

OPRM1 gene variants modulate amphetamine-induced euphoria in humans

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The μ -opioid receptor is involved in the rewarding effects of not only opioids like morphine but also psychostimulants like amphetamine. This study aimed to investigate associations between subjective response to amphetamine and genetic polymorphisms and haplotypes in the μ -opioid receptor including the exonic variant rs1799971 (Asp40Asn). One hundred and sixty-two Caucasian volunteers participated in three sessions receiving either placebo or d-amphetamine (10 and 20 mg). Associations between levels of self-reported Euphoria, Energy and Stimulation [Addiction Research Center Inventory 49-item questionnaire (ARCI-49)] after d-amphetamine ingestion and polymorphisms in *OPRM1* were investigated. The intronic single nucleotide polymorphisms (SNPs) rs510769 and rs2281617 were associated with significantly higher ratings of Euphoria, Energy and Stimulation after 10 mg amphetamine. Feelings of Euphoria, Energy and Stimulation were also found to be associated with a two-SNP haplotype formed with rs1799971 and rs510769 and a three-SNP haplotype formed with rs1918760, rs2281617 and rs1998220. These results support the hypothesis that genetic variability in the μ -opioid receptor gene influences the subjective effects of amphetamine and may suggest new strategies for prevention and treatment of psychostimulant abuse.

Keywords: Amphetamine, energy, euphoria, stimulation, inter-individual differences, μ -opioid receptor

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Amphetamine, a prototypic drug of abuse, increases feelings of euphoria, energy and attention in most people (Brauer & de Wit 1996). However, some individuals experience adverse effects such as increased anxiety or dysphoria (de Wit *et al.* 1986). There is evidence that some of the variations in subjective responses to amphetamine may be genetic in origin. For example, monozygotic twins have a higher concordance in subjective response to amphetamine compared to dizygotic twins (Crabbe *et al.* 1983; Nurnberger *et al.* 1982), and in healthy volunteers, polymorphisms in dopaminergic and other receptor mechanisms have been found to be associated with acute response to amphetamine (Dlugos *et al.* 2007; Mattay *et al.* 2003; Palmer *et al.* 2005).

Amphetamine acts mainly by inhibiting reuptake of presynaptic dopamine, norepinephrine and serotonin via interactions with membrane transporters involved in neurotransmitter vesicular storage and reuptake (Schuldiner *et al.* 1993; Wallace & Connell 2008). However, its reinforcing effects are also modulated by μ -opioid receptors (OPRM1). OPRM1 are densely located in the mesolimbic dopamine system, which is composed of projections from the ventral tegmental area to the striatal nucleus accumbens (NAc) (Mathon *et al.* 2006). Amphetamine increases synaptic dopamine levels, which activate striatal neuropeptides in postsynaptic neurons acting on opioid receptors, including OPRM1 (Dalia *et al.* 1998; Florin *et al.* 1998; Wang *et al.* 1998). The stimulation of OPRM1, in turn, inhibits GABA release and disinhibits dopamine neuronal firing. This results in further increases in extracellular dopamine levels in the NAc and striatum (Gerrits *et al.* 2003; Kieffer *et al.* 1992; Spanagel *et al.* 1992). The idea that endogenous opioids contribute to amphetamine responses is consistent with the observation that μ -receptor agonists and psychostimulants taken together produce strong euphorogenic effects ('speedball') (Leri *et al.* 2003).

Controlled studies with rats and humans further support the hypothesis that responses to amphetamine are mediated by the opioid system. *Oprm1*-knockout mice show reduced reward from psychostimulants (Hall *et al.* 2004), and opioid receptor antagonists attenuate amphetamine-induced dopamine release in the striatum and NAc (El Daly *et al.* 2004; Hooks *et al.* 2004). In healthy humans and in amphetamine-dependent patients, the opioid receptor antagonist naltrexone decreases the subjective effects of amphetamine (Jayaram-Lindström *et al.* 2004a,b). Additionally, imaging studies show that human cocaine users have increased OPRM1 binding in several brain regions, and this increased binding is positively correlated with self-reported cocaine craving (Gorelick *et al.* 2005; Zubieta *et al.* 2005). OPRM1 binding also predicted time to relapse in the cocaine

users (Gorelick *et al.* 2008). Thus, several lines of evidence suggest that the endogenous opioid system contributes to the rewarding effects of stimulant drugs.

We hypothesized that genetic variations at the *OPRM1* locus may influence subjective response to amphetamine. Two single nucleotide polymorphisms (SNPs) were of particular interest. The non-synonymous *OPRM1* SNP rs1799971 (Asp40Asn) is reportedly a risk allele for addiction. Studies have shown that it is associated with methamphetamine-induced psychosis (Ide *et al.* 2006), alcoholism (Schinka *et al.* 2002), heroin addiction (Drakenberg *et al.* 2006) and incidence of completed suicide (Hishimoto *et al.* 2008). The intronic SNP rs510769 appears to be associated with vulnerability to heroin addiction (Levrán *et al.* 2008). In addition, we performed association analyses between *OPRM1* polymorphisms and physiological measures, such as blood pressure and heart rate, to determine whether *OPRM1* polymorphisms might also influence physiological measures after amphetamine. We hypothesized that SNPs that modulate the subjective responses to drugs would also modulate physiological responses. In total, we investigated the impact of seven SNPs in the *OPRM1*, including rs1799971 and rs510769, on physiological as well as prototypic behavioral and subjective responses to amphetamine: Euphoria, Energy and Stimulation.

Materials and methods

Subjects

Seventy-two female and 90 male healthy volunteers, aged 18–35, were recruited by advertisements and posters. All subjects were of self-reported Caucasian origin, which was confirmed by the analysis of ancestry informative markers in the sample (see *Genotyping* paragraph below). All participants took part in a screening that included a structured clinical psychiatric interview, 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 a physical examination by a physician and obtained an electrocardiogram. Subjects 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 to be a contraindication for amphetamine (e.g. hypertension, abnormal EKG), or any current Axis I psychiatric disorder (American Psychiatric Association 1994). To reduce variability due to withdrawal or tolerance from caffeine and nicotine, volunteers were excluded if they consumed more than three cups of coffee per day, or smoked more than 10 cigarettes per week. 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, if they were not fluent in English, if they had less than a high school education, or if they worked a night shift. Women who were pregnant or lactating or planning to become pregnant during the study were also excluded. Women were only tested in their follicular phase as response to amphetamine is dampened during the luteal phase of the menstruation cycle because of altered pharmacodynamic effects (White *et al.* 2002).

Design

The study used a three-session crossover design. Each subject received placebo and d-amphetamine (10 and 20 mg), under double-blind conditions and in randomized order. After obtaining baseline measures, behavioral and physiological measures were recorded at regular intervals over 4 h after capsule administration. Genotyping was performed blind to all behavioral data. A subset of subjects

($N = 101$) also 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 the smaller sample size.

Procedure

Subjects first attended an orientation session to provide informed consent. They practiced behavioral tests and questionnaires, completed a personality questionnaire (data not presented) and gave a 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. Volunteers were allowed to read during the sessions and watch emotionally neutral movies when no measurements were being taken. This study was approved by the Institutional Review Board of The University of Chicago and was carried out in accordance with the Helsinki Declaration of 1975.

Sessions were conducted from 0900 to 1300 h, at least 48 h apart. At the beginning of each session, subjects provided breath samples to confirm their abstinence from nicotine and alcohol and a urine sample that was tested for recent use of marijuana, cocaine, benzoylcegonine, phencyclidine, opiates, methamphetamine, Ecstasy, methadone, amphetamine, barbiturates and benzodiazepines. Subjects testing positive for these substances were excluded from the study. Volunteers completed baseline mood questionnaires of predrug subjective effects. At 0930 h, they ingested a capsule containing either placebo or d-amphetamine (10 or 20 mg) administered with 100 ml of water. Compared with clinically recommended daily doses of up to 40 mg of amphetamine for school-aged children with attention deficit hyperactivity disorder (ADHD) (Greenhill *et al.* 2002; Spencer *et al.* 2006) we used relatively low doses of amphetamine, in part to minimize risk to subjects and in part to enhance our ability to detect genotype-dependant differences. The doses were sufficient to produce measurable subjective and behavioral effects in the participating healthy volunteers. Subjective, behavioral and physiological measures including heart rate and blood pressure were obtained 30, 60, 90, 150 and 180 min after capsule intake.

Dependent measures

To assess subjective drug effects, subjects completed the Addiction Research Center Inventory 49-item questionnaire (ARCI-49) (Martin *et al.* 1971). We examined the association between polymorphisms in *OPRM1* and subjective response to amphetamine using the ARCI-49 in the present study. The ARCI-49 consists of 49 yes–no questions that relate to five factors corresponding to typical effects of psychoactive drugs. It is a sensitive instrument for determining subjective drug effects, and consists of five scales assessing (1) Euphoria (Morphine-Benzedrine Group scale), (2) intellectual Efficacy and Energy (Benzedrine Groups scale; hereafter referred to as Energy), (3) Stimulation (Amphetamine Group scale), (4) Sedation (Pentobarbital-Chlorpromazine-Alcohol Group scale) and (5) Dysphoria (Lysergic Diethylamide scale). The ARCI scales Euphoria, Energy and Stimulation were selected as primary outcome measures to capture the prototypic effects of amphetamine. Sedation was included as secondary outcome measure to examine whether ratings of Sedation decreased when ratings of Euphoria, Energy and Stimulation increased after amphetamine. In order to reduce variability between and within subjects, peak change scores were calculated by subtracting the predrug baseline scores from the measured scores after drug ingestion. The value with the greatest distance from baseline was chosen as peak change score. In case of equal positive and negative maximum distances, 0 was used as peak change score.

Genotyping

Genotyping was performed 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

Table 1: Allele and Genotype frequencies of the *OPRM1* gene polymorphisms

	Chromosome Position	Gene Position	Allele		Genotype			HWE*
			1	2	1/1	1/2	2/2	P-Value
rs1799971A/G [†]	151924380	355	282	40	125	32	4	0.41
rs510769 A/G [†]	151925602	1577	86	238	13	60	89	0.27
rs660756A/C	151979108	54992	206	118	63	80	19	0.53
rs1918760 A/G	152045410	121724	195	129	58	79	25	0.99
rs2281617C/T	152050664	126978	280	44	121	38	3	1.0
rs1998220A/G	152069596	145985	141	183	31	79	52	1.0
rs9371781A/C	152115349	191764	226	98	78	70	14	0.94

*Hardy–Weinberg Equilibrium: *P*-values assessed by Haploview software version 4.0.

[†]Significant associations with methamphetamine-induced psychosis (Ide *et al.* 2006), alcoholism (Schinka *et al.* 2002), heroin addiction (Drakenberg *et al.* 2006), completed suicide (Hishimoto *et al.* 2008) and HPA axis activation (Hernandez-Avila *et al.* 2003; Wand *et al.* 2002) ($p < 0.05$).

[‡]Significant associations with vulnerability to heroin addiction (Levrán *et al.* 2008) ($p < 0.05$).

and anxiety (Hodgkinson *et al.* 2008). Criteria for sample exclusion and classification as genotyping failure were previously described (Hodgkinson *et al.* 2008). Of 162 original subjects, only a single sample was uncalleable at rs1799971; all other genotypes were successfully obtained. Genotyping accuracy was determined based on genotype concordance between DNA replicates (genotype error rate: 0%, 27 replicates). Subjects were genotyped at seven selected polymorphisms in the *OPRM1* gene (Table 1) and were assigned to one of the three genotype groups at five of the seven loci: homozygotes for the first or second allele and heterozygotes. There were only four subjects with genotype G/G at rs1799971 and three subjects with genotype T/T at rs2281617. Thus, participants with genotype G/G and A/G at rs1799971 and subjects with genotype T/T and C/T at rs2281617 were pooled into one group.

A panel of 186 ancestry informative SNPs was selected as previously described (Hodgkinson *et al.* 2008). It was analyzed with Structure 2.1 (Pritchard *et al.* 2000) considering 1051 CEPH reference subjects to confirm the participants' self-reported and experimenter-observed Caucasian designations. Proportions of membership for each subject in clusters corresponding to seven geographic regions (Africa, Europe, Middle East, Central Asia, Far East Asia, America and Oceania) were estimated. Score means were compared between genotype groups at rs2281617, which was associated with amphetamine response.

Haplotype reconstruction

Haplotypes were derived using the program Phase 2.1.1 (stephenslab.uchicago.edu/software.html). Criteria to assess neighboring SNPs together as haplotypes were as follows: pairwise standardized linkage disequilibrium coefficient $D' > 0.95$ between polymorphisms; probability of reconstructed pairs of haplotypes for each individual $D' \geq 0.95$ and haplotype frequency in the sample $N > 10$. On the basis of these criteria, three haplotypes of rs1799971 and rs510769 and four haplotypes of rs1918760, rs2281617 and rs1998220 were identified. The pairwise standardized linkage disequilibrium coefficients were $D' < 0.95$ between the markers rs660756 and rs9371781 and the ones included in the haplotype analysis. Thus, both SNPs were not included in the haplotype analysis. The haplotypes were coded as having two copies (2), as having one copy (1), or as not present (0) in an individual. Hardy–Weinberg equilibrium for each marker and linkage disequilibrium between the markers were analyzed using the Haploview software version 4.0 (<http://www.broad.mit.edu/mpg/haploview/>). Haploview was also used to generate a linkage disequilibrium map of the *OPRM1* gene with the available HapMap data (The International HapMap Genome Browser B36).

Statistical analyses

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

was performed using dose (0, 10 and 20 mg of drug) and time [five time-points after capsule ingestion minus predrug (baseline) scores] as within-subject factors for each dependent measure. Possible confounding variables (age, BMI, gender and baseline responses) were assessed by performing separate two-way analyses of covariance (ANCOVAs) with peak change scores as within-subject factors. A threshold of $P < 0.05$ was used; confounding variables exceeding this threshold were included 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 each outcome measure. Genotype or number of haplotype copies were used as grouping factors and peak change scores for placebo, 10 and 20 mg amphetamine were chosen as within-subjects factors, comprising two-way ANOVAs and ANCOVAs. Assessing drug by genotype and haplotype interactions Lavene's test for equality of error variances was always included in the analyses. Greenhouse–Geisser correction was used when Lavene's test for equality of error variances was significant. To investigate main effects of genotype groups further, and to determine whether differences in drug response were because of placebo or amphetamine, *post hoc* analyses were conducted by performing one-way ANOVAs or ANCOVAs with peak change scores as dependent measures. Alpha was set at $P < 0.05$ (two-tailed) for all analyses. However, because we examined seven SNPs and three primary outcome measures (Euphoria, Energy and Stimulation), a Bonferroni–Holm correction would require a *P*-value of 0.002 for statistical significance. Because both the SNPs and phenotypes are correlated with one another, 0.002 is likely to be overly conservative.

Results

Demographic and genetic characteristics of the subjects

All subjects were of Caucasian origin. They were aged 18–35 years and reported 12–20 years of formal education. Table 2 shows demographic characteristics including current and lifetime substance abuse of the overall sample.

One hundred and sixty-two subjects completed ARCI questionnaires for all three sessions. Genotype and allele frequencies for the investigated SNPs are shown in Table 1. All SNPs were in Hardy–Weinberg equilibrium. Allele frequency proportions did not significantly differ from those given in

Table 2: Demographic characteristics for all subjects

Demographic characteristics	Overall
Overall, <i>n</i>	162
Age [mean (years) ±SEM]	22.8 ± 0.3
Gender (%female)	44.1
BMI (mean (kg/m ²) ±SEM)	22.7 ± 0.2
Current substance use	
Alcohol (mean drinks/week)	4.5 ± 0.3
Cigarettes (mean cigarette/week)	0.8 ± 0.1
Caffeine (mean cups/week)	7.3 ± 0.5
Marijuana (mean times/month)	0.9 ± 0.2
Lifetime substance use	
Stimulants (% ever used)	52.2
Sedatives (% ever used)	6.2
Opiates (% ever used)	21.7
Marijuana (% ever used)	44.1
Hallucinogens (% ever used)	28.6
Inhalants (% ever used)	9.3

Comparisons across genotype groups for all *OPRM1* SNPs were made using one-way ANOVA for continuous data and χ^2 for frequency data (* $P < 0.05$).

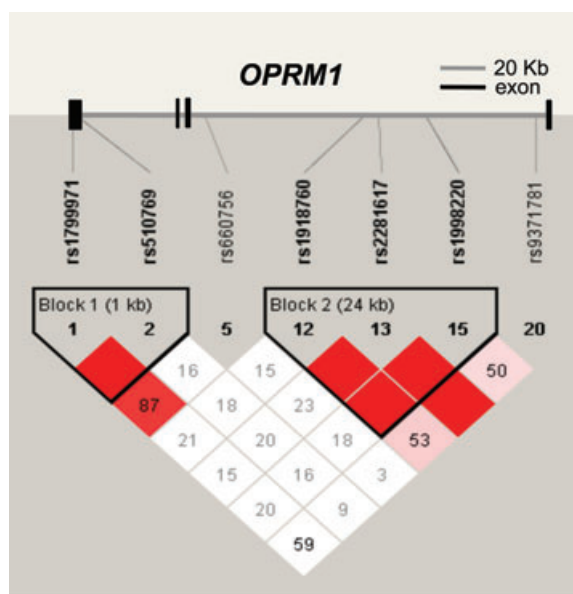


Figure 1: Genomic structure of *OPRM1* gene, mapped to chromosome 6, is shown to scale including four exons spanning 207.6 kb as well as results of linkage disequilibrium analyses: D' values of SNPs along the *OPRM1* gene, illustrating two haplotype blocks. D' values were calculated by Haploview version 4.0.

the Hapmap project (The International HapMap Genome Browser B36). Polymorphisms within each gene were in high intermarker linkage disequilibrium forming two haplotype blocks. Haplotype blocks and D' values between the genetic variants are shown in Fig. 1. Rs1799971 and rs510769 were in high intermarker linkage disequilibrium

well as the SNPs rs1918760, rs2281617 and rs1998220 ($r^2 < 1.0$). These observations were consistent with the HapMap data (The International HapMap Genome Browser B36). Analysis of ancestry informative markers (Structure 2.1.) confirmed self-reported Caucasian origin in all study participants. Caucasian origin was confirmed by score means of the analyzed seven clusters corresponding to seven geographic regions (Africa, Europe, Middle East, Central Asia, Far East Asia, America and Oceania). The genotype groups at locus rs2281617 did not differ in ethnicity, and because all subjects were confirmed to be Caucasian we did not repeat the ethnicity check with other polymorphisms.

Correlation analyses for all demographic information with SNPs and haplotypes were performed to determine whether demographic measures should be included as covariates in statistical analyses. Genotype groups for each SNP and haplotype were similar on most demographic information. However, subjects with genotype C/C at rs660756 reported significantly higher lifetime opioid use ($P < 0.001$, $\chi^2 = 36.60$, $df = 2$) and higher lifetime sedative use ($P < 0.001$, $\chi^2 = 36.60$, $df = 2$). Participants with genotype A/G at rs1799971 reported higher marijuana consumption per month (one-way ANOVA: $P < 0.05$, $F_{2,152} = 3.90$) than people of the other genotype groups. In a separate analysis we found no relationship between lifetime opiate use or marijuana use per month and the outcome measures. Response to placebo did not differ across any of the genotype groups.

Genotype-independent effects of amphetamine

The ARCI scales Euphoria, Energy and Stimulation were selected as primary outcome measures to capture the prototypic drug effects. Sedation was included as secondary outcome measure to examine whether ratings of Sedation decreased when ratings of Euphoria, Energy and Stimulation increased. Amphetamine produced the expected effects on the outcome measures with dose-dependent increases in Euphoria, Energy, Stimulation and decreases in Sedation (drug main effect from two-way ANOVA/ANCOVA, Euphoria: $P < 0.001$, $F_{2,320} = 64.36$, Energy: $P < 0.001$, $F_{2,320} = 60.55$, Stimulation: $P < 0.001$, $F_{2,322} = 83.23$, Sedation: $P < 0.001$, $F_{2,322} = 44.94$). Amphetamine also had dose-dependent effects increasing blood pressure and heart rate (drug main effect from two-way ANOVA/ANCOVA; systolic blood pressure: $P < 0.001$, $F_{2,328} = 159.03$, diastolic blood pressure: $P < 0.001$, $F_{2,326} = 81.28$, heart rate: $P < 0.001$, $F_{2,326} = 40.25$). For most measures the effect of capsule ingestion was significant by 60 min post-administration and peaked between 90 and 120 min post-administration. Males scored significantly higher on Energy than females (main effect of sex from two-way ANOVA: $P < 0.05$, $F_{1,160} = 5.92$). Therefore, sex was used as a covariate and included as a between-subject factor in further analyses. Further demographic measures did not affect the self-reported outcome measures. Baseline scores did not differ between genotype groups.

OPRM1 gene polymorphisms and ARCI scales

To reduce variability within and between subjects we calculated peak change scores within each session for the primary outcome measures Euphoria, Energy, Stimulation

Table 3: Results of ANOVA's for responses to d-amphetamine for individual OPRM1 gene polymorphisms

	rs510769A/G	rs2281617C/T	rs1998220A/G
Euphoria			
F-value (DF)*	2.69 (4)	4.73 (2)	0.89 (4)
P-value	0.031 [†]	0.010 [†]	0.468
<i>Post hoc</i> (10 mg):			
F-value (DF) [‡]	2.59 (2)	15.33 (1)	—
P-value	0.078	0.0001	—
Energy[§]			
F-value (DF)*	1.33 (4)	4.92 (2)	2.91 (4)
P-value	0.257 [†]	0.008 [†]	0.022
<i>Post hoc</i> (10 mg):			
F-value (DF) [‡]	—	12.88 (1)	1.32 (2)
P-value	—	0.0003	0.271
Stimulation			
F-value (DF)*	2.99 (4)	2.28 (2)	2.67 (4)
P-value	0.019	0.104 [†]	0.032 [†]
<i>Post hoc</i> (10 mg):			
F-value (DF) [‡]	4.74 (2)	—	1.35 (2)
P-value	0.010	—	0.263
Sedation			
F-value (DF)*	0.80 (4)	2.76 (2)	0.89 (4)
P-value	0.525	0.065 [†]	0.469
<i>Post hoc</i> (10 mg):			
F-value (DF) [‡]	—	—	—
P-value	—	—	—

Statistical significant p-values (< 0.05) are listed in bold.
 *Drug by genotype interactions: P-values assessed by two-way ANOVA/ANCOVA.
[†]Greenhouse–Geisser correction.
[‡]Post hoc analysis: P-values assessed by one-way ANOVA/ANCOVA (10 mg dose).
[§]Energy adjusted for gender (included as between-subject covariate in statistical analyses).

and the secondary outcome measure Sedation (ARCI). These were calculated by subtracting the predrug baseline scores from the highest or lowest measured scores after drug ingestion. Significant associations between SNPs in OPRM1 and the peak change scores for Euphoria, Energy, Stimulation and Sedation after amphetamine ingestion are reported in Table 3. Genotype A/A of rs510769 was associated with lower Stimulation and Euphoria scores after amphetamine (two-way ANOVA/ANCOVA, Euphoria: $P < 0.05$, $F_{4,318} = 2.69$; Stimulation: $P < 0.05$, $F_{4,318} = 2.99$). Figure 2 shows peak change scores of Euphoria and Stimulation (ARCI) between the three genotype groups of the rs510769 polymorphism. Post hoc comparisons using peak change scores of each session respectively showed that genotype groups scored significantly different on the Stimulation scale in response to the 10-mg dose (one-way ANOVA, $P = 0.01$, $F_{2,162} = 4.74$). A similar trend was apparent for Euphoria (one-way ANOVA, $P < 0.1$, $F_{2,162} = 2.59$) after the 10-mg dose.

Subjects with genotype C/C of rs2281617 felt significantly more Euphoric and Energetic after amphetamine (10 mg) compared to participants with genotypes C/T and T/T (Genotype × Drug interaction on two-way ANOVA/ANCOVA, Euphoria: $P = 0.01$, $F_{2,320} = 4.73$; Energy: $P < 0.01$, $F_{2,316} = 4.92$).

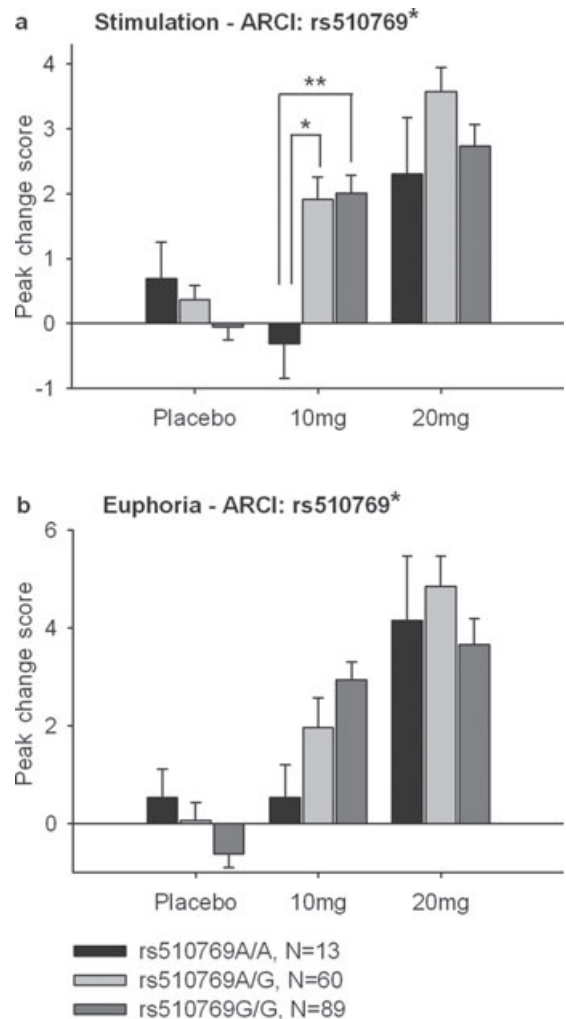


Figure 2: Mean ± SEM peak change scores on (b) Euphoria and (a) Stimulation (ARCI) in the three genotype groups at rs510769 after placebo, 10 and 20 mg of amphetamine. Amphetamine (10 mg) increased Euphoria and Stimulation more in the rs510769A/G and G/G group than in the 510769A/A group. (*Significant drug × genotype interaction, two-way ANOVA, not significant after adjustment for multiple testing. **Post hoc one-way ANOVA multiple comparisons between genotypes with Bonferroni correction $P < 0.05$).

Figure 3 shows peak change scores of Euphoria and Energy (ARCI) between the genotype groups. Post hoc analyses (one-way ANOVAS or ANCOVAS, Euphoria: $P < 0.001$, $F_{1,163} = 15.33$; Energy: $P < 0.001$, $F_{1,163} = 12.88$) showed that genotype groups significantly differed in Euphoria and Energy after 10 mg amphetamine. To clarify the source of peak change scores, time–courses of Euphoria, Energy, Stimulation and Sedation are descriptively shown in Fig. 4 as changes from baseline for the genotype groups after the 10-mg dose. Locus rs1998220 was in strong linkage disequilibrium with rs2281617 ($D' = 1.0$) and showed to be also significantly associated with response to amphetamine. Subjects with

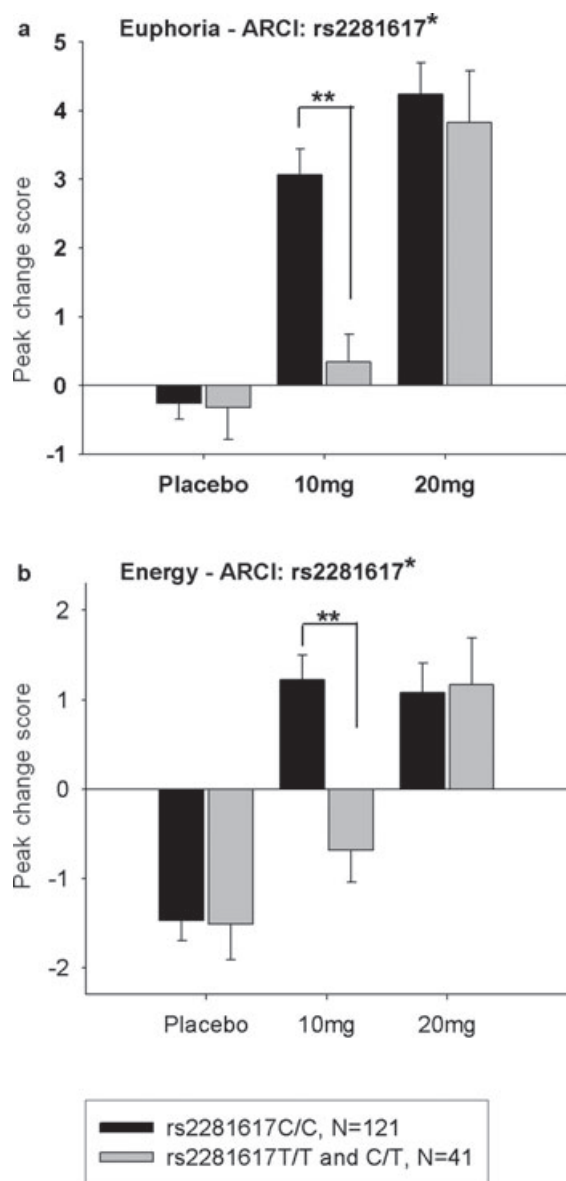


Figure 3: Mean ± SEM peak change scores on (a) Euphoria and (b) Energy (ARCI) in the two genotype groups at rs2281617 (C/C: N = 121; C/T and T/T: N = 41) after placebo, 10 and 20 mg of amphetamine. After 10 mg of amphetamine (10 mg) the rs2281617C/C group reported greater increases in Euphoria, Energy and Stimulation and lower levels of Sedation compared to the 2281617C/T and T/T group. (*Significant drug × genotype interaction, two-way ANOVA, not significant after adjustment for multiple testing. **Post hoc one-way ANOVA multiple comparisons between genotypes with Bonferroni correction $P < 0.05$).

genotype A/A at rs1998220 had significantly higher Energy levels and felt more stimulated than subjects with alleles A/G or G/G at rs1998220 (Genotype × Drug interaction on two-way ANOVA/ANCOVA, Energy: $P < 0.05$, $F_{4,312} = 2.91$;

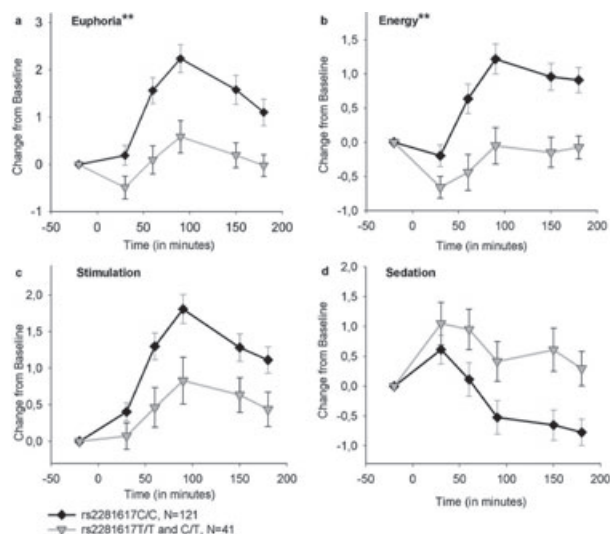


Figure 4: Time-courses of (a) Euphoria, (b) Energy, (c) Stimulation and (d) Sedation (ARCI) after 10 mg of amphetamine administration for the rs2281617 groups. The rs2281617C/C group reported a significantly greater increase in Euphoria and Energy compared to the T/T and C/T group in *post hoc* analyses (one-way ANOVA/ANCOVA). *Post hoc* analyses were not carried out for Stimulation and Sedation, as these scales were not significantly associated with rs2281617 genotype groups in primary analyses (two-way ANOVA/ANCOVA). Data are mean (SEM) ratings as change from predrug baseline. The groups did not significantly differ on baseline scores (** $P < 0.01$).

Stimulation: $P < 0.05$, $F_{4,318} = 2.67$), but there was no significant association between rs1998220 and Energy in *Post hoc* analyses (one-way ANOVA/ANCOVA). If we used a Bonferroni–Holm correction for the number of statistical tests performed, none of the results would remain significant.

There were no significant Genotype × Drug interactions on the outcome measures for the genotype groups at rs1799971, rs660756, rs1918760 and rs9371781. No significant associations were found between any of the seven investigated SNPs and placebo or the 20-mg amphetamine condition.

OPRM1 haplotypes and ARCI scales

Linkage disequilibrium (LD) measures (D' values) between the seven investigated polymorphisms are shown in Fig. 1 (Haploview software version 4.0). Because high pairwise LD measures ($D' = 1.0$) were found between rs1799971 and rs510769 as well as between rs1918760, rs2281617 and rs1998220, haplotype pairs from the linked SNPs were estimated for each individual. The probability of all haplotype estimates was ≥ 0.99 , except for one subject, that had an undetermined genotype at locus rs1799971. This subject was excluded from the relevant haplotype analyses. Seven reconstructed haplotypes were assessed for association with the ARCI scales by performing a two-way ANOVA/ANCOVA using peak change scores for Euphoria, Energy, Stimulation

Table 4: Association of ARCI scores Euphoria, Energy, Stimulation and Sedation after amphetamine with OPRM1 gene haplotypes

	Haplotype rs1799971-A rs510769-G	Haplotype rs1799971-A rs510769-A	Haplotype rs1799971-G rs510769-G	Haplotype rs1918760-A rs2281617-C rs1998820-G	Haplotype rs1918760-A rs2281617-C rs1998220-A	Haplotype rs1918760-A rs2281617-T rs1998220-A	Haplotype rs1918760-G rs2281617-C rs1998220-G
Frequencies							
Number of copies	2: N = 60 1: N = 74 0: N = 26	2: N = 13 1: N = 59 0: N = 88	2: N = 4 1: N = 32 0: N = 124	2: N = 0 1: N = 12 0: N = 149	2: N = 30 1: N = 80 0: N = 51	2: N = 3 1: N = 38 0: N = 120	2: N = 23 1: N = 78 0: N = 60
Euphoria							
F-value (DF)*	2.23 (4)	3.34 (4)	0.21 (4)	0.03 (2)	1.62 (4)	3.47 (4)	0.68 (4)
P-value	0.066	0.012[†]	0.932	0.970	0.169	0.009[†]	0.610
Post hoc (10 mg):							
F-value (DF) [‡]	—	3.26 (2)	—	—	—	8.17 (2)	—
P-value	—	0.041	—	—	—	0.000	—
Energy [§]							
F-value (DF)*	1.50 (4)	1.41 (4)	0.56 (4)	0.15 (2)	0.54 (4)	3.18 (4)	2.78 (4)
P-value	0.201	0.230	0.691	0.859	0.709	0.014	0.027
Post hoc (10 mg):							
F-value (DF) [‡]	—	—	—	—	—	7.01 (2)	2.21 (2)
P-value	—	—	—	—	—	0.001	0.113
Stimulation							
F-value (DF)*	2.90 (4)	3.38 (4)	0.73 (4)	0.25 (2)	1.52 (4)	2.34 (4)	1.73 (4)
P-value	0.022	0.010	0.571	0.781	0.195	0.054 [†]	0.142
Post hoc (10 mg):							
F-value (DF) [‡]	5.36 (2)	4.76 (2)	—	—	—	—	—
P-value	0.006	0.010	—	—	—	—	—
Sedation							
F-value (DF)*	0.88 (4)	0.92 (4)	0.48 (4)	0.14 (2)	0.44 (4)	0.48 (4)	1.56 (4)
P-value	0.484	0.450	0.751	0.874	0.780	0.060 [†]	0.184 [†]
Post hoc (10 mg):							
F-value (DF) [‡]	—	—	—	—	—	—	—
P-value	—	—	—	—	—	—	—

*Drug by genotype interactions: P-values assessed by two-way ANOVA/ANCOVA.

[†]Greenhouse–Geisser correction.

[‡]Post hoc analysis: P-values assessed by one-way ANOVA/ANCOVA (10 mg dose).

[§]Energy adjusted for gender (included as between-subject covariate in statistical analyses).

and Sedation as within-subject factors. Post hoc analyses were carried out conducting one-way ANOVAS/ANCOVAS using peak change scores for each session. Results are shown in Table 4. Group peak change score means and standard error of means (SEM) for the haplotype groups after the 10-mg dose are shown in Table S1.

Haplotype AA from rs1799971 and rs510769 was found to be significantly associated with levels of Euphoria and Stimulation (two-way ANOVA, Euphoria: $P < 0.05$, $F_{4,310} = 3.34$; Stimulation: $P = 0.01$, $F_{4,310} = 3.38$) and haplotype AG from rs1799971 and rs510769 was associated with Stimulation scores (two-way ANOVA, $P < 0.05$, $F_{4,310} = 2.90$). Both of these findings were confined to the 10-mg dose condition (one-way ANOVA, $P < 0.05$, $F_{2,157} = 5.36$). Subjects with zero copies of haplotype AG scored lower on the associated ARCI scale than subjects with one or two haplotype copies. Participants with two copies of haplotype AA felt less euphoric and stimulated after 10 mg of amphetamine than subjects with one or zero haplotype copies (one-way ANOVA, Euphoria: $P < 0.05$, $F_{2,157} = 3.26$; Stimulation: $P = 0.01$, $F_{2,157} = 4.76$).

Haplotype ATA rs1918760, rs2281617 and rs1998220 was significantly associated with Euphoria and Energy (two-way ANOVA/ANCOVA, Euphoria: $P < 0.01$, $F_{4,312} = 3.47$; Energy: $P < 0.05$, $F_{4,312} = 3.18$) after the 10-mg dose (one-way ANOVA/ANCOVA, Euphoria: $P < 0.001$, $F_{2,158} = 8.17$; Energy: $P = 0.001$, $F_{2,155} = 7.01$). People having zero copies of haplotype ATA from rs1918760, rs2281617 and rs1998220 felt significantly more energetic and euphoric after 10 mg amphetamine than participants with one or two copies. Along the same lines the haplotype GCG was significantly associated with increased Energy (two-way ANCOVA, $P < 0.05$, $F_{4,306} = 2.78$) after 10 mg amphetamine; however, this effect was not significant in any of the post hoc analyses.

There were few associations between the haplotypes and baseline scores. Haplotype ATA groups from rs1918760, rs2281617 and rs1998220 differed at baseline on the Euphoria and Stimulation scales (two-way ANOVA, Euphoria: $P < 0.05$, $F_{4,312} = 2.69$; Stimulation: $P < 0.01$, $F_{4,312} = 3.77$). Subjects with two copies of haplotype ATA scored higher on Euphoria and Stimulation at baseline at the 10-mg session,

which was significant in *post hoc* analyses for Euphoria only (one-way ANOVA, $P < 0.05$, $F_{2,158} = 3.37$). Haplotype GCG groups from rs1918760, rs2281617 and rs1998220 differed at baseline on the Sedation scale (two-way ANOVAs, $P < 0.05$, $F_{4,312} = 2.85$). Subjects with two copies of haplotype GCG appeared to score higher on Sedation at baseline of the 10- and 20-mg amphetamine session. However, these differences were not significant in *post hoc* analyses.

OPRM1 gene polymorphisms, haplotypes and physiological measures

Association analyses were performed between peak change scores of heart rate, systolic and diastolic blood pressure at the different doses and the investigated SNPs and haplotypes (two-way ANOVA/ANCOVA). Because subjects with a higher BMI had significantly higher diastolic blood pressure BMI was included as a covariate in analyses involving this measure.

The SNP rs2281617 and one haplotype were associated with some of the physiological measures (Table S2). Specifically, genotype C/C of rs2281617 was associated with higher diastolic blood pressure after 10 mg amphetamine (two-way ANCOVA, $P < 0.05$, $F_{2,310} = 3.84$, *post hoc* analysis: one-way ANCOVA, 10 mg: $P < 0.05$, $F_{1,159} = 5.76$). Increases in diastolic blood pressure after 10 mg amphetamine were also significantly associated with haplotype ATA from rs1918760, rs2281617 and rs1998220 (two-way ANCOVA, $P < 0.05$, $F_{4,298} = 3.07$; *post hoc* analysis: one-way ANCOVA, 10 mg: $P < 0.05$, $F_{2,153} = 3.45$) and haplotype GCG (two-way ANCOVA, $P = 0.05$, $F_{4,296} = 3.27$, *post hoc* analysis: one-way ANCOVA, 10 mg, $P < 0.05$, $F_{2,153} = 4.58$). There was a significant Drug \times Genotype interaction between haplotype AA from rs1799971 and rs510769 and diastolic blood pressure (two-way ANOVA, $P < 0.05$, $F_{2,296} = 2.89$), but *post hoc* analyses showed that haplotype groups significantly differed after placebo only (one-way ANOVA, $p < 0.05$, $F_{2,155} = 3.79$). Other polymorphisms and haplotypes did not differ at baseline, after placebo, 10 mg or 20 mg dose for any of the investigated physiological measures.

Discussion

The main finding of the study is that subjects with genotype C/C at rs2281617 scored significantly higher on Euphoria and Energy after 10 mg amphetamine than subjects with genotypes C/T and T/T, and trends were also observed with measures of Stimulation and (lower) Sedation. Volunteers with genotypes A/G and G/G at rs510769 felt significantly more stimulated and euphoric after 10 mg amphetamine than participants with genotype A/A at rs510769; this SNP is in strong LD with the functional exonic variant rs1799971. These primary findings were extended in the haplotype analysis (Table 3). Haplotypes AA and AG from rs1799971 and rs510769 as well as haplotypes ATA and GCG from rs1918760, rs2281617 and rs1998220 were related to feelings of Euphoria, Energy and Stimulation in the expected directions (Table 3). But, as noted previously, none of these results would remain significant if they were adjusted for multiple testing.

Interestingly, we also found several associations between genotypes and blood pressure responses. First, the associated genotype C/C at locus rs2281617 and haplotype ATA from rs1918760, rs2281617 and rs1998220 were significantly associated with increases in diastolic blood pressure after 10 mg (Table S2). Participants with the C/C genotype at rs2281617 or ATA haplotype exhibited greater blood pressure responses after amphetamine than other genotypic groups, which was consistent with the associations observed with subjective responses. Second, we found a significant interaction between haplotype AA from rs1799971 and rs510769 and diastolic blood pressure on the placebo session only. Subjects with two haplotype copies had the lowest diastolic blood pressure, whereas subjects with one haplotype copy had the highest and subjects with zero copies were in between. These modest associations with amphetamine-induced changes in blood pressure, and possibly in the drug-free state, suggest that opioid mechanisms may also be involved in the cardiovascular response to amphetamine.

The present results add to a growing literature on the genetic influences of *OPRM1* on differences in sensitivity or rewarding effects of different drugs (Ide *et al.* 2006; Levran *et al.* 2008; Ray *et al.* 2004; Schinka *et al.* 2002). Our findings have implications for individual differences in susceptibility to substance abuse. In studies with rodents, individual differences in sensitivity to stimulant drugs are predictive of future self-administration (Piazza *et al.* 1989). Adolescents who report positive reactions to early use of cannabis are at increased risk of later cannabis dependence (Fergusson *et al.* 2003) and drug users report that their initial subjective responses to psychostimulants and opioids predicted their future use (Haertzen *et al.* 1983). It is possible that people carrying the common genotype C/C at rs2281617 or genotypes A/G and G/G at rs510769 might be more likely to progress to excessive use of amphetamine because they experience more positive effects from the drug. Recent studies have shown that the opioid receptor antagonist naltrexone attenuates reinstatement of amphetamine drug-seeking in the rat and Jayaram-Lindström *et al.* (2004a,b) showed the efficacy of naltrexone in reducing amphetamine use in amphetamine-dependent humans in placebo-controlled clinical trials. Our finding showing that *OPRM1* significantly influences response to amphetamine suggests that opioid antagonists may be of value in the treatment of stimulant addiction. However, we found that *OPRM1* polymorphisms only influenced response to the lower 10-mg dose and not for the 20-mg dose, while drug abusers typically use high doses of drug. We previously reported a similar finding (Veenstra-VanderWeele *et al.* 2006), in which associations with subjective responses to amphetamine were observed for the lower dose (10 mg) but not the higher dose (20 mg). This suggests that certain 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.

It is not clear whether rs2281617 itself alters the regulation of *OPRM1* by modulating gene transcription, mRNA stability, processing and splicing or whether it is instead in strong LD with some other functional variant, for example any 3' functional variant that regulates gene transcription rate.

Rs2281617 is located in transcription-factor binding sites, according to the TRANSFAC program providing matrix families the identified transcription-factor binding sites belong to (MatInspector; Genomatix Software version 2.0, <http://www.Genomatix.de/index.html>). The analyses showed that subjects with the associated C allele at rs2281617 have additional predicted transcription-factor binding sites from the matrix families V\$Hand (Twist subfamily of class B bHLH transcription factors), V\$ZBPF (Zinc binding protein factors) and V\$GLIF (GLI zinc finger family) compared to subjects carrying the T allele. Matrix families V\$ZBPF and V\$GLIF are found in the central nervous system. However, to our knowledge in the literature, there are no findings about the families of the predicted binding sites that might modulate and enhance transcription rate.

One SNP in our study, rs1799971, is known to cause an amino acid substitution in *OPRM1*. This SNP was previously reported to result in an increased beta-endorphin binding density and in an impairment of receptor signaling (Befort *et al.* 2001; Mague *et al.* 2009). We did not find any significant interaction between the rs1799971 genotype groups and amphetamine response. However, our subjects with genotype G/G ($N = 4$) and A/G ($N = 32$) were pooled into one group, whereas only subjects with genotype G/G are likely to differ in their response to amphetamine compared to participants that are heterozygous or homozygous for the A allele. Genotype G/G at rs1799971 was in strong LD with genotype A/A at rs510769 in our sample ($D' = 1.0$). Thus, significant effects of genotype A/A could reflect the influence of the highly linked but less frequent functional rs1799971G/G genotype. Genotype alleles A/A at rs510769 were associated with a lower response to amphetamine compared to both heterozygous A/G and homozygous G/G groups, who reported very similar drug effects (Fig. 2). Overall, Euphoria and Stimulation peak change scores of only G/G subjects ($N = 4$) at rs1799971 were lower than peak change scores of A/G and A/A subjects, suggesting that genotype G/G at rs1799971 might have an influence on amphetamine response similar to genotype A/A at rs510769. Zhang *et al.* (2005) found the G allele at rs1799971 to have deleterious effects on both mRNA and protein yield in an experimental environment and the rs1799971G variant has been found to be associated with decreased potency of opioids such as morphine, its active metabolite morphine-6-glucuronide and alfentanil (Caraco *et al.* 2001; Lötsch *et al.* 2002; Romberg *et al.* 2003, 2005; Skarke *et al.* 2003). In addition, humans carrying the G allele at rs1799971 were found to have decreased clinical responses to the OPRM1 agonist levomethadone (Lötsch *et al.* 2006); in their analysis, the association was only statistically significant when subjects homozygous for the G allele were included as a separate group. Thus, the effect of the G/G genotype needs to be examined in larger samples. As only subjective primary outcome measures were included in this study, imaging studies would be helpful to further elucidate how and in which brain regions the investigated genetic variations modulate response to amphetamine.

There were minor differences in the demographic characteristics of some of the genotypic groups in our study. People with genotypes G/G and A/G of rs1799971 reported significantly higher marijuana consumption per month than

subjects with genotype A/A, and people with genotype C/C allele of rs660756 reported higher lifetime opioid and sedative use compared to subjects with genotypes A/A and A/C. It is unlikely that these differences are related to our primary findings. Separate correlation analyses showed that habitual use of marijuana, opioids and sedatives was not related to responses to amphetamine in the entire sample. Locus rs660756 was not in high LD with any of the other investigated markers ($D' < 0.21$), was not associated with any of the outcome measures and not included in any haplotype analysis. Finally, both of these apparent correlations were of marginal significance.

An important consideration in this and related drug challenge studies concerns the problem of multiple testing. Because we tested several specific hypotheses about the effects of genetic polymorphisms (Dlugos *et al.* 2009, 2010) using this same sample, the most stringent approach would be to set a criteria for significance that limits the chance of a single false-positive to 5% in any past, present or future study. However, when sample size and thus the corresponding power is limited, such a stringent threshold for significance greatly increases the likelihood of false-negative (type 2) errors, and reduces the likelihood of detecting a real effect. Because we have not used such stringent criteria, our results must be interpreted with appropriate caution until confirmed with larger numbers of subjects. However, despite the limitations related to sample size, we believe that the detailed and well-controlled phenotypes collected in this study are extremely valuable for elucidating genetic factors related to individual differences in drug responses.

In summary, our study provides support that genetic variations of the *OPRM1* are related to quantitative amphetamine response in healthy human volunteers. This finding elucidates possible reasons for inter-individual susceptibility becoming addicted to the drug and potency of opioid antagonists as pharmacological targets in amphetamine treatment.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Mean peak change ARCI scores (SEM) of Euphoria, Energy, Stimulation and Sedation after 10 mg amphetamine for OPRM1 gene haplotype groups.

Table S2: Results of ANOVA of blood pressure and heart rate with the significantly associated rs2281617 and two haplotypes.

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