habitual caffeine intake and caffeine consumption status ('frequent' versus 'infrequent' consumption) in relation to rs5751876 genotype group. The primary hypotheses were that administration of caffeine would increase anxiety to a greater extent in infrequent consumers of caffeine (lack of tolerance) and in individuals carrying the rs5751876 TT genotype, and that this genotype would be associated with caffeine avoidance (especially avoidance of drinks containing higher amounts of caffeine, e.g., coffee) or at least relatively low habitual caffeine consumption. Associations between both the anxiogenic and alerting effects of caffeine and other *ADORA2A* SNPs and *ADORA1* SNPs were also examined. These further SNPs were selected according to their potential functional

relevance, and to extend the findings of Alsene *et al* (2003) and Childs *et al* (2008). A secondary objective of the study was to test the withdrawal reversal hypothesis (James and Rogers, 2005), alluded to above, which predicts that administration of caffeine to frequent caffeine consumers after acute (overnight) caffeine deprivation will increase their alertness, but not to above the level of alertness experienced by infrequent caffeine consumers not given caffeine. In view of the ubiquitous consumption of caffeine worldwide, the potential impact on human well-being of the alerting effects of caffeine and vulnerability to its anxiogenic action is potentially very significant.

Caffeine was administered in two doses, 100 mg late-morning, and 150 mg 90 minutes later. This was done to test the anxiogenic and alerting effects of the amount of caffeine relevant to the consumption of caffeine-containing drinks (e.g., a cup of ground coffee). The second dose ensured that systemic caffeine concentration later in the test session modelled that expected for frequent caffeine consumers. Finally, it is worth noting that the target sample size was 400, which is substantially higher than the number of participants tested in typical studies of the behavioural effects of caffeine.

## **MATERIALS AND METHODS**

### **Participants and Randomization**

The participants were 218 women and 198 men. Many (44%) were recruited from a list of respondents to a postal survey of caffeine consumption habits and health carried out during the previous year (Heatherley *et al*, 2006b). These respondents were provisionally divided into infrequent and (more) frequent caffeine consumers (caffeine intake of <40 mg/d and  $\geq$ 40 mg/d) and younger and older participants (<30 years and  $\geq$ 30 years), and within these groups they were

selected for contact at random. The aim of this was to achieve similar numbers of infrequent and frequent caffeine consumers of similar ages in the final sample, despite the fact that only 10% of this population consumed less than <40 mg caffeine daily, and older participants tended to have higher habitual caffeine intake. The remaining participants were recruited using local advertisements and by word-of-mouth, targeting infrequent and frequent caffeine consumers, as required. Suitability for the study was assessed in a telephone or face-to-face interview. Key inclusion criteria were: age between 18 and 65 years, good general physical and mental health, availability and willingness to attend an experiment lasting 7 h which might include consumption of caffeine, willingness to give a blood sample (for genotyping), being a non- or a light-smoker ( $\leq 5$  cigarettes or equivalent a day), normal blood pressure, not pregnant, not planning to become pregnant, and not breastfeeding.

Randomization to receive caffeine or placebo on the test day was stratified according to self-reported habitual caffeine intake of <40 mg/d and  $\geq 40$  mg/d and age (<30 years and  $\geq 30$  years) recorded during the recruitment interview. Final assignment to caffeine group (see Data Analysis below) was done on the basis of information recorded in a caffeine intake questionnaire completed during the week preceding testing, and analysed after the participant had been tested. This assessed frequency of consumption of teas, coffees, colas, other caffeine-containing drinks (e.g., Red Bull) and products (e.g., Pro-plus and Anadin-Extra), and chocolate. Mean daily caffeine intake was calculated from these data using dietary and manufacturers' information on caffeine content (Heatherley *et al*, 2006a); for example, instant coffee 54 mg, ground coffee 105 mg, tea (bags, loose leaf, instant and green) 40 mg.

The research was presented to participants as a study on ‘genetic variation, caffeine consumption habits and caffeine effects.’ It was reviewed and approved by the University of Bristol’s, Department of Experimental Psychology Human Research Ethics Committee.

## **Design and Procedure**

This was a double-blind, parallel groups, repeated measures study. After overnight caffeine abstinence, participants received one of two treatments: either 100 mg of caffeine followed 90 minutes later by a further 150 mg of caffeine, or placebo on both these occasions. This two-stage dosing regimen was used to model, as far as practicable, real-life consumption of caffeine and to allow assessment of effects at lower and higher plasma caffeine concentrations. Various measurements were made pre-treatment, again after the first dose of caffeine or placebo, and finally twice more after the second dose of caffeine or placebo.

The procedures are summarised in Table 1. The behavioural data described here are self-rated anxiety, alertness and headache assessed using the Mood, Alertness and Physical Sensations Scales (MAPSS, see below). This was presented as part of a battery of ‘computer tasks’ programmed using E-Prime 1.0 (Psychology Software Tools, Science Plus Group bv, 9747 AA Groningen, The Netherlands), which also included tests of psychomotor performance, memory, attention and vigilance. Results for these latter tests and for other measures (hand steadiness, heart rate and blood pressure, taste sensitivity, etc.) will be reported elsewhere.

On any single day, between two and six participants were tested. They were previously told not to consume alcohol or caffeine-containing products from 19.00 h on the evening before testing, to replace any caffeine-containing drinks with water, and to eat their normal breakfast. If they were a smoker they were asked not to smoke on the test day until after they left the



laboratory. They were informed that compliance with the instruction to avoid caffeine would be assessed via measurement of the concentration of caffeine present in their saliva at the start of testing (saliva sample taken at 11.10 h). The initial briefing session was held in a communal room in the laboratory, and this same room was used for rest periods, lunch and debriefing. Blood collection and the computer tasks were carried out in rooms close by. For the computer tasks, each participant was accommodated in a separate, private booth within the larger of these rooms. Lunch consisted of a sandwich, a small cake and fruit (total energy content 580-740 kcal). Participants had access to bottled water throughout their stay in the laboratory, and the amount they consumed was recorded.

### **Drug Administration**

Caffeine BP (caffeine anhydrous powder, Courtin and Warner Ltd, Lewes, East Sussex, UK) and placebo (cornflour) were administered in white, size 1 cellulose capsules (Capsuline Inc, Pompano Beach, Florida, FL 33066, USA). These caffeine and placebo capsules were identical in appearance, and were swallowed with 50 ml of room temperature water. Each dose was contained in a single capsule.

### **Mood, Alertness and Physical Sensations Scales (MAPSS)**

MAPSS was used to measure anxiety, alertness and headache. It was adapted from similar instruments used in previous studies on the effects of caffeine (Rogers *et al*, 2005; Rogers *et al*, 2008). It comprised 24 items (single or groups of descriptors – e.g. I feel mentally alert / attentive / able to concentrate / observant; I feel tense / anxious / nervous / on edge; My head aches / I feel headachy), which were rated on a 9-point unipolar scale using the horizontal

number pad on the computer keyboard, where 1 represented ‘not at all’ and 9 represented ‘extremely’ (adjusted to a 0 to 8 scale for the presentation of results here). Participants were instructed: ‘There are no right or wrong answers. Do not spend too much time on any one statement but give the rating which seems to best describe your present feeling.’ The order of presentation of the items was determined randomly for each participant on each occasion. See Supplementary Materials and Methods for full details of MAPSS.

### **SNP Selection and Genotyping**

SNP selection was based on previous studies (Alsene *et al*, 2003; Childs *et al*, 2008; Deckert *et al*, 1998), and their regulatory potential (UCSC (<http://genome.ucsc.edu>), linkage disequilibrium (LD) and tagging capabilities (Hapmap, <http://www.hapmap.org>), and minor allele frequencies. Eight SNPs were selected to cover the 25 kb *ADORA2A* variant resulting in mRNA X68486 (rs5751862, rs5760405, New3, rs11704959, rs2298383, rs3761422, rs2267076, rs5751876), and nine SNPs were selected to cover the 76 kb *ADORA1* variant resulting in mRNA L22214 (rs9660662, rs1874142, rs10920568, rs12135643, rs3766566, rs3766560, rs3753472, rs16851030, rs12744240). Participants were genotyped by custom TaqMan SNP genotyping assays (Applied Biosystems, Darmstadt, Germany) for all SNPs except rs2298383 and rs10920568 (see below), with PCR setup (5 µl reactions) as recommended by the manufacturer and performed on a Genesis Workstation RSP150 (Tecan, Crailsheim, Germany). The ABI Prism 7900 Sequence Detection System with SDS software version 2.1 (Applied Biosystems) was used for PCR amplification and allelic discrimination. To minimize the risk of genotyping errors, about 10% of randomized participants (n=45) were additionally genotyped for all SNPs by RFLP assays using independent primer-sets different to those used for the TaqMan assays,

which resulted in concordance rates of 100%. Genotyping assay conditions and primer-probe sequences from all TaqMan and RFLP assays are available on request. *ADORA2A* SNP rs2298383 and *ADORA1* SNP rs10920568, which failed TaqMan assay design, were genotyped by RFLP assays as previously described (Deckert *et al*, 1998; Freitag *et al*, 2010). Overall, genotyping resulted in averaged call rates of 100% (range 99.5-100.0), and all genotypes from TaqMan and RFLP analysis were assigned blind with respect to group assignment and the measured phenotypic characteristics of the participants.

### **Collection of Saliva and Analysis of Methylxanthine Concentrations**

Saliva collections were made at the times shown in Table 1. Caffeine and its metabolites, paraxanthine, theophylline and theobromine, were analysed using an HPLC method adapted from Hartley *et al* (1985). The limit of detection for all analytes was 0.02 µg/ml. For full details see Supplementary Materials and Methods.

### **Data Analysis**

Initial assessment of influences of habitual level of caffeine consumption (consumer status) was conducted by dividing the <40 mg caffeine per day participants into two groups based on a median split of consumption level to produce ‘non-consumer’ (N) and ‘low’ (L) consumer groups, and doing the same for ≥40 mg/d participants to produce ‘medium’ (M) and ‘high’ (H) consumer groups. Subsequent analyses involving consumer status were conducted on a <40 mg/d (NL) versus ≥40 mg/d (MH) split.

ANOVA, run using SPSS 12.0.1 for Windows, was used to analyse the data on anxiety, alertness and headache. Age as a covariate and gender as a factor were included in all these

analyses. Additionally, for certain analyses of the effects of caffeine versus placebo on anxiety, baseline (pre-treatment) anxiety score was also included as a covariate. This is similar to the approach of calculating change from baseline scores (e.g., Childs *et al*, 2008), and was done to control for pre-existing individual differences in anxiety in these analyses. Baseline differences in anxiety as a function of consumer status and genotype group were small (see Results).

For genotype data, Hardy-Weinberg equilibrium and LD analyses were performed using Haploview 4.0 (Barrett *et al*, 2005). Because Childs *et al* (2008) found that 150 mg but not a higher dose of caffeine differentially increased anxiety in *ADORA2A* rs5751876 genotype groups, initial analysis of anxiety effects in relation to this SNP was carried out separately for post-treatment session 1 data (collected after administration of 100 mg of caffeine) alone. In analyses which included dose/session as a (within subjects) factor, data for post-treatment sessions 2 and 3 were meaned so as to give equal weight to the data collected after administration of the first (session 1) and second doses (sessions 2 and 3) of caffeine. Dose/session was included in the analyses involving caffeine and consumer status, and caffeine, consumer status and genotype. In the latter analyses, data from *ADORA2A* rs5751876 CC and CT genotype groups (which were similar in their behaviour) were combined in order to simplify the presentation of the results.

The Bonferroni procedure was used to adjust for multiple testing at eight *ADORA2A* loci ( $\alpha=0.00625$ ) and seven *ADORA1* loci ( $\alpha=0.00714$ ). Two of the nine *ADORA1* SNPs (rs9660662 and rs10920568) were excluded from the analyses due to deviation from Hardy-Weinberg expectations (see results section). Bonferroni t (Howell, 1997) was used following ANOVA for making multiple paired comparisons (caffeine versus placebo within consumer and/or genotype group).

## RESULTS

Ethnic origin of the participants was predominantly (95%) white European. Four hundred and sixteen individuals were randomised to receive caffeine or placebo. Genetic data were not available for four participants due to problems encountered in extracting DNA. Fifteen NL participants were excluded because their baseline caffeine sample contained  $>0.2 \mu\text{g/ml}$  caffeine and/or paraxanthine. This concentration of caffeine and its major metabolite, paraxanthine, suggested that caffeine consumption the previous day was substantially higher than the  $<40 \text{ mg}$  criterion. Five MH participants were excluded because their baseline caffeine sample contained  $>2 \mu\text{g/ml}$  caffeine. This high salivary concentration of caffeine suggested that they had failed to comply with the instruction to be overnight caffeine abstinent. The same criterion was used in a previous study (Rogers *et al*, 2005). Of the remaining participants, eight withdrew after having received at least the first caffeine or placebo capsule, and there were missing data for five participants due to equipment malfunction. Of the eight participants who withdrew, five were from the MH group, all of whom received placebo (reasons given for withdrawing were headache and feeling sick ( $n=4$ ), no reason ( $n=1$ )), and three were from the NL group, of whom two received caffeine and one received placebo (no reasons given for withdrawing). There was no difference in the *ADORA2A* rs5751876 genotype distribution for the 33 participants excluded, who withdrew or had missing data compared with the remaining 379 participants for whom genetic data were available ( $\text{CC}=13$ ,  $\text{CT}=14$ ,  $\text{TT}=6$  vs  $\text{CC}=146$ ,  $\text{CT}=168$ ,  $\text{TT}=65$ ;  $\chi^2=0.05$ ,  $\text{df}=2$ ,  $P>.1$ ). All analyses reported below were carried out on these 379 participants or on subsets of these participants.

## **Participant Characteristics**

Participant characteristics, summarised by consumer status group, are shown in Table 2.

Participants were aged between 18 and 62 years, 47% were male and 16.5% were smokers. Age varied among the groups, with L and M participants being somewhat younger. Smokers tended to be overrepresented in the higher caffeine consumption groups. The lowest recorded mean daily caffeine intake was 0 mg (43 participants), and the highest was 778 mg, with 55 participants consuming over 300 mg/d. Lowest mean daily caffeine intake in group H was 203 mg.

## **Systemic Caffeine Concentrations**

Mean  $\pm$  SD baseline salivary caffeine concentrations for N, L, M and H participants were respectively  $0.014 \pm 0.031$ ,  $0.024 \pm 0.040$ ,  $0.18 \pm 0.26$ , and  $0.39 \pm 0.45$   $\mu\text{g/ml}$ . For participants who received caffeine, mean  $\pm$  SD salivary caffeine concentrations 90 minutes after the first dose of caffeine and 90 minutes after the second dose of caffeine were respectively  $1.64 \pm 0.68$  and  $2.86 \pm 1.21$   $\mu\text{g/ml}$ .

## **Consumer Status by Caffeine Effects**

The analyses of the effects of caffeine as a function of level of habitual caffeine consumption (four consumer status groups) revealed no effects ( $P > 0.05$ ) involving dose/session for either anxiety, alertness or headache, so the results shown in Figure 1 are meaned across post-treatment sessions (session 1 + ((session 2 + session 3)/2)/2). For all three variables there was a caffeine by consumer status interaction effect (anxiety,  $F(3,362)=7.03$ ,  $P=0.0001$ ; alertness,  $F(3,362)=6.06$ ,  $P=0.0005$ ; headache,  $F(3,362)=9.52$ ,  $P<0.0001$ ). Additionally, there was an effect of treatment

for anxiety ( $F(1,362)=12.37$ ,  $P=0.0005$ ) and alertness ( $F(1,362)=17.94$ ,  $P<0.0001$ ), and there was a consumer status effect for alertness ( $F(3,362)=6.30$ ,  $P=0.0004$ ). Because smoking tended to be associated with caffeine intake (Table 2), and smoking was not permitted on the test day, analyses were conducted to examine differences in anxiety, alertness and headache between smokers and non-smokers. Smokers did not report more headache or anxiety than non-smokers, but they were less alert (data not shown). Therefore, the analysis of effects of consumer status and caffeine on alertness was repeated with smoking additionally included as a covariate. Controlling for smoking in this way had little effect on the outcome (caffeine by consumer status interaction  $F(3,357)=6.31$ ,  $P=0.0004$ ).

Figure 1 shows that caffeine increased anxiety in N and L participants. It did not affect anxiety in either M or H participants. In contrast, alertness declined with increasing level of habitual caffeine consumption in participants who received placebo, but not in those who received caffeine. Caffeine did not increase alertness in any group above that of the non-consumers (N) who received placebo. Headache was increased in H participants who received placebo, and increased by caffeine in L participants.

At baseline there were consumer status effects for anxiety ( $F(3,370)=2.66$ ,  $P=0.048$ ), alertness ( $F(3,365)=5.74$ ,  $P=0.001$ , smoking also included as a covariate) and headache ( $F(3,370)=5.05$ ,  $P=0.002$ ). Higher habitual caffeine intake was associated with greater anxiety, lower alertness and more headache at baseline. The placebo group in Figure 1 shows continuation and worsening of these effects.

### **Genotype Distributions and Genotype by Caffeine Effects**

Genotype frequencies conformed to Hardy-Weinberg expectations for all eight *ADORA2A* SNPs, (smallest  $P=0.075$ ; Table 3) and for seven *ADORA1* SNPs (smallest  $P=0.093$ ; Table S1).

Genotype frequencies did not conform to Hardy-Weinberg expectations for *ADORA1* rs9660662 and rs10920568 ( $P=0.029$  and  $P=0.023$ , respectively), therefore these SNPs were excluded from subsequent analyses. LD analysis revealed one block of high LD in *ADORA2A* spanning the eight SNPs, and two blocks of high LD in *ADORA1*, spanning three and four SNPs, respectively (Figure 2).

None of the *ADORA2A* or *ADORA1* SNPs were significantly associated with baseline anxiety, headache or alertness (largest F ratios were for anxiety and *ADORA2A* rs3761422,  $F(2,372)=2.84$ ,  $P=0.060$ ; and for anxiety and *ADORA2A* rs5751876,  $F(2,372)=2.63$ ,  $P=0.073$ ; and 5751876; uncorrected for multiple tests).

For the *ADORA2A* rs5751876 SNP, which has previously been found to be associated with caffeine-induced anxiety (see Introduction), there was an effect of caffeine ( $F(1,366)=8.97$ ,  $P=0.003$ ), an effect of genotype ( $F(2,366)=7.12$ ,  $P=0.0009$ ) and a marginally insignificant caffeine by genotype interaction ( $F(2,366)=2.72$ ,  $P=0.067$ ) for anxiety in session 1 (after 100 mg caffeine). Figure 3 shows that caffeine increased anxiety in the TT genotype group but not in the CC or CT genotype groups. When baseline anxiety was included as a covariate in the analysis, the caffeine by genotype interaction was significant (Table 3). Similar results were found for rs3761422 (Table 3), with the TT genotype group showing the largest increase in anxiety after caffeine (mean  $\pm$  SE for caffeine =  $1.65 \pm 0.15$  and for placebo =  $0.95 \pm 0.17$ ,  $P<0.01$ ). The LD analysis showed rs5751876 and rs3761422 to be in strong LD ( $D'=1$ ,  $R^2=0.92$ ; Figure 2, top panel).



The effects involving genotype described above remained significant when the analysis was confined to participants who were of European descent ( $n=361$ ).

None of the *ADORA1* SNPs were associated with caffeine-induced anxiety ( $P>0.1$ , data not shown), and none of the *ADORA1* or *ADORA2A* SNPs were associated with the effects of caffeine on alertness or headache ( $P>0.05$ , data not shown).

### **Consumer Status by *ADORA2A* Genotype by Caffeine Effects: Anxiety**

Given that consumer status and *ADORA2A* genotype were both found to modify the anxiogenic response to caffeine, these factors were included together in a further analysis of the effects of caffeine on anxiety. This consumer status (NL versus MH) by *ADORA2A* rs5751876 genotype (CC and CT versus TT) by caffeine by dose/session analysis (baseline anxiety included as a covariate) revealed an effect of caffeine ( $F(1,361)=28.36$ ,  $P<0.0001$ ) and a caffeine by consumer status interaction ( $F(1,361)=7.00$ ,  $P=0.009$ ). The effect of genotype ( $F(1,361)=6.44$ ,  $P=0.012$ ) and the caffeine by genotype interaction ( $F(1,361)=7.16$ ,  $P=0.008$ ) were marginally insignificant after correction for multiple testing ( $\alpha=0.00625$ ). The consumer status by genotype by caffeine interaction was not significant ( $F<1$ ). Because of the unequal variances in the combined CC and CT genotype group versus the TT genotype group, planned comparisons investigating the above effects were conducted following ANOVA performed separately for these two groups. These analyses showed that caffeine increased anxiety in NL participants in both TT and combined CC and CT genotype groups (Figure 4). Although caffeine also somewhat increased anxiety in MH participants possessing the TT genotype, this effect was not significant ( $P>0.1$ ) in this relatively small sample (Figure 4, lower panel).

Effects involving the above variables (caffeine, consumers status and genotype) and dose/session were not significant ( $P>0.1$ ) after controlling for multiple testing (Figure 4).

Results were similar for the *ADORA2A* rs3761422 SNP (data not shown).

### ***ADORA2A* Genotype and Habitual Caffeine Consumption**

*ADORA2A* rs5751876 genotype distribution did not differ between the NL (CC=62, CT=70, TT=30) and MH (CC=84, CT=98, TT=35) groups (chi-square=0.39, df=2,  $P=0.82$ ; for CC and CT combined versus TT, chi-square=0.37, df=1,  $P=0.54$ ). Genotype distribution in the H group was: CC=43, CT=46, TT=19. In the participants who habitually consumed at least moderate amounts of caffeine (i.e., the MH group), caffeine intake from coffee was higher in the TT genotype group compared with the combined CC and CT group ( $F(1,212)=5.91$ ,  $P=0.016$ ; adjusting for multiple comparisons  $\alpha=0.017$ ), but tea intake was somewhat, though not significantly, lower in the TT group ( $F(1,212)=2.89$ ,  $P=0.09$ ) (Figure 5). As a result, total caffeine intake did not differ between genotype groups ( $P>0.1$ ) (Figure 5). Again, results were similar for rs3761422, including higher habitual coffee consumption in the TT genotype group (mean  $\pm$  SE for CC and CT combined =  $106 \pm 10$  mg/d and for TT =  $181 \pm 24$  mg/d;  $F(1,212)=8.03$ ,  $P=0.005$ ; adjusting for multiple comparisons  $\alpha=0.017$ ).

## **DISCUSSION**

The present results confirm that the *ADORA2A* rs5751876 SNP is associated with variation in the anxiogenic response to caffeine (Alsene *et al*, 2003; Childs *et al*, 2008). Furthermore, they show that this differential response of the TT versus CC and CT genotypes is apparent at a fairly modest dose of caffeine (100 mg – approximately the amount of caffeine in a cup of ground

coffee). The effect was observed after 150 mg, but not after 50 mg and 450 mg, by Alsene *et al* (2003) and Childs *et al* (2008). Similar results were found for the nearly completely linked SNP rs3761422, and less certainly so for rs2298383 (not significant after adjustment for multiple testing, Table 3). In the present population rs2298383 was completely linked with rs4822492 ( $D'=1$ ,  $R^2=0.995$ ) and the latter was therefore dropped from the analysis. These results are in partial agreement with Childs *et al* (2008), who found rs5751876, rs2298383 and rs4822492, but not rs3761422 to predict caffeine-induced anxiety. SNPs rs2298383 and rs3761422 are potentially functional variants reflected by rs5751876, which is coding but does not cause an amino acid exchange and therefore is unlikely to represent the causal variant. Both rs2298383 and rs3761422 are located in potential promoter regions upstream of several newly identified variants of *ADORA2A* exon 1 (Yu *et al*, 2004) near the corresponding transcription start sites. SNPs located within or immediately around these sites have been highlighted as having high potential to alter gene function (Veyrieras *et al*, 2008).

In contrast to *ADORA2A* SNPs, no association of caffeine-induced anxiety was found with *ADORA1* SNPs. This is consistent with and extends the findings of Alsene *et al* (2003) who investigated 716T>G (rs10920568), a synonymous SNP located within the first coding exon. The present study included further SNPs of high potential to regulate gene function, however none of them moderated caffeine-induced anxiety.

Another key finding from the present study is that a clear anxiogenic effect of caffeine, larger in individuals with the *ADORA2A* rs5751876 TT genotype, was observed only for people who habitually consumed little or no caffeine (the N and L groups). Higher caffeine consumption appears to lead to substantial tolerance to this effect. Previously, Evans and Griffiths (1992) found that caffeine consumers chronically withdrawn from caffeine responded to a caffeine

challenge with increased anxiety, whereas caffeine consumers maintained on 900 mg of caffeine daily did not. What is striking about the present results is that substantial tolerance to caffeine-induced anxiety appears to occur at much lower daily caffeine intakes than this. Indeed, caffeine (100 + 150 mg) failed to increase anxiety even in the medium (M) consumer group (Figure 1, top panel), whose habitual caffeine consumption averaged just 128 mg/d of caffeine, which is equivalent to, for example, a little more than 1 cup of ground coffee or 3 cups of tea a day. It is worth noting that 90% of the population from which many of the present participants were recruited (Heatherley *et al*, 2006a) had habitual caffeine intakes within the range represented by the MH group (40-778 mg/d), and only 10% had caffeine intakes within the range represented by the NL group (<40 mg/d).

Whilst frequent caffeine consumers experience minimal increased anxiety after caffeine consumption, they are at risk of at least two clear adverse effects of acute caffeine abstinence, namely low alertness and increased headache. These are the most commonly found symptoms of caffeine withdrawal (reviewed by Juliano and Griffiths, 2004). In the present study both symptoms were evident after less than 24 h caffeine abstinence in the group with the highest level of caffeine consumption (H), whose average caffeine intake was 346 mg/d (equivalent to about 3 cups of ground coffee per day). The caffeine withdrawn M group showed only decreased alertness. It is noteworthy that all of the five MH participants who dropped out during the test day had been given placebo, and four of these were high consumers who complained of headache or related symptoms. If caffeine was consumed, the adverse effects of lowered alertness and headache were avoided, but even after 100 +150 mg of caffeine their alertness was not raised above the level of alertness displayed by non-consumers of caffeine (group N) who received placebo (Figure 1, middle panel). This result is similar to that from an early study

comparing responses to caffeine of coffee drinkers and abstainers (Goldstein *et al*, 1969), and is consistent with the claim, supported by a variety of subsequent findings, that regular caffeine consumption provides little or no net benefit for alertness or performance on tests of vigilance (James and Rogers, 2005; Sigmon *et al*, 2009). Another interpretation could be that frequent caffeine consumers are ‘constitutionally’ less alert or more fatigued, and they use caffeine to remedy this state of affairs. This, though, does not readily explain why caffeine failed to increase alertness in individuals consuming little or no caffeine (i.e., group N and L participants in Figure 1, middle panel).

Vasodilation leading to increased cerebral blood flow appears to be the cause of headache that occurs on withdrawal of caffeine in frequent caffeine consumers (e.g., Couturier *et al*, 1997). It may be that vasoconstriction and reduced cerebral blood flow was responsible for the increased headache observed in the present study in the group N participants given caffeine (Figure 1, bottom panel).

Critical to the above conclusions concerning the alerting (and headache) effects of caffeine and caffeine withdrawal is the assignment of caffeine consumer status. In several previous studies, participants who were classified variously as ‘infrequent caffeine users’, ‘light, nondependent caffeine users’ and ‘non-consumers’, based on their self-reported intake of caffeine-containing drinks and foodstuffs, have been found to respond to caffeine versus placebo with increased alertness and improved cognitive performance (Alsene *et al*, 2003; Childs and de Wit, 2006; Haskell *et al*, 2005; Rogers *et al*, 2003). Although the studies differ in various ways, the discrepancy between these results and those of the present study could be explained if these groups included at least some participants who nonetheless habitually consumed significant amounts of caffeine. Evidence in support of this comes from pre-treatment salivary caffeine

concentrations measured in two of the studies. The mean concentrations were 0.11 µg/ml (Childs and de Wit, 2006) and 0.36 µg/ml (Haskell *et al*, 2005). The corresponding results for the N and L groups in the present study, after exclusion of 15 individuals with values >0.2 µg/ml (see above), were 0.014 and 0.024 µg/ml, indicating much lower dietary caffeine intakes in these participants. Indeed, the value reported by Haskell *et al* (2005) is twice of that of the present M group (0.18 µg/ml). It would seem, therefore, that data on caffeine intake can prove an unreliable guide to consumer status. Similarly, using salivary caffeine concentration to verify compliance with intake restrictions in frequent consumers is important for accurate determination of the effects of acute caffeine withdrawal. These effects will be underestimated if even a small minority of participants do not abstain as instructed.

As discussed above, individuals carrying the TT genotype of the *ADORA2A* SNP rs575187 were more susceptible to caffeine-induced anxiety than individuals carrying the CC and CT genotypes. However, they were not, as hypothesised, less likely to be frequent caffeine consumers. Indeed, among frequent caffeine consumers (MH group), TT individuals' caffeine intake from coffee was greater than that of CC and CT individuals, and they had a slightly greater (not statistically significant) total caffeine intake (Figure 5). In other words, the anxiogenic effect of caffeine does not appear to deter individuals from becoming or being caffeine consumers. There are several possible reasons for this. First, although reliable, the increase in anxiety after a dose of caffeine equivalent to one cup of ground coffee is not large. In the present study, after 100 mg of caffeine (versus placebo) anxiety measured on an 8-point scale (ranging from 'not at all' to 'extremely') increased by an average of 0.67 points in the TT genotype group and 0.11 points in the combined CC and CT genotype group (Figure 3). The statistically significant effects of caffeine (150 mg) on anxiety in TT individuals observed by

Alsene *et al* (2003) and Childs *et al* (2008) were similarly modest in size. Second, lower doses of caffeine, which are more likely to correspond to early experiences of caffeine-containing drinks (e.g., cola or weak milky coffee or, in the UK, tea), may have very little or no effect on anxiety even in individuals with the TT genotype (Childs *et al*, 2008). Third, the anxiety effect of caffeine might be outweighed by withdrawal reversal or an (unidentified) positive effect of caffeine. Fourth, anxiety-related feelings experienced after caffeine might be appraised positively (e.g., as a ‘buzz’ or ‘excitement’). This would be consistent with Thayer’s conceptualisation of mood and arousal (1989) which sees a modest level of ‘tense arousal’, resulting from an external threat or challenge, or drug, as pleasant. Indeed, notwithstanding the second point above, this might explain why among the present sample of frequent caffeine consumers, individuals with the TT genotype consumed more caffeine from coffee than CC and CT individuals. If 100 mg is around the ‘threshold’ dose for anxiety induction, then coffee is the only widely consumed caffeine-containing drink that would produce such an effect – albeit a very modest effect in frequent caffeine consumers. Caffeine avoidance, predicted by caffeine-induced anxiety, is readily observed only at higher acute doses (300 mg) (Evans and Griffiths, 1992; Stern *et al*, 1989).

A caveat to the above arguments is that ‘impression management’ may have caused participants to be reluctant to report their feelings of anxiety. However, because caffeine was administered double blind, it is unlikely that this can account even for the differences between infrequent and frequent caffeine consumers’ anxiety response to caffeine, rather it would have reduced the size of this effect (and the size of the effect of genotype).

While the various considerations above help explain why the *ADORA2A* rs5751876 TT genotype was not associated with lower dietary caffeine intake, this is different from the result

reported recently by Cornelis *et al* (2007). They found that individuals with the rs5751876 TT genotype were less likely to be heavier (>200 mg per day) caffeine consumers than CC and CT individuals. There are several potentially crucial differences between this and the current study. The Cornelis *et al* (2007) study population was a large sample (n=2,735) of Hispanic Americans who were survivors of a first acute myocardial infarction between 1994 and 2004 and their case controls. The frequency of the rs5751876 TT genotype was much higher in this population (30.6%) than in the present sample (17.2%), and the proportion of current smokers was higher (36% versus 16.5% with those smoking more than 5 cigarettes or equivalent per day being excluded). The latter might be especially important, as Cornelis *et al* (2007) found the association between rs5751876 genotype and caffeine intake to be stronger in smokers. On the other hand, in the present study the small number of caffeine-consuming (MH group) smokers with the TT genotype, like the whole sample of caffeine-consuming TT individuals, consumed more caffeine from coffee than their CC and CT counterparts (287 mg/d versus 123 mg/d,  $P<0.05$ , after adjusting for multiple comparisons).

Another possible source of bias in the present study is that anticipated anxiety or a related effect in susceptible individuals (i.e., individuals with the rs5751876 TT genotype compared with individuals with the rs5751876 CC or CT genotype) might have deterred them from taking part, thus leading to an underrepresentation of these susceptible individuals in the sample. Again, though, there is evidence against this, as in both the infrequent (NL) and frequent (MH) caffeine consumer groups the TT genotype frequency did not differ from Hardy-Weinberg predictions (observed = 30 and 35, expected = 26 and 33, for NL and MH groups, respectively). Furthermore, very few (<2%) known infrequent caffeine consumers who were contacted but not successfully recruited to the study indicated concern about possible adverse effects of caffeine as



a reason for not wanting to take part. The difference between the present study and that of Cornelis *et al* (2007) in relation to association between caffeine intake and *ADORA2A* rs5751876 genotype, therefore, remains unexplained.

In conclusion, the present results are consistent with the proposal that frequent caffeine consumption is maintained by avoidance of the negative effects of withdrawal (negative reinforcement), and they also show that caffeine consumption is little affected by the tendency of caffeine to increase anxiety, at least in part because substantial tolerance develops to this effect even at modest levels of habitual intake and even in susceptible individuals.

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## **DISCLOSURE/CONFLICT OF INTEREST**

The authors declare that over the past three years: PJR has received consulting fees from Unilever; and grants for research from Barry Callebaut, Cadbury, DSM, GSK, and Unilever. DJN has received consulting fees from Pfizer, GSK, MSD, BMS, Esteve, Novartis, Asahi, Organon, Cypress, Lilly, Janssen, Takeda, Pharmacia, Therasci, Passion for Life, Hythiam, Servier, Roche, Sanofi Aventis, Actelion, Lundbeck, and Wyeth; speaker's fees from Reckitt-Benckiser, and Cephalon; grants for research or clinical trial payments from MSD, GSK, Novartis, Servier, Janssen, Yamanouchi, Lundbeck, Pfizer, Wyeth, Organon, AZ, Cephalon, P1vital, MoD, and NHS; and he has worked for the UK Government's Committee on Safety of Medicines, the Advisory Council on the Misuse of Drugs, and the British National Formulary.

JD has received speaker's fees for educational seminars from Astra Zeneca, BMS, GSK, Janssen-Cilag, Pfizer and Wyeth. The other authors declare that they have no potential conflicts of interest to disclose.

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**Table 1** Test Day Schedule

<b>Time</b>	<b>Activity</b>
9.30 h <sup>a</sup>	Briefing and consent Blood pressure, height, weight Venous blood sample Practice computer tasks <sup>b</sup> and hand-steadiness
10.30 h	<i>Baseline (pre-treatment) test session</i> Computer tasks <sup>b</sup> , hand-steadiness, blood pressure, saliva sample (11.10 h)
11.15 h	<b>100 mg caffeine / placebo</b> Rest break
12.00 h	<i>Post-treatment test session 1</i> Computer tasks <sup>b</sup> , blood pressure, hand steadiness
12.45 h	Saliva sample <b>150 mg caffeine</b> (if caffeine at 11.15h) / <b>placebo</b> (if placebo at 11.15h) Lunch
13.15 h	Attention and impulsivity tasks
13.45 h	<i>Post-treatment test session 2</i> Computer tasks <sup>b</sup> , saliva sample (14.15 h), hand steadiness, blood pressure, sweet and bitter taste sensitivity Rest break
15.00 h	<i>Post-treatment test session 3</i> Computer tasks <sup>b</sup> , attentional bias for caffeine-related stimuli Rest break
16.00 h	Debriefing, participants paid £50 each
16.15 h	Saliva sample, participants leave

<sup>a</sup>Caffeine intake was prohibited from 19.00 h the previous evening.

<sup>b</sup>This battery of tasks included MAPSS, which measured anxiety, alertness and headache. MAPSS was completed after a tapping task, which lasted 30 seconds.

**Table 2** Participant Characteristics by Level of Habitual Caffeine Consumption

Participant characteristic	Caffeine consumer status group				Statistic
	Non-consumers (N)	Low (L)	Medium (M)	High (H)	
n	81	81	109	108	
Age (years)	34.1±13.3	29.9±11.2	32.5±12.4	35.4±13.1	F(3,375)=3.23 P=0.023
Gender (male/female)	35/46	39/42	49/60	57/51	$\chi^2=2.10$ df=3, P>0.1
Weight (kg)	70.6±12.3	71.2±16.3	74.4±15.1	73.9±15.3	F(3,375)=1.47 P>0.1
Non-smokers/smokers	73/7	67/13	87/22	86/20	$\chi^2=4.99$ df=3, P>0.1
Habitual caffeine intake (mg/d)	1.3±1.7	19 ±10	128±46	346±129	

Data are means ± SD, and n for gender and smoking (data for smoking were missing for 4 participants).



**Table 3** Genotype Distributions of *ADORA2A* Polymorphisms and their Association with Caffeine-induced Anxiety

Polymorphism	Genotype			Anxiety at session 1, genotype by treatment effect <sup>a</sup>
rs5751862	105 (GG)	184 (AG)	90 (AA)	F(2,365)=1.10, $P>0.1$
rs5760405	234 (CC)	133 (CT)	12 (TT)	F(2,365)<1
rs new3	310 (GG)	68 (AG)	1 (AA) <sup>b</sup>	F(1,368)=1.12, $P>0.1$
rs11704959	328 (CC)	46 (AC)	5 (AA) <sup>b</sup>	F(1,364)=3.24, $P=0.073$
rs2298383	140 (TT)	167 (CT)	72 (CC)	F(2,365)=4.08, $P=0.018$
rs3761422	150 (CC)	170 (CT)	59 (TT)	F(2,365)=5.58, $P=0.004$
rs2267076	156 (CC)	176 (CT)	47 (TT)	F(2,365)=1.04, $P>0.1$
rs5751876	146 (CC)	168 (CT)	65 (TT)	F(2,365)=6.57, $P=0.002$

<sup>a</sup>After 100 mg caffeine. Pre-treatment (baseline) anxiety included as covariate.  $\alpha=0.00625$  after correction for multiple tests.

<sup>b</sup>These rare genotypes were excluded from the analyses of the effects of genotype and treatment.

## Titles and legends to figures

**Figure 1** Effects of caffeine on anxiety, alertness and headache as a function of level of habitual caffeine consumption (corresponding to the non- (N), low (L), medium (M) and high (H) consumer groups described in Table 2). The data are for session 1 (after 100 mg caffeine) pooled with sessions 2 and 3 (after a second dose, 150 mg, of caffeine). Note that smoking was included as a covariate in the analysis of the data for alertness (see text for rationale). \*\* $P < 0.01$  and \* $P < 0.05$  for caffeine versus placebo within consumer group (Bonferroni t).

**Figure 2** Genomic organization and linkage disequilibrium (LD) structure of the *ADORA2A* gene (NM\_000675), and the *ADORA1* (NM\_000674) gene. SNP positions relative to the 5' promoter region, exons (numbered consecutively; *ADORA2A* coding exons are 4 and 5, and *ADORA1* coding exons are 5 and 6) and introns are shown by arrows. Shades of gray represent extent of LD (black denotes  $D' = 1$ ) and numbers in boxes give R-squared values ( $> .5$  denotes high LD,  $> .9$  denotes nearly complete LD in bold).

**Figure 3** Effect of 100 mg caffeine (session 1 data, see text) on anxiety as a function of *ADORA2A* rs5751876 genotype group. \*\* $P < 0.01$  for caffeine versus placebo within TT genotype group (Bonferroni t).

**Figure 4** Effect of caffeine on anxiety as a function of habitual level of caffeine consumption and *ADORA2A* rs5751876 genotype group (with baseline anxiety included as a covariate). The data are for session 1 (after 100 mg caffeine) pooled with sessions 2 and 3 (after a second dose,

150 mg, of caffeine). NL = non- and low consumers, MH = medium and high consumers. Top panel: CC and CT genotype groups combined. Bottom panel: TT genotype group.  $**P<0.01$  and  $*P<0.05$  for caffeine versus placebo within consumer and genotype group (Bonferroni t).

**Figure 5** Habitual caffeine consumption as a function of *ADORA2A* rs5751876 genotype group.

‘Other’ sources of caffeine were cola, energy drinks and chocolate.  $*P<0.05$  (adjusted for multiple tests).

## **SUPPLEMENTARY MATERIALS AND METHODS**

### **The Mood, Alertness and Physical Sensations Scales (MAPSS)**

MAPSS comprised 24 items (single or groups of words/descriptors) describing moods and physical sensations, which were rated on a 9-point scale anchored at the left hand end with ‘not at all’ and the right hand end with ‘extremely’ Factor analysis (using the method of principal factoring with varimax rotation) carried out on the present data set identified three main factors, on the basis of which three scales were formed. The ‘alertness’ scale comprised the following items: I feel energetic / active / lively; I feel mentally alert / attentive / able to concentrate / observant; I feel revitalized; I feel clearheaded; I feel friendly / sociable; I feel cheerful / happy / contented; I feel sleepy / drowsy / half awake (-ve); I feel fatigued / exhausted / worn out (-ve). The ‘anxiety’ scale comprised: I feel tense / anxious / nervous / on edge; I feel jittery / shaky; I feel stressed; I feel scared; My heart is pounding / racing. The ‘headache’ scale comprised: My head aches / I feel headachy; I feel dizzy / light headed / faint; I feel muzzy / dazed; I feel muddled; I feel nauseous / sick / queasy; I feel miserable / depressed / dejected. Reliability of these three scales was good (Cronbach’s  $\alpha = 0.89, 0.82$  and  $0.83$ , for the alertness, anxiety and headache scales, respectively). Four items (I feel relaxed / calm / at ease; I feel irritable / angry / cross; I feel bored / unmotivated; I feel hot / sweaty) were not included because they did not load strongly on any of the three main factors and they reduced the reliability of the scales.

### Collection of Saliva and Analysis of Methylxanthine Concentrations

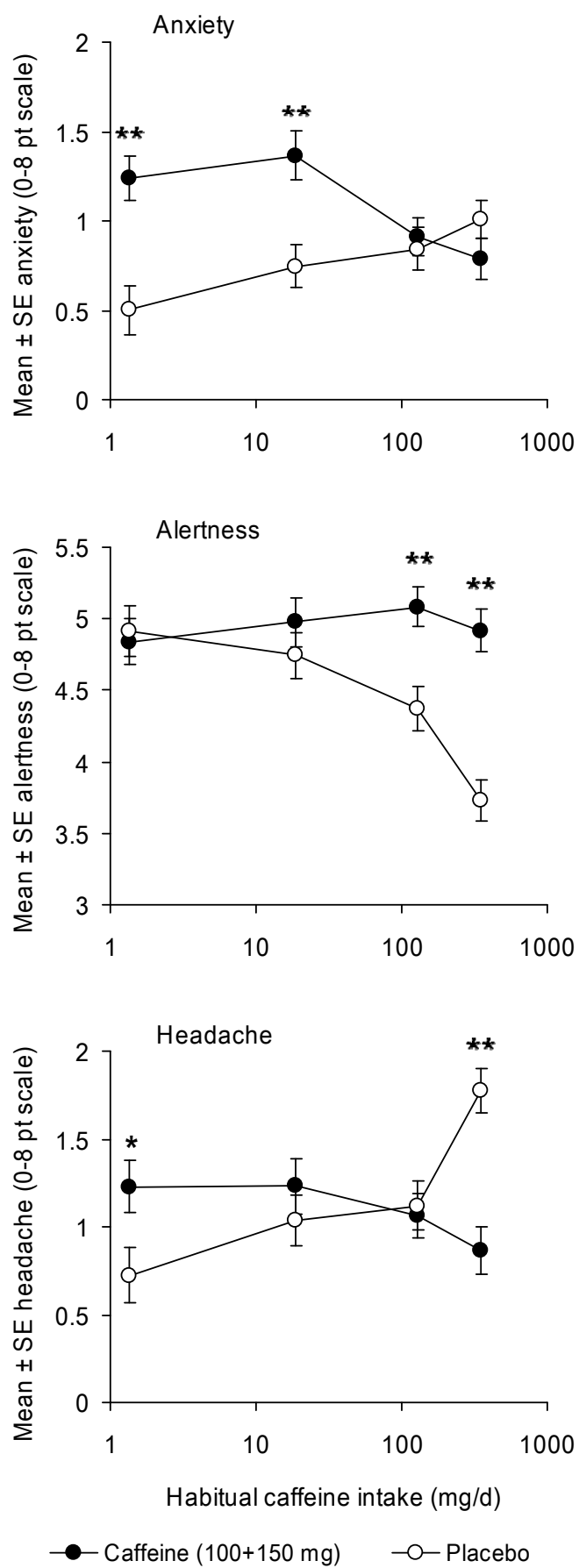
Saliva collections were made at the times shown in Table 1. Participants were required to ‘spit’ into a 6 ml plastic tube labelled with their ID number, seal it with the screw top provided and place it in a clear plastic envelope. They were asked to provide at least 1.5 ml of saliva (this level was marked on the tube). The samples were frozen within 10 min of collection at -20°C.

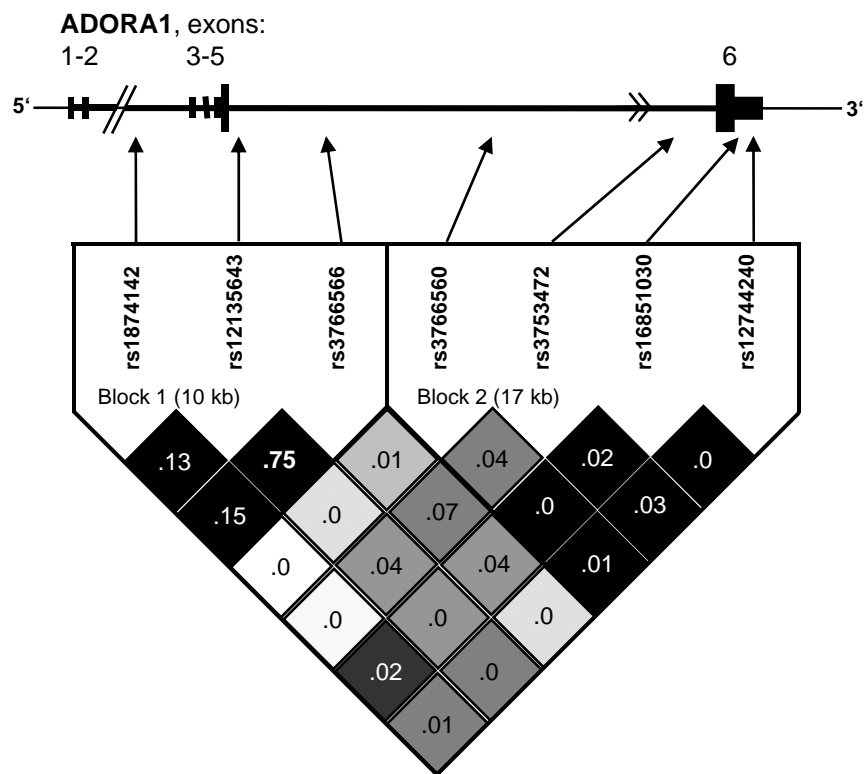
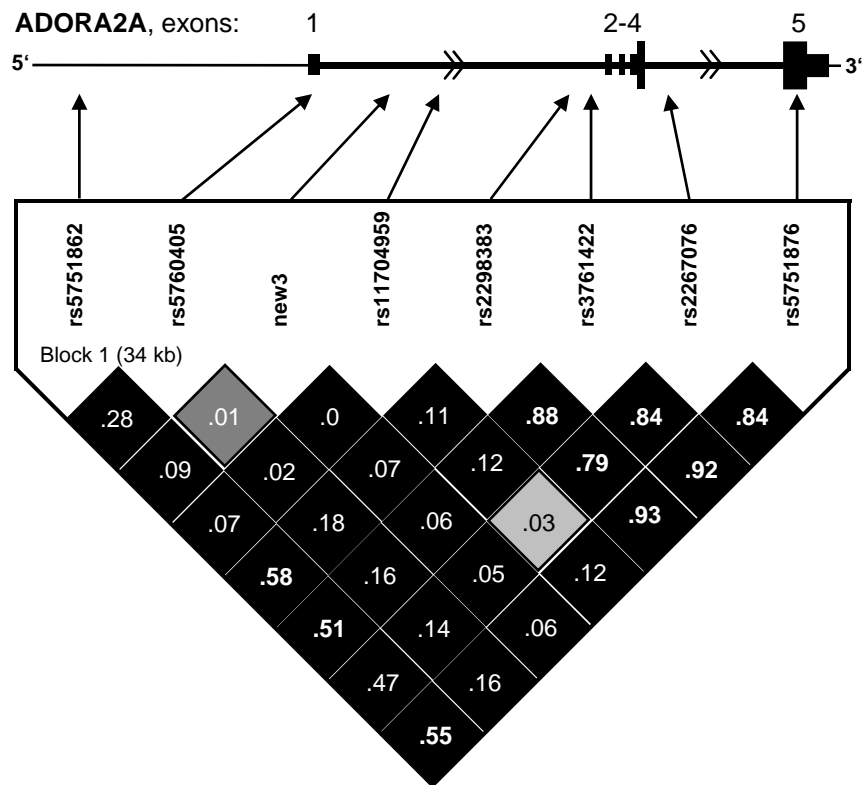
Caffeine and its metabolites, paraxanthine, theophylline and theobromine, were analysed using a high-performance liquid chromatography (HPLC) method adapted from Hartley *et al* (1985). Following the addition of ammonium sulphate (400 mg) and  $\beta$ -hydroxy-ethyltheophylline (internal standard), saliva samples (300  $\mu$ l) were extracted with dichloromethane/isopropanol (85:15, v/v) (1 ml x 3) and the organic phase evaporated under nitrogen. The extracts were dissolved in a mobile phase of 1% acetic acid and methanol (87:13 v/v) prior to analysis by reversed phase HPLC. All samples were quantified using a Hewlett Packard 1050 HPLC in conjunction with a fixed wavelength UV absorbance detector and separation was achieved on a Supelco Discovery C18 column (5  $\mu$ m, 250 $\times$ 4.6 mm). The eluent flow rate was 1.5 ml/min and target analytes were detected at a UV wavelength of 276 nm. Calibration standards containing all four xanthines were prepared in 1% acetic acid or phosphate buffers at concentrations of 4, 1, 0.2  $\mu$ g/ml (caffeine) and 2, 0.5, 0.1  $\mu$ g/ml (paraxanthine, theophylline and theobromine). The limit of detection for all analytes was 0.02  $\mu$ g/ml.

**Table S1** Genotype Distributions of *ADORA1* Polymorphisms

Polymorphism		Genotype	
rs1874142	162 (GG)	163 (AG)	54 (AA)
rs12135643	248 (CC)	115 (AC)	16 (AA)
rs3766566	236 (GG)	120 (AG)	23 (AA)
rs3766560	275 (CC)	96 (CT)	8 (TT) <sup>a</sup>
rs3753472	157 (TT)	186 (CT)	36 (CC)
rs16851030	344 (CC)	33 (CT)	1 (TT) <sup>a</sup>
rs12744240	328 (GG)	51 (GT)	0 (TT) <sup>a</sup>

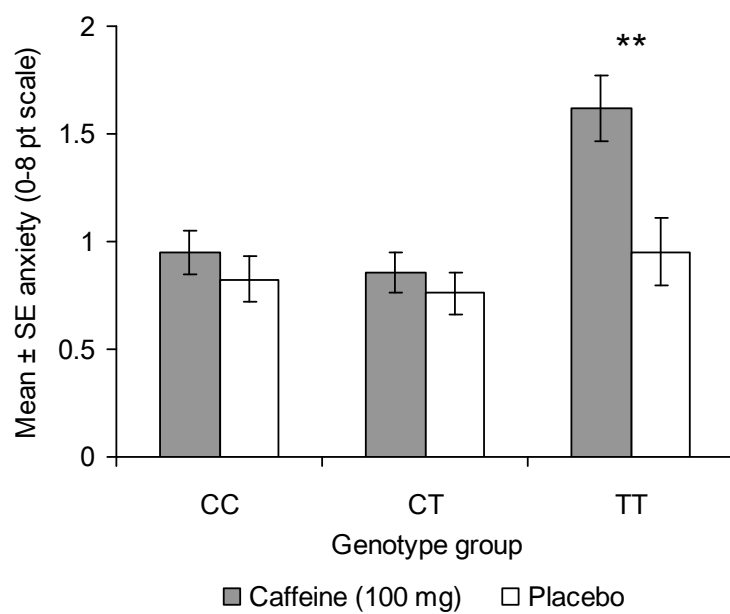
**Figure 1**



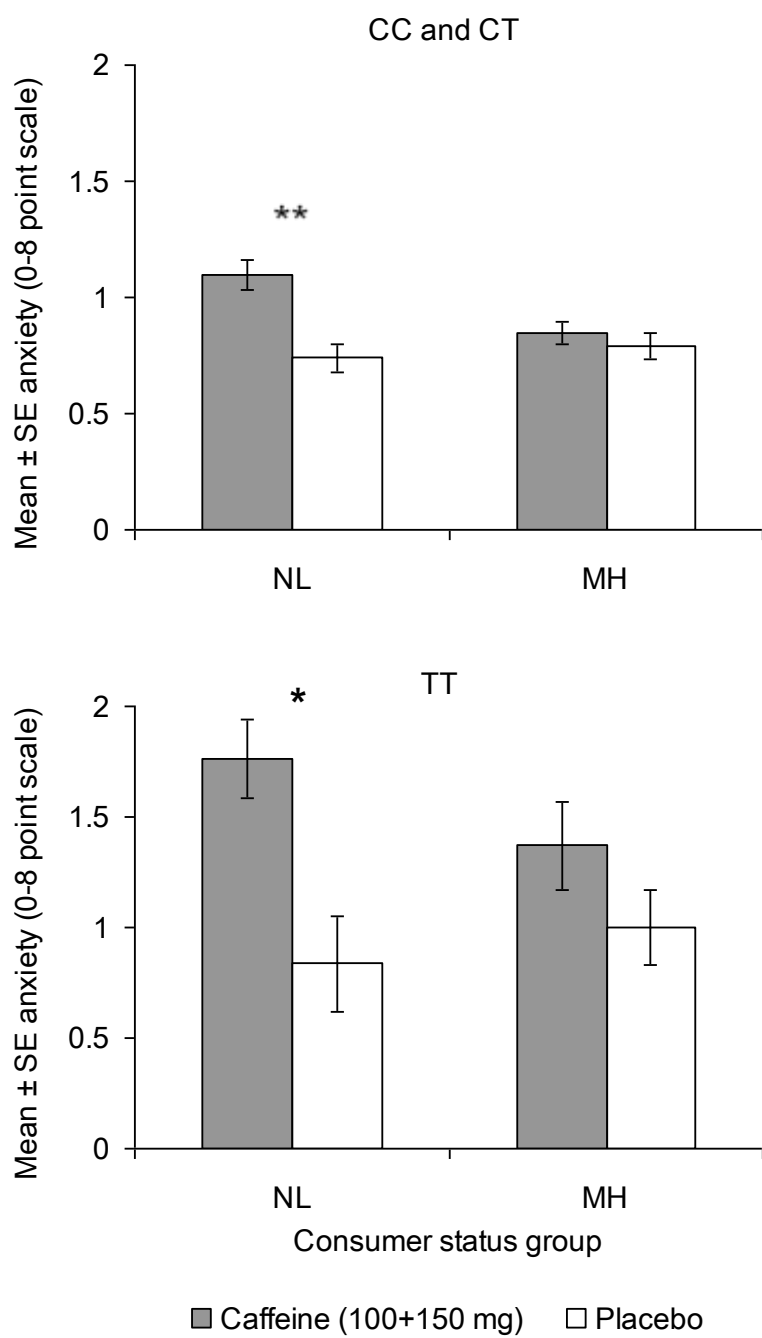




**Figure 3**



**Figure 4**



**Figure 5**

