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More Aroused, Less Fatigued: Fatty Acid Amide Hydrolase Gene Polymorphisms Influence Acute Response to Amphetamine

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Amphetamine is a stimulant drug that enhances attention and feelings of alertness. Amphetamine's effects are known to be modulated by endogenous cannabinoids, which are degraded by the enzyme fatty acid amide hydrolase (FAAH). In this study we investigated inter-individual differences in mood response to amphetamine in relation to four polymorphisms in the FAAH gene, including the FAAH missense variant rs324420C→A (Pro129Thr), which was previously found to be associated with street drug use and addictive traits. One hundred and fifty-nine healthy Caucasian volunteers participated in a three-session, double-blind crossover study receiving either placebo or oral d-amphetamine (10 and 20 mg). Associations between individual genotypes and levels of self-reported Arousal (Profile of Mood States) after d-amphetamine ingestion were investigated using two-way ANOVAs/ANCOVAs. Association analyses for haplotypes were performed using the adaptive permutation approach implemented in PLINK. Genotypes at rs3766246 and rs2295633 were significantly associated with increased ratings of Arousal ($p<0.05$) and Fatigue ($p<0.01$) after the 10-mg dose. Fatigue levels were also found to be associated with the haplotypes CCC and TAT formed from rs3766246, rs324420, and rs2295633 ($p<0.05$). These data suggest that the endocannabinoid system influences variation in subjective response to amphetamine. This has important implications for understanding the role of endogenous cannabinoids in response to amphetamine, studies of poly-substance abuse, and understanding the genetic determinants of inter-individual differences in stimulant effects and risk of abuse.

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INTRODUCTION

Amphetamine is a psychostimulant drug that increases feelings of arousal and euphoria, decreases fatigue, modulates attention, and enhances cognitive performance (Barch and Carter, 2005; Bishop *et al*, 1997; Brauer and de Wit, 1996; Caldwell *et al*, 2003). It is used clinically for treating chronic fatigue syndrome, attention-deficit/hyperactivity disorder (ADHD), and narcolepsy, but is also abused. Although subjective and behavioral effects of amphetamine are well characterized, it is also known that individuals vary in their responses to the drug. One-third of ADHD patients do not respond to treatment with amphetamine (Wilens *et al*, 2002), and healthy volunteers vary in their responses to acute administration of the drug in ways that could affect their vulnerability to develop a substance abuse disorder (de Wit *et al*, 1986; Gabbay, 2003; Kuhar *et al*, 2001). Although many individuals report pleasurable effects from amphetamine, some report adverse effects of stimulant drugs such as anxiety or panic attacks (de Wit *et al*, 1986; Williamson *et al*, 1997).

Monozygotic twins have a higher concordance in subjective response to amphetamine as compared with dizygotic twins, showing that subject drug response is a heritable genetic trait (Crabbe *et al*, 1983; Nurnberger *et al*, 1982). We and others have identified specific genes and polymorphisms that influence acute behavioral and subjective response to amphetamine in healthy human volunteers (Dlugos *et al*, 2007; Hohoff *et al*, 2005; Lott *et al*, 2005; Mattay *et al*, 2003; Veenstra-VanderWeele *et al*, 2006).

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Amphetamine produces its effects primarily by inducing release and inhibiting reuptake of dopamine, norepinephrine, and serotonin at their respective presynaptic transporter proteins (Cheng and Wooten, 1982; Gainetdinov *et al*, 1999; Sulzer *et al*, 2005; Taylor and Ho, 1978). Recent evidence suggests that responses to psychostimulants such as amphetamine and cocaine are also influenced by the endocannabinoid system. The endocannabinoid system modulates responses to stimulants, as well as conditioned drug seeking and relapse (De Vries and Schoffelmeer, 2005; Hill *et al*, 2005; Maldonado *et al*, 2006; Thiemann *et al*, 2008). CB1-receptor antagonists alter amphetamine-induced activity and Arousal as well as behavioral and locomotor sensitization in mice and Cebus monkeys (Madsen *et al*, 2006; Thiemann *et al*, 2008). The endocannabinoid system is also implicated in responses to stress, which may share some mechanisms with stimulant drugs. Stress and exogenous glucocorticoids increase endocannabinoid levels in the amygdala and hypothalamus in mice, leading to the idea that endocannabinoids function as a stress-dampening system after HPA-axis activation (Di *et al*, 2003; Hill *et al*, 2005; Patel *et al*, 2004). The HPA system can also be activated by amphetamine administration (Swerdlow *et al*, 1993).

Synaptic levels of the endocannabinoids are in part controlled by fatty acid amide hydrolase (FAAH), which terminates the activity of several endogenous cannabinoids binding to central cannabinoid receptors (CB1) (McKinney and Cravatt, 2005). In studies with rodents, FAAH inhibitors such as URB597 exert anxiolytic (Kathuria *et al*, 2002; Moreira *et al*, 2008; Rubino *et al*, 2008) and wake-modulating effects such as alertness and Arousal (Murillo-Rodríguez *et al*, 2007). Mice lacking *FAAH* exhibit CNR1-dependent behavioral responses such as analgesia, catalepsy, and hypomotility (Cravatt *et al*, 2001). Just as genetic deletion of *FAAH* in mice results in higher concentrations of endocannabinoids in the brain, polymorphisms in the human *FAAH* gene may also affect levels of these signaling lipids and consequently behavioral responses in humans (Sipe, 2004). The *FAAH* gene has been proposed to influence addiction and reward through its effect on endocannabinoids and dopaminergic pathways in the CNS (Morita *et al*, 2005; Sipe, 2004). Specifically, the FAAH common single-nucleotide polymorphism (SNP) rs324420C→A produces a missense substitution (Pro129Thr) (Sipe *et al*, 2002). The A allele at rs324420 was found to be associated with frequency of street drug use and problem drug use (Sipe *et al*, 2002), addictive traits (Flanagan *et al*, 2006), and frequency of overweight and obesity in recent case/control studies (Sipe *et al*, 2005). Another study demonstrated significantly greater increase in withdrawal scores after marijuana abstinence in individuals homozygous for the C allele (Haughey *et al*, 2008).

Based on these data, we hypothesized that acute behavioral and subjective responses of healthy volunteers to controlled administration of amphetamine would be influenced by polymorphisms in *FAAH*. In this study we focused on self-reported levels of Arousal, because it is known to be modulated by amphetamine and the endocannabinoid system. Four SNPs, including rs324420 and the common FAAH haplotypes, were examined in relation to levels of Arousal induced by amphetamine.

MATERIALS AND METHODS

Subjects

Seventy two female and 90 healthy male volunteers, aged 18–35 years, were recruited. Subjects were of self-reported Caucasian origin (confirmed via ancestry-informative markers (AIMs) as described under section Genotyping). Volunteers were excluded if they consumed more than three cups of coffee per day, or smoked more than 10 cigarettes per week. All subjects underwent a screening that included structured clinical psychiatric interview, several screening questionnaires, a psychiatric symptom checklist (SCL-90; Derogatis, 1983), the Michigan Alcoholism Screening Test (Selzer, 1971), and a health questionnaire with a detailed section on current and lifetime drug use. Volunteers received physical examination and obtained an electrocardiogram. Volunteers were excluded from participation if their body mass index (BMI) was less than 18 or greater than 26, if they had any current medical condition requiring medication or current or past medical condition that was considered a contraindication for amphetamine, any current Axis-I psychiatric disorder (American Psychiatric Association, 1994; DSM IV). Subjects were not included if they had been treated for a substance use disorder or had a history of legal, personal, or employment problems related to drug use; were not fluent in English; had less than high school education; or if they worked in night shift. Women were tested in the follicular phase of the menstrual cycle because of dampened response to amphetamine during the luteal phase (White *et al*, 2002).

Design

The study used a three-session crossover design. Each subject received placebo and d-amphetamine (10 and 20 mg), in randomized order and under double-blind conditions. Subjective, physiological, and behavioral effects of amphetamine were recorded over 4 h after drug administration. A subset of subjects ($n = 101$) took part in a fourth session in which a 5-mg dose was used; data from this session are not included in this analysis because of smaller sample size. This study was approved by the Institutional Review Board of The University of Chicago and was conducted in accordance with the Helsinki Declaration of 1975. For genetic analysis we genotyped the *FAAH* missense variant rs324420, three additional SNPs to tag the common FAAH haplotypes, and a set of AIMs.

Procedure

Subjects attended an orientation in which they provided consent. They practiced tests and questionnaires, completed a personality questionnaire (data not presented), and gave blood sample for genotyping. Participants were instructed to abstain from taking drugs, including alcohol, nicotine, or caffeine, for 24 h before each session and to fast from midnight the night before the sessions. Subjects were tested individually in a comfortably furnished room with television and reading materials for the 4-h session. Subjective and behavioral tasks were administered via computer.

Sessions were conducted from 9:00 AM to 1:00 PM, at least 48 h apart. At the beginning of each session, subjects

provided breath and urine samples to confirm abstinence from drugs and alcohol. Volunteers completed measures and baseline mood questionnaires of pre-drug subjective effects. At 9:30 AM, subjects ingested a capsule containing either placebo or d-amphetamine (10 or 20 mg). Clinically recommended daily doses of amphetamine for school-aged children with ADHD range as high as 40 mg (Greenhill *et al*, 2002; Spencer *et al*, 2006), based on these criteria the doses used in this study, are relatively low. This allowed us to minimize risk to subjects and the doses were sufficient to produce measurable effects in the participants. Subjective, behavioral, and physiological measures were obtained 30, 60, 90, 150, and 180 min after capsule intake.

Dependent Measures

To assess subjective drug effects, subjects completed three standardized questionnaires: The Drug Effects Questionnaire (DEQ), the Addiction Research Center Inventory (Martin *et al*, 1971), and the Profile of Mood States (POMS; Johanson and Uhlenhuth, 1980; McNair *et al*, 1971). In the present study we focused on the POMS to examine the association between *FAAH* gene polymorphisms and amphetamine mood response. The POMS indicates current subjective drug effects and is highly sensitive to the effects of drugs in samples of healthy volunteers. Seventy-two adjectives are used to describe momentary mood states on eight primary scales (Anger, Anxiety, Confusion, Depression, Elation, Fatigue, Friendliness, and Vigor) and two composite scales (Positive mood and Arousal) by using a five-point scale ranging from 'extremely' (4) to 'not at all' (0). The composite scale for Arousal was investigated as primary outcome measure for amphetamine's effects. This outcome measure was chosen with a clear hypothesis based on the literature. The endogenous cannabinoid system is known to modulate the levels of Arousal (Madsen *et al*, 2006; Thiemann *et al*, 2008) and amphetamine increases the levels of Arousal and alertness (Bishop *et al*, 1997; Brauer and de Wit, 1996). The Arousal scale is a composite of four different subscales. Its score is calculated using the following equation (POMS; Johanson and Uhlenhuth, 1980; McNair *et al*, 1971):

$$\text{Arousal} = [(\text{Anxiety} + \text{Vigor}) - (\text{Fatigue} + \text{Confusion})]$$

In order to reduce variability within and between subjects over time, levels of Arousal were assessed by using the Area und the Curve (AUC) providing a stable measure of individual differences in the magnitude of effect. The AUC as an integral function was assessed using the trapezoidal rule, a numerical integration method. To calculate this integral accurately, the time course has been split into smaller subintervals from time point to time point, approximating these regions under the graphs as trapezoids. Trapezoid areas have been calculated for each of the intervals and have been added. The AUC can have negative values, if POMS scores mainly decrease over time compared to baseline.

Of 162 original Caucasian study participants, 159 subjects completed the POMS for all sessions and were included in statistical analyses.

Selection of Polymorphisms and Genotyping

Four *FAAH* SNPs, including the non-synonymous coding variant rs324420, were selected and genotyped (Figure 1) using the Addictions Array (Hodgkinson *et al*, 2008). The Addictions Array aimed to develop a panel of markers able to extract full haplotype information for candidate genes in alcoholism, other addictions and disorders of mood and anxiety (Hodgkinson *et al*, 2008).

Genotyping was performed blind to all phenotypic data and with an Illumina GoldenGate, 96-well format, Sentrix array as described (Hodgkinson *et al*, 2008). Of 162 original study participants, genotype was undetermined for a single subject at a single SNP (rs3766246); the genotyping error rate was less than 1% based on concordance between duplicate samples. Subjects were genotyped at four polymorphisms in the *FAAH* gene and were assigned to one of three genotype groups: homozygotes for the first or second allele and heterozygotes. As there were only four subjects with the A/A genotype at rs324420, the A/A and C/A groups were combined for this locus.

A panel of 186 AIMs was selected for this array (Hodgkinson *et al*, 2008). To confirm participants' self-reported Caucasian ethnicity and to rule out ethnic stratification between high and low responders, genotypes of these AIMs were analyzed with Structure 2.1 (Pritchard *et al*, 2000) and in relation to a worldwide diversity panel consisting of 51 geographically defined reference populations, making a total of 1051 individuals. Ethnic proportions for each of seven worldwide factors corresponding to the geographic regions of Africa, Europe, Middle East, Central Asia, Far East Asia, America, and Oceania were estimated for each individual. Ethnic factor scores were compared between genotype groups at rs3766246 and rs2295633, because both SNPs were associated with amphetamine response.

Statistical Analyses of Polymorphisms

To assess genotype-independent main effects of placebo and amphetamine (10 and 20 mg) a two-way ANOVA was

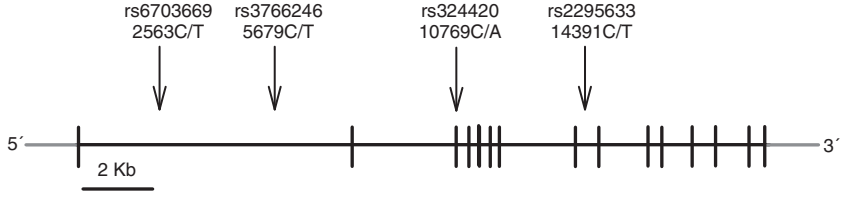

Figure 1 Genomic structure of *FAAH* gene, mapped to chromosome 1,46,632,526-46,652,107, is shown to scale, including 15 exons spanning 19.52 kb. The gene surrounding area is indicated in gray. The four polymorphisms genotyped in this study are indicated by arrows.

performed using dose (0, 10, and 20 mg of drug) and time (five time points after capsule ingestion minus pre-drug baseline scores) as within subject factors for the dependant measure. Possible confounding variables (age, BMI, gender, and baseline responses) were assessed by performing separate two-way ANCOVAs with AUC scores as within-subject factors. A p-value less than 0.05 was set as a threshold for association of POMS scales with possible confounding variables and for their inclusion as covariates in further statistical analyses. Demographic characteristics for the different genotype groups, such as gender, BMI, education in years, age, current substance abuse, and lifetime substance use were compared using ANOVA or χ^2-tests.

To analyze the impact of genotypes on drug response, either separate two-way ANOVAs or two-way ANCOVAs (SPSS 16.0) were performed for the outcome measure. Genotype was used as grouping factors and AUC scores for placebo, 10, and 20 mg amphetamine administration were chosen as within-subject factors, comprising two-way ANOVAs and ANCOVAs. When assessing drug by genotype interactions, Levene's test for equality of error variances was always included in the analyses. Greenhouse–Geisser correction was used when Levene's test for equality of error variances was significant. *Post hoc* analyses were conducted by performing one-way ANOVAs or ANCOVAs with AUC scores as dependent measures. Alpha was set at $p<0.05$ (two-tailed) for all analyses.

Haplotype Analyses

Hardy–Weinberg equilibrium for each marker and linkage disequilibrium (LD) between the markers were analyzed using Haploview version 4.1 (http://www.broad.mit.edu/mpg/haploview/). Haploview was used to generate an LD map of *FAAH* with the data of our sample and the available HapMap data (The International HapMap Genome Browser B36). Haplotype blocks were identified and captured with the Haploview software using CEU-HapMap data and our data, which were consistent. Haplotype pairs were estimated and correlation analyses between haplotypes and the outcome measures were performed using PLINK. Empirical p-values were calculated using the adaptive permutation approach implemented in PLINK, which corrects for testing multiple haplotypes, giving up permuting haplotypes that are clearly going to be non-significant more quickly than haplotypes that look interesting.

RESULTS

Subjects

Genotype frequencies for the *FAAH* SNPs (Table 1) were in Hardy–Weinberg equilibrium and allele frequencies were consistent with those of HapMap Caucasians (The International HapMap Genome Browser B36). The *FAAH* SNPs were in high LD (Figure 4), consistent with HapMap data. *FAAH* genotypes did not predict demographic measures (Table 2) or placebo response. Analysis of ancestry informative markers (Structure 2.1.) confirmed self-reported Caucasian origin in all study participants. Ethnic factor scores did not differ between genotype groups at the loci that were found to be associated with the primary outcome measure: rs3766246 and rs2295633. One hundred and fifty-nine subjects completed POMS questionnaires for each session. Three subjects had missing POMS data and were, therefore, excluded from the analyses.

Table 1 Allele and Genotype Frequencies of the *FAAH* Polymorphisms

	Position	Allele		Genotype			HWE[a]
		1	2	1/1	1/2	2/2	*p*-value
rs6703669 (C/T)	2563	232	88	81	68	10	0.44
rs3766246 (C/T)	5679	203	115	65	71	22	0.74
rs324420 (C/A)	10769	257	63	101	53	5	0.79
rs2295633 (C/T)	14391	206	114	67	70	22	0.62

FAAH, fatty acid amide hydrolase; HWE, Hardy–Weinberg equilibrium.
[a]Hardy–Weinberg equilibrium: *p*-values assessed using Haploview software version 4.0.

Table 2 Demographic Characteristics for all Subjects

Demographic characteristics	Overall
N	159
Age (years) (mean ± SEM)	22.8+3.6
Gender (% female)	44
BMI (mean ± SEM)	22.7+2.2
Education (years) (mean ± SEM)	15.1+1.4
Current substance use	
Alcohol (drinks/week) (mean ± SEM)	4.5+3.7
Cigarettes (cigarettes/week) (mean ± SEM)	0.8+1.8
Caffeine (cups/day) (mean ± SEM)	7.3+7.0
Marijuana (times/month) (mean ± SEM)	0.9+2.3
Lifetime substance use	
Stimulants (ever used) (%)	51.6
Sedatives (ever used) (%)	6.3
Opiates (ever used) (%)	22.0
Marijuana (ever used) (%)	44.2
Hallucinogens (ever used) (%)	28.9
Inhalants (ever used) (%)	9.4

Comparisons across genoptype groups for all FAAH SNPs were made using one-way ANOVA for continuous data and χ^2-test for frequency data; none of these tests yielded significant results.

Genotype-Independent Effects of Amphetamine

We first examined the effects of amphetamine independent of genotype. Amphetamine produced the expected increases in Anxiety and Vigor and decreases in Fatigue and Confusion ($p<0.001$, drug main effect from repeated-measures ANOVA). These four primary scales were used to calculate Arousal. Amphetamine also increased the levels

of Arousal dose dependently ($p<0.001$, drug main effect from repeated-measures ANOVA). Amphetamine's effects on Arousal and Fatigue levels at the three sessions are shown in Supplementary Figure 1 (S1). For most measures, drug effect appeared by 60 min and peaked between 90 and 120 min after capsule ingestion. Males scored significantly higher on Arousal than females in all sessions ($p=0.039$). Therefore, sex was used as a covariate and included as a between-subject factor in further analyses. Baseline scores did not differ between genotype groups.

FAAH Gene Polymorphisms and the POMS-Scale Arousal

Associations of the four *FAAH* SNPs with the POMS scale Arousal are reported in Table 3a. The C/C genotype groups for both rs3766246 and rs2295633 showed higher Arousal levels after amphetamine ingestion (Genotype × Drug interaction on two-way ANOVA/ANCOVA). When we examined each dose individually we found that these effects were significant at the 10-mg dose, but not for the placebo or 20-mg conditions. Figure 2a shows AUC scores of Arousal (POMS) between the three rs2295633 genotype groups and Supplementary Figure 2a shows that between the three rs3766246 genotype groups. *Post hoc* comparisons (one-way ANOVA/ANCOVA) revealed that genotype groups were significantly different only in their response to the 10-mg dose of amphetamine, although a similar trend was apparent after the 20-mg dose. FAAH genotypes did not predict placebo response. The time courses of the three genotype groups at rs25295633 for Arousal after 10 mg amphetamine administration are shown in Figure 3a. To clarify the source of AUC scores, time courses of Arousal are descriptively shown for rs2295633 and rs3766246 in Figure 3a and Supplementary Figure 3a as changes from baseline for the genotype groups after the 10-mg dose. Genotype × Drug (placebo, 10 mg, and 20 mg) interactions on Arousal at rs6703669 and the rs324420 variant were not significant.

Post Hoc Analyses of the Primary Scales Fatigue, Anxiety, Confusion, and Vigor

In order to better understand the source of the significant genotype effect on Arousal, we examined the relationship between genotype and dose on the four sub-scales Vigor, Fatigue, Anxiety, and Confusion used to calculate Arousal scores. These *post hoc* analyses were performed for all four SNPs.

Analogous to findings for Arousal, the C/C genotype groups for rs3766246 and rs2295633 showed significantly lower Fatigue levels after amphetamine ingestion (Table 3b) (Genotype × Drug interaction on two-way ANOVA/ANCOVA). The AUC scores of Fatigue (POMS) between the three rs2295633 and the three rs3766346 genotype groups are shown in Figure 2b and Supplementary Figure 2b. Again, *post hoc* comparisons (one-way ANOVA/ANCOVA) showed that genotype groups were significantly different only in their response to the 10-mg dose of amphetamine. Genotype groups did not differ after placebo. The time courses for Fatigue for the three genotype groups at rs25295633 after 10 mg amphetamine administration are descriptively shown in Figure 3b and for the three genotype groups at rs3766246 in Supplementary Figure 3b. There were no significant Genotype × Drug (placebo, 10 mg, 20 mg) interactions at rs6703669 and the rs324420 variant on the Fatigue scale; however, the *p*-value for an association between genotypes at rs324420 and

Table 3 Association of POMS Scores Arousal and Fatigue after Amphetamine with Individual *FAAH* Polymorphisms

	rs6703669 (C/T)	rs3766246 (C/T)	rs324420 (C/A)	rs2295633 (C/T)
(a) Arousal[a]				
F-value (dF)	1.65 (4)	3.37 (4)	1.58 (2)	3.04 (4)
p-value[b]	0.162	**0.010***	0.208	**0.018***
Post hoc (10 mg)	—			
F-value (dF)	—	3.3 (2)	—	3.9 (2)
p-value[c]	—	**0.038***	—	**0.021***
(b) Fatigue				
F-value (DF)	1.11 (4)	3.53 (4)	2.42 (2)	3.41 (4)
p-value[b]	0.350	**0.009**[d]**	0.091	**0.009****
Post hoc (10 mg)				
F-value (dF)	—	3.7 (2)	7.4(1)	4.82 (2)
p-value[c]	—	**0.028***	**0.007****	**0.009****

FAAH, fatty acid amide hydrolase; POMS, Profile of Mood States.
[a]Arousal adjusted for gender (included as between subject covariate in statistical analyses).
[b]Drug-by-genotype interaction effects: *p*-values assessed by two-way ANOVA/ANCOVA.
[c]*Post hoc* analysis: *p*-values for main effect of genotype performed using one-way ANOVA/ANCOVA for the 10 mg dose only (placebo and 20 mg doses were not significant).
[d]Greenhouse–Geisser Correction.
*$p<0.05$.
**$p<0.01$.

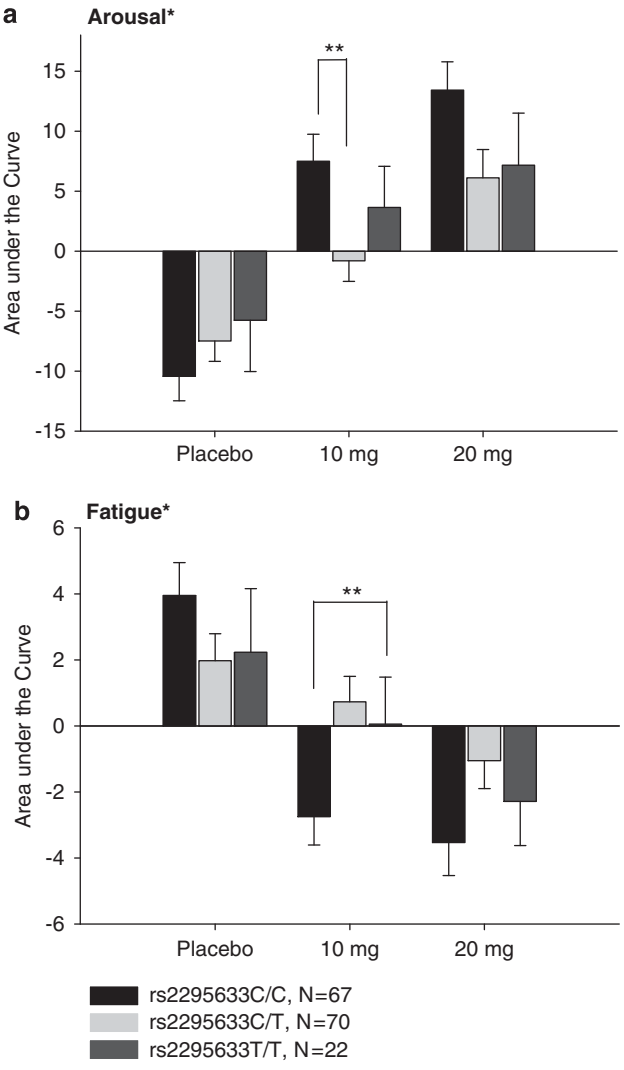

Figure 2 Mean ± SEM AUC scores on Arousal (a) and Fatigue (b) (POMS) between the three genotype groups at rs2295633 (T/T: $n = 22$; C/T: $n = 70$; C/C: $n = 67$) after placebo and after 10 and 20 mg of amphetamine administration. Amphetamine (10 mg) increased Arousal and decreased Fatigue, relative to placebo, in the rs2295633C/C group as compared with the 2295633C/T and T/T groups. These results are analogous to findings for rs3766246. (*, significant two-way ANOVA/ANCOVA; **, *post hoc* multiple comparisons between genotypes with Bonferroni correction $p < 0.05$).

Fatigue was $p < 0.1$. To further investigate this finding, exploratory *post hoc* analyses were performed. Although Genotype × Drug interaction did not reach significance for the rs324420 variant, subjects with the C/C genotype showed significantly greater decrease in Fatigue after 10 mg amphetamine administration as compared with the other two genotypes.

There was no significant association between Confusion, Vigor, and Anxiety and any of the genotypes. We conclude that the effect on Arousal was mainly driven by decreases in Fatigue and was not directly related to the other three subcomponents (Anxiety, Vigor, and Confusion). Thus, whereas amphetamine treatment had significant effects on all four sub-scales, only Fatigue interacted with genotypes in *FAAH*.

FAAH Haplotypes and the POMS-Scale Arousal and Fatigue

D′-values between the four polymorphisms in our sample are shown in Figure 4 (Haploview version 4.1). LD parameters between SNPs in our sample did not significantly differ from those given in the HapMap project, but the CEU-HapMap database did not provide the LD parameters for rs2295633. Using either our data or the CEU-HapMap data Haploview identified a single haplotype block formed from the three SNPs rs3766246, rs324420, and rs2295633 (Figure 4). Haplotype pairs were estimated for each individual using PLINK, which allows for uncertainty of haplotype phases. Three reconstructed haplotypes: TAT (Frequency (F): 0.1906), TCT (F: 0.1563), and CCC (F: 0.641) were assessed for correlation analyses with AUC scores of the associated POMS-scale Arousal and Fatigue at the 10-mg dose. As associations between the investigated gene polymorphisms and amphetamine response was most distinct at the 10-mg dose, this condition was chosen for haplotype analyses.

Results of the analyses are shown in Table 4. Haplotypes CCC and TAT, but not haplotype TCT, were significantly associated with levels of Fatigue. Haplotype TAT was associated with higher Fatigue scores ($p < 0.05$), whereas haplotype CCC ($p < 0.01$) was correlated with lower Fatigue levels after 10 mg amphetamine administration. These findings are consistent with the results of the SNP association analyses. There were no significant associations between any haplotypes and Arousal.

FAAH Gene Polymorphisms and Physiological Measures

Association analyses were performed between AUC scores of heart rate, systolic, and diastolic blood pressure at the different doses and the investigated SNPs and haplotypes (two-way ANOVA/ANCOVA). As subjects with higher BMI had significantly higher diastolic blood pressure, BMI was included as a covariate in analyses involving this measure. There was no association between any of the physiological measures and the genetic markers.

DISCUSSION

The main finding of this study is that the SNPs rs3766246 and rs2295633 were associated with higher self-reported Arousal in response to amphetamine. *Post hoc* analyses of the subcomponents of Arousal revealed that subjects with genotypes C/C at rs3766246 and rs2295633 and also the functional SNP rs324420 showed significantly greater decrease in Fatigue after 10 mg amphetamine administration compared with the other two genotypes. In addition, the CCC haplotype from rs3766246, rs324420, and rs2295633 was significantly related to lower ratings of Fatigue after amphetamine. Haplotype TAT, but not haplotype TCT, was significantly correlated with higher Fatigue scores after 10 mg amphetamine administration. Thus, these three SNPs, and in particular rs324420, showed effects on Fatigue both alone and when combined to form a haplotype. The three investigated *FAAH* haplotypes were not significantly associated with levels of Arousal. However, a trend was apparent in line with our findings for Fatigue.

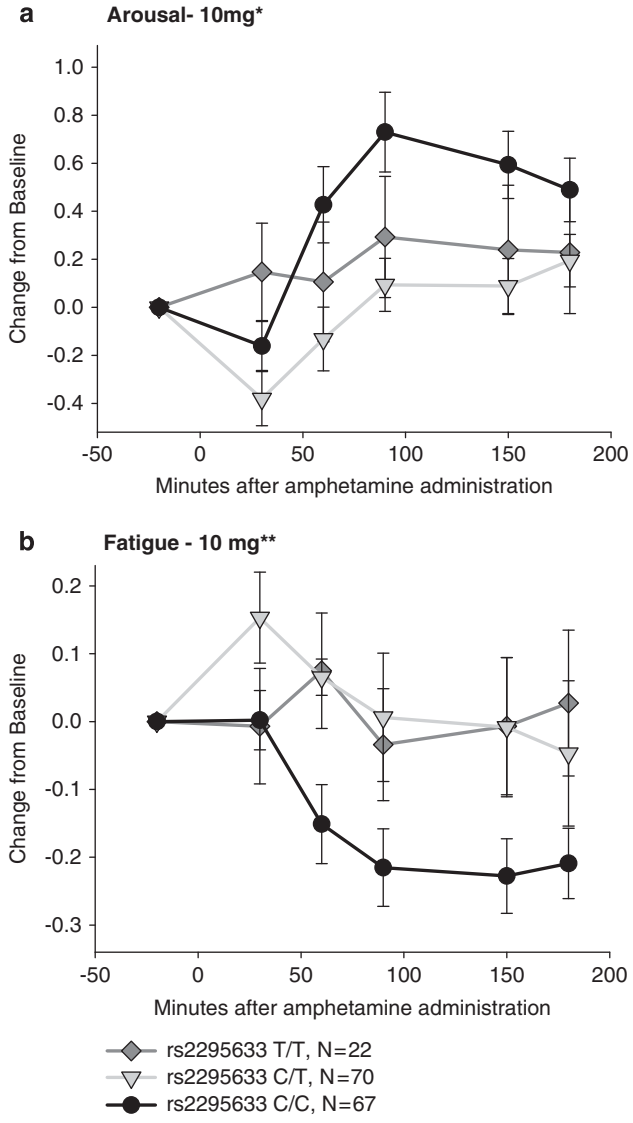

Figure 3 Time course of Arousal (a) and Fatigue (b) (POMS) after 10 mg amphetamine administration for the three genotype groups. The 2295633 C/C group reported a greater increase in Arousal and a greater decrease in Fatigue compared with the other two groups. Data are mean (SEM) ratings of Arousal and Fatigue as change from pre-drug baseline. The groups did not significantly differ on baseline scores. These results are analogous to findings for rs3766246. (*, $p<0.05$; **, $p<0.01$, assessed by one-way ANOVA/ANCOVA with Bonferroni correction).

Subjects with the CCC haplotype from rs3766246, rs324420, and rs2295633 showed higher ratings of Arousal and subjects with haplotype TAT scored lower on Arousal after 10 mg amphetamine administration ($p<0.1$).

When specific doses were examined, only the 10-mg dose showed an effect of genotype groups on Arousal and Fatigue. It is possible that *FAAH* genotype effects on response to amphetamine are apparent only at lower, marginally effective doses, and that the genetic differences are overcome by higher doses of the drug. A similar relationship between a SNP in the gene CSNK1E and dose of amphetamine was observed in a previous study (Moreira *et al*, 2008) supporting the idea that certain drug–genotype interactions might be more evident at lower doses. *FAAH* genotypes were not related to any demographic factors or to POMS-scale baseline mood scores. These findings demonstrate a pharmacogenetic difference, indicating that variations in the *FAAH* gene influence acute responses to d-amphetamine.

The only SNP examined in this study with a known functional consequence is rs324420. *In vitro* and *in vivo* studies have shown reduced cellular activity and expression of the human A/A variant (Chiang *et al*, 2004). Thus, the *FAAH* C/C variant may result in higher FAAH enzyme activity and consequent lower endocannabinoid levels due to greater degradation of endocannabinoids by FAAH (Doehring *et al*, 2007). This might underlie the observed lower levels of fatigue, higher levels of Arousal, and levels of feeling stimulated after 10 mg amphetamine administration. In line with our suggestions, Murillo-Rodriguez (2008) found activation of the endocannabinoid system via the CB1 receptor to induce sleep and to modulate wakefulness (Murillo-Rodriguez 2008). *FAAH*-knockout mice process higher values of slow wave sleep and more intense episodes of slow wave sleep as compared with wild-type animals (Huitron-Resendiz *et al*, 2004). However, in another study oleoylethanolamide, palmitoylethanolamide, and an FAAH antagonist were found to enhance waking (Murillo-Rodríguez *et al*, 2007).

Further, Tyndale *et al* (2007) found that subjects ($n=749$) with the A/A genotype of rs324420 were at significantly reduced risk for being THC-dependent as compared with those with the C/A or C/C genotype. The C/C variant of rs324420 is also associated with higher craving for marijuana after abstinence (Haughey *et al*, 2008) and with lower frequencies of obesity (Sipe *et al*, 2005). However, the A/A genotype (not C/C) of rs324420 is more prevalent in problem drug users (Sipe *et al*, 2002) and in persons having addictive traits (Flanagan *et al*, 2006). Thus, while our results and multiple prior studies have suggested that this SNP is functionally important, neither the C nor the A alleles can be easily identified as the 'risk allele' for drug abuse and dependence when considering all available data.

Our data suggest that, in addition to the non-synonymous variant rs324420 two other polymorphisms (rs3766246 and rs2295633) located in the same haplotype block are also associated with differences in subjective response to amphetamine. It is conceivable that either of these intronic variants may be associated with differences in mRNA processing, stability, splicing, or changes of transcription rates. Interestingly, in a study of response to cold pain, rs2295633, but not rs324420, was significantly associated with pain sensitivity (Kim *et al*, 2006). As these three SNPs form a haplotype block (Figure 4), it is difficult to determine which (if any) of them are causally related to the observed phenotypes. Thus, some uncertainty remains about whether the coding difference caused by the variant rs324420 or changes by one of the intronic variants is the causal polymorphism at this locus.

In an effort to investigate the possibility that a difference in gene expression, rather than a coding difference, was reasonable for the associations detected in this study, we investigated the possible associations between the SNPs surveyed in this study and gene expression. We used expression data from HapMap CEPH immortalized

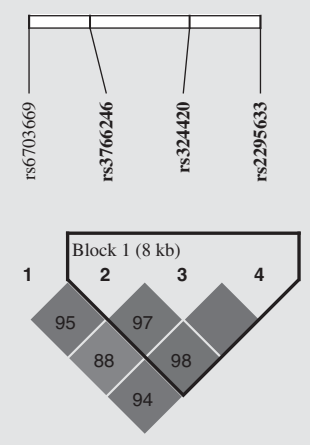

Figure 4 LD analyses: *D′*-values of SNPs along the *FAAH* gene, illustrating one haplotype block. *D′*-values were calculated using Haploview version 4.0.

Table 4 Associations Between the POMS-Scale Arousal and Fatigue and *FAAH* three-SNP Haplotypes from rs3766246, rs324420, and rs2295633

Haplotypes (10-mg dose)	Beta[a]	R^{2b}	STAT[c]	NP[d]	Corr. Emp. *p*-value[e]
Arousal					
TAT ($F^f = 0.102$)	−3.847	0.016	−1.620	168	0.0947
TCT (F = 0.0807)	−1.388	0.002	−0.572	7	0.75
CCC (F = 0.485)	3.682	0.023	1.941	300	0.0565
Fatigue					
TAT ($F^f = 0.102$)	2.217	0.031	2.238	826	**0.0206***
TCT (F = 0.0807)	0.687	0.002	0.674	10	0.5625
CCC (F = 0.485)	−2.054	0.041	−2.601	2316	**0.0078****

FAAH, fatty acid amide hydrolase; POMS, Profile of Mood States.
[a]Regression coefficient.
[b]Proportion of phenotypic variability explained by haplotype.
[c]Wald test (based on *t*-distribtion).
[d]Number of permutations performed for this haplotype.
[e]Empirical *p*-value (adaptive).
[f]Frequency.
*$p < 0.05$.
**$p < 0.01$.

lymphoblast cell lines (Stranger *et al*, 2007; Veyrieras *et al*, 2008) for this purpose. We identified a highly significant (log(Bayes factor) >10) association between rs6703669, which is located in intron 1 of the *FAAH* gene, and expression of NSUN4 (probe ID hmm8232) (Veyrieras *et al*, 2008). A similar observation is reported in the Supplementary materials of Stranger *et al* (2007) (Supplementary Table S2, probe ID hmm8232). *FAAH* is immediately adjacent to the *NSUN4* gene. cDNAs that include exons from NSUN4 and *FAAH* have been detected (www.ncbi.nlm.nih.gov/IEB/Research/Acembly), suggesting that these two genes constitute a single gene complex (Thierry-Mieg and Thierry-Mieg, 2006). However, rs6703669 was not significantly associated with any of our outcome measures (Table 3), and, conversely, none of the three SNPs in Tables 3 and 4 were significantly associated with differential gene expression. In summary, while we did identify an SNP in *FAAH* that is associated with differential gene expression, this SNP was not associated with the sensitivity to amphetamine as measured by Arousal or Fatigue in the present study.

The quality and magnitude of subjective responses that individuals experience from their first experience with the drug is related to subsequent abuse or dependence (Di Franza *et al*, 2004; Fergusson *et al*, 2003; Haertzen *et al*, 1983). Thus, our findings may help to predict individual differences in susceptibility to misuse amphetamine. Individuals carrying C alleles of rs3766246, rs2295633, or haplotype CCC from rs3766246, rs324420, and rs2295633, may be possibly more likely to use amphetamine repeatedly as a recreational drug because of their greater sensitivity to its stimulating subjective effects. As we only found *FAAH*-genotype-dependent differences in response to the low amphetamine dose, it remains unclear whether our finding has an impact on frequency of higher dosage drug abuse and has to be investigated in future studies. Alternatively, in cases where amphetamine is being clinically used to counteract fatigue, lower doses might be sufficient in individuals with high-sensitivity genotypes. Thus, in the context of clinical use of amphetamine, it may be possible to identify patients who are at risk for amphetamine abuse, or to better calibrate the dose required when using amphetamine for its stimulant properties. In the former case, special precautions may be taken when prescribing this drug to patients at risk for abuse (Shastry, 2006). However, it should be noted that genetic variation only accounts for a fraction of individual differences, and *FAAH* is only one of the many genes involved in the genetics of amphetamine sensitivity (Dlugos *et al*, 2007; Lott *et al*, 2005, 2006; Palmer *et al*, 2005; Veenstra-VanderWeele *et al*, 2006). Thus, clinically useful predictions will require accounting for multiple genetic loci.

This study has several limitations. Our findings need confirmation by performing larger replication studies. Although this complex pharmacological study used a substantial number of subjects, the inherent level of variability in the outcome measures and the complexity of the gene–environment interactions call for replication to confirm these observations. Further, we only included subjective outcome measures to assess the effects of amphetamine, and functionally intermediate measures such as functional MRI or SPECT (Mattay *et al*, 2003; Rohde *et al*, 2003) would help to reinforce our observations. Such studies would further elucidate how and in which brain regions the investigated genetic variations might modulate the endocannabinoid system after amphetamine consumption. Another limitation of the study is the fact that the minor-allele frequency of the functional variant for rs324420 was low, (0.225 HapMap CEU sample), requiring us to pool subjects with genotypes A/A and A/C into a single group. Replication in a larger sample may allow separate analyses of all three genotypes.

In summary, our study provides novel evidence that genetic variation in the *FAAH* gene is associated with specific mood responses after amphetamine administration. These data add to evidence that the endogenous cannabinoid system is related to response to a stimulant drugs in humans, which may lead to improvements in preventing and treating amphetamine abuse disorder.

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DISCLOSURE

The authors declare no conflict of interest.

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