

Further evidence of association between amphetamine response and *SLC6A2* gene variants

Andrea M. Dlugos · Ajna Hamidovic ·
Abraham A. Palmer · Harriet de Wit

Received: 15 March 2009 / Accepted: 17 July 2009
© Springer-Verlag 2009

Abstract

Background and rationale We previously found that the intronic norepinephrine transporter gene (*SLC6A2*) polymorphism rs36017 modulates feelings of elation after administration of 20 mg D-amphetamine in healthy volunteers.

Objectives In this study, we further investigated the association between D-amphetamine response and 11 *SLC6A2* single-nucleotide polymorphisms (SNPs), including rs36017, in an extended sample of Caucasian young adults. **Methods** One hundred fifty-nine healthy volunteers participated in a three-session double-blind crossover design receiving either placebo or oral D-amphetamine (10 and 20 mg). Based on our previous results, we examined the associations between levels of self-reported elation and vigor after D-amphetamine administration and SNPs and SNP haplotypes in *SLC6A2*.

Results Consistent with our previous findings, SNPs rs36017 and rs1861647 were associated with significantly higher ratings of elation and vigor after 20 mg D-amphetamine. Ratings of vigor after 20 mg D-amphetamine were also associated with a two-SNP haplotype formed with rs1861647 and rs5569 and a three-SNP haplotype formed with rs36017, rs10521329, and rs3785155.

Conclusions These results provide further evidence that genetic variants in the *SLC6A2* gene are involved in acute

response to D-amphetamine, which may influence progression to amphetamine abuse. Identifying sources of variation in acute drug responses could lead to better prevention and treatment of psychostimulant abuse and may be valuable in the therapeutic use of stimulants.

Keywords Amphetamine · Norepinephrine · Transporter · Gene · Reward · Human

Introduction

Amphetamine is a psychoactive drug that increases attention, alertness, and cognitive performance (Brauer and de Wit 1996). It is used clinically for treatment of chronic fatigue syndrome, narcolepsy, and attention-deficit/hyperactivity disorder (ADHD). However, it is also one of the most abused substances in the world. Individuals are known to vary in their responses to the drug; some people report adverse effects such as anxiety or panic attacks after amphetamine intake (de Wit et al. 1986; Williamson et al. 1997). The sources of these interindividual differences in amphetamine response are partially genetic in origin. Monozygotic twins have a higher concordance in subjective response to D-amphetamine compared to dizygotic twins, showing that subjective drug response is a heritable genetic trait (Crabbe et al. 1983; Nurnberger et al. 1982). We and other authors have identified specific genes and polymorphisms that influence response to D-amphetamine in healthy human volunteers (Comings et al. 1997; Kirley et al. 2003; Lott et al. 2005).

Amphetamine produces its effects primarily by inducing release and inhibiting reuptake of norepinephrine and dopamine from the synaptic cleft at their respective presynaptic transporter proteins (Cheng and Wooten 1982;

A. M. Dlugos · A. Hamidovic · A. A. Palmer · H. de Wit (✉)
Department of Psychiatry and Behavioral Neuroscience,
The University of Chicago,
5841 South Maryland Avenue,
MC3077 Chicago, IL, USA
e-mail: hdew@uchicago.edu

A. A. Palmer
Department of Human Genetics, The University of Chicago,
Chicago, IL, USA

Gainetdinov et al. 1999; Taylor and Ho 1978). One of these transporter proteins is the Na^+/Cl^- -dependent norepinephrine transporter that removes mostly norepinephrine, but also dopamine, from the synaptic cleft (Horn 1973; Raiteri et al. 1977).

Norepinephrine is involved in many aspects of mood, alertness, and behavior. Tricyclic antidepressants exert their therapeutic actions in part by inhibiting reuptake of norepinephrine and increasing noradrenergic metabolite levels (Leonard 1997; Ressler and Nemeroff 1999). Norepinephrine plays a critical role in response to stress and maintaining attention and vigilance; dysregulation of this system is thought to contribute to ADHD, posttraumatic stress disorder, and anorexia nervosa (Biederman and Spencer 1999; Heim and Nemeroff 2001; Ricca et al. 1999). Several polymorphisms in the noncoding and coding regions of *SLC6A2* have been studied and found to modulate individual differences in behavior, mood, and drug responses (Brookes et al. 2006; Inoue et al. 2004; Kim et al. 2006, 2008). We previously reported that two highly linked ($D'=1.0$, $r^2=1.0$) *SLC6A2* polymorphisms were associated with subjective response to acute D-amphetamine in a sample of 56 Caucasian healthy volunteers (Dlugos et al. 2007). In the present study, we extended the Caucasian subsample to 159 Caucasian individuals. We examined the relationship between nine new *SLC6A2* gene polymorphisms and two of the previously investigated polymorphisms (rs36017 and rs239771). Some of the genetic variants investigated in this study have been previously found to be associated with either drug response or other psychiatric traits. rs5569 has been implicated in the response to methamphetamine (Yang et al. 2004). rs239771 has been associated with panic disorder without agoraphobia (Lee et al. 2005). rs3785155 was found to influence performance task phenotypes in ADHD children and families (Kollins et al. 2008). Finally, rs3785143 was associated with the etiology of ADHD in an International Multi-centre ADHD Genetics project (Brookes et al. 2006; Kim et al. 2008).

In our previous study (Dlugos et al. 2007), genotype groups at *SLC6A2* locus rs36017 differed on the composite scale positive mood (positive mood=elation–depression; Profile of Mood States [POMS]; Johanson and Uhlenhuth 1980; McNair et al. 1971) after 20 mg D-amphetamine in 56 healthy volunteers. Post hoc analyses revealed that this association was due to the elation subcomponent of positive mood (Dlugos et al. 2007). Therefore, we chose the elation scale as the primary outcome measure to extend the findings in larger sample. We chose the vigor scale (POMS) to investigate another prototypic effect of amphetamine known to be affected by norepinephrine (Verhoeff et al. 2003). We focused on locus rs36017 to detect genotype-dependent differences in amphetamine response and per-

formed exploratory analyses for ten additional polymorphisms. We hypothesized that acute behavioral and euphorogenic subjective responses to D-amphetamine would be associated with rs36017, as in our previous study.

Materials and methods

The sample consisted of 162 (male=90 and female=72) volunteers, aged 18–35 years. All subjects were of self-reported Caucasian origin, which was confirmed using genotypes as described below. This sample included the 56 Caucasians reported previously (Dlugos et al. 2007). Volunteers were excluded from participation if their body mass index (BMI) was less than 18 or greater than 26, if they consumed more than three cups of coffee a day or smoked more than ten cigarettes a week, if they had less than a high school education or lack of fluency in English. They were also excluded if they had any serious medical condition, current axis I psychiatric disorder including substance abuse or dependence (DSM-IV; American Psychiatric Association 1994) or any current or past medical condition that was considered to be a contraindication for amphetamine administration. Women were tested in their follicular phase of the menstrual cycle (White et al. 2002). Further details on screening procedures are described in Dlugos et al. (2007).

Design

This 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 D-amphetamine were recorded over 4 h after drug administration. Subjects were genotyped after they had completed the behavioral phase of the study. Genotyping was performed blind to behavioral data.

Procedure

Subjects first attended an orientation session to provide informed consent. They practiced 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. 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 9:00 A.M. to 1:00 P.M., at least 48 h apart. At the beginning of each session, subjects provided breath and urine samples to confirm their drug and alcohol abstinence. Volunteers completed measures and baseline mood questionnaires of predrug subjective effects. At 9:30 A.M., subjects ingested a capsule containing either placebo or D-amphetamine (10 or 20 mg). The clinically recommended daily doses of D-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 POMS (Johanson and Uhlenhuth 1980; McNair et al. 1971). In this study, we focused on the POMS to assess genotype-dependent effects of amphetamine. The POMS indicates current subjective drug effects and is highly sensitive to the effects of drugs in samples of healthy volunteers. This version of the POMS consists of 72 adjectives describing 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). We calculated peak change scores by subtracting the predrug baseline scores from the highest or lowest value after baseline. In case of equal positive and negative maximum values, 0 was used as peak change score. Elation and vigor scales were chosen as primary outcome measures based on our previous findings (Dlugos et al. 2007). Of 162 original Caucasian study

participants, 159 subjects completed the POMS for the investigated outcome measures in all sessions and were included in statistical analyses.

Selection of polymorphisms and genotyping

Eleven *SLC6A2* single-nucleotide polymorphisms (SNPs) were selected and genotyped (Fig. 1) using the Addictions Array (Hodgkinson et al. 2008). The Addictions Array was designed to provide 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). It included 130 genes assessed with Illumina GoldenGate genotyping protocols on 96-well format Sentrix arrays. Arrays were imaged using an Illumina Beadstation GX500 and the data analyzed using GenCall v6.2.0.4 and GTS Reports software v5.1.2.0 (Illumina). Criteria for sample exclusion and classification as genotyping failure were previously described (Hodgkinson et al. 2008). Of 159 participants included in the analyses, genotype was undetermined for a single subject at a single SNP (rs1861647); the genotyping error rate was <1% based on concordance between duplicate samples. Subjects were genotyped at 11 polymorphisms in the *SLC6A2* gene and were assigned to one of three genotype groups: homozygotes for the first or second allele and heterozygotes. Because there were only two subjects with the T/T genotype at rs3785143, four subjects with the T/T genotype at rs3785152, and two subjects with the A/A genotype at rs10521329, these genotype groups were combined with the subjects heterozygous at the respective loci.

A panel of 186 ancestry-informative SNPs was selected as previously described (Hodgkinson et al. 2008). Proportions of membership for each subject in seven clusters corresponding to seven geographic regions (Africa, Europe, Middle East, Central Asia, Far East Asia, America, and Oceania) were estimated. Structure 2.1 (Pritchard et al. 2000) was used for analyses considering 1,051 Centre d'Etude du Polymorphisme Humain subjects as reference to confirm self-reported and experimenter-observed Caucasian designations for study participants.

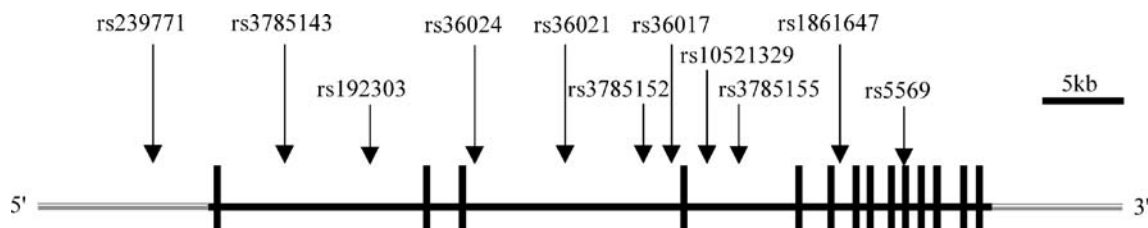


Fig. 1 Genomic structure of *SLC6A2* gene, mapped to chromosome 16, 54,248,057–54,295,199, is shown to scale including 14 exons spanning 47.14 kb. Gene surrounding area is indicated by gray color. The 11 polymorphisms genotyped in this study are indicated with arrows

Statistical analyses

Because gene–drug interactions are not always systematically related to dose of the drug (Hohoff et al. 2005; Lott et al. 2005; Veenstra-VanderWeele et al. 2006), we examined gene–drug interactions with 10 and 20 mg D-amphetamine in separate analyses. To assess genotype-independent main effects of placebo and D-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 the dependent measure. Possible confounding variables (age, BMI, gender, and baseline responses) were assessed by performing separate two-way analysis of covariance (ANCOVA) with peak change scores as within-subject factors. A p value of <0.05 was set as a threshold for the 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 chi-square tests. Considering that multiple demographic characteristics were tested (Table 2), a p value of <0.01 was set as a threshold for significant demographic differences between genotype groups.

To analyze associations between genotypes and drug response, 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 and 10 and 20 mg D-amphetamine were within-subjects factors, comprising two-way ANOVAs and ANCOVAs. Lavenne's test for equality of error variances was included in drug by genotype or drug by haplotype analyses.

Greenhouse–Geisser correction was used when Lavenne's test for equality of error variances was significant. 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.

Haplotype analyses

Hardy–Weinberg equilibrium for each marker and linkage disequilibrium (LD) between the markers were analyzed using the Haploview software version 4.1 (<http://www.broad.mit.edu/mpg/haploview/>). Haploview was also used to generate a LD map of *SLC6A2* with the data of our sample and the available HapMap data (The International HapMap Genome Browser B36). LD parameters between SNPs in our sample were generally similar to those observed in the CEU HapMap samples. Haplotype blocks identified by the Haploview software using data from our sample were used for our haplotype analyses. Correlation analyses between haplotypes and ratings of vigor and elation were performed using PLINK. Empirical p values were calculated and 5,000 permutations were specified and performed for each haplotype to calculate empirical p values adjusted for testing more than one haplotype.

Results

Subjects

One hundred fifty-nine subjects completed the POMS questionnaires for all three sessions. All investigated SNPs were in Hardy–Weinberg equilibrium. Allele and genotype frequencies for the investigated SNPs are shown in Table 1.

Table 1 Allele and genotype frequencies of the *SLC6A2* polymorphisms

SNP ID	Allele		Genotype			HWE p value	
	1	2	1/1	1/2	2/2		
rs2397771 (C/G)	193	125	54	85	20	0.19	
rs3785143 (C/T)	282	36	125	32	2	1.00	
rs192303 (C/G)	82	236	13	56	90	0.40	
rs36024 (C/T)	183	135	54	75	30	0.75	
rs36021 (A/T)	128	190	27	74	58	0.78	
rs3785152 (C/T)	279	39	124	31	4	0.38	
rs36017 (C/G)	164	154	47	70	42	0.17	
rs10521329 (A/C)	58	260	2	54	103	0.13	
rs3785155 (A/G)	32	286	0	32	127	0.35	
<i>HWE</i> Hardy–Weinberg equilibrium (p values were assessed by Haploview software version 4)	rs1861647 (A/G)	114	202	19	76	63	0.75
	rs5569 (C/T)	222	96	76	70	13	0.75

Allele frequency proportions did not significantly differ from the ones given in the HapMap project (The International HapMap Genome Browser, CEU population, B36). Consistent with the HapMap data, rs2397771 and rs3785143 were in high intermarker LD, as were the SNPs rs36017, rs10521329, and rs3785155 and the SNPs rs1861647 and rs5569 (Fig. 4). Analysis of ancestry-informative markers (Structure 2.1.) confirmed self-reported Caucasian origin in all study participants.

Genotype groups for the SNPs rs239771, rs192303, rs36021, rs3785152, rs36017, rs10521329, rs3785155, rs1861647, and rs5569 did not differ on any demographic characteristics ($p > 0.01$). However, the genotype groups for SNP rs3785143 significantly differed for lifetime marijuana use (C/C, 80.3%; C/T and T/T, 19.7%; $\chi^2 = 0.005$). rs36024 genotype groups differed on weekly caffeine use [means (\pm SD): C/C, 5.70 (\pm 6.9); C/T, 10.03 (\pm 8.2); T/T, 5.14 (\pm 4.2); one-way ANOVA: $p = 0.005$]. In separate analyses, we found no relationship between the demographic measures and the outcome measures except that gender influenced levels of elation. Gender was included as a between-subject factor in the analyses related to elation. Prior stimulant use was not correlated with vigor and elation scores after amphetamine and did not contribute to the results. The rigorous exclusion criteria (above) limited the variability of demographic factors in the sample (Table 2).

Table 2 Demographic characteristics of the overall sample

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

Comparisons across genotype groups for all *SLC6A2* SNPs were made using one-way ANOVA for continuous data and chi-square test for frequency data

Genotype-independent effects of D-amphetamine

D-Amphetamine produced the expected effects on the two primary outcome measures with dose-dependent increases in elation and vigor ($p < 0.001$, drug main effect from two-way ANOVA). These effects were significant 60 min post administration and peaked between 90 and 120 min. Males scored significantly higher on elation than females (main effect of sex: $p = 0.03$). Therefore, sex was used as a covariate and included as a between-subject factor in further analyses. Baseline scores differed between genotype groups at rs3785152 ($p < 0.05$). There were no genotype group differences on baseline scores for the other investigated SNPs.

SLC6A2 gene polymorphisms and the POMS scales elation and vigor

Several significant associations between the SNPs in *SLC6A2* and the POMS scales elation and vigor after drug administration were observed (Table 3). Subjects with genotype A/A at rs1861647 scored higher on elation and vigor after drug administration (genotype \times drug interaction on two-way ANOVA/ANCOVA). As in the previous study, subjects carrying the C/C allele of SNP rs36017 scored significantly higher on vigor after amphetamine, compared to subjects with other genotypes (genotype \times drug interaction on two-way ANOVA/ANCOVA). Figures 2 and 3 show peak change scores of vigor and elation (POMS) between the three genotype groups at the rs1861647 and rs36017 polymorphisms, which are highly correlated with each other ($D' = 0.97$, $r^2 = 0.5$). Post hoc comparisons (one-way ANOVA) of both scales and the two associated SNPs revealed that genotype groups differed on ratings of vigor and elation after 20 mg D-amphetamine. No significant associations were found at the 10-mg dose, and there were no significant genotype \times drug interactions on the outcome measures for the genotype groups at the other nine investigated loci.

Post hoc analyses in only new study subjects

Locus rs36017 was previously investigated in a subsample of 56 participants who were included here (Dlugos et al. 2007). To determine whether the same associations were also observed in the 103 new subjects, we conducted a separate analysis with only the added subjects for the associated loci rs36017 and rs1861647. Both rs186167 and rs36017 were significantly associated with levels of vigor (rs1861647: drug \times genotype (two-way ANOVA): $p = 0.002$; rs36017: drug \times genotype (two-way ANOVA): $p = 0.006$) in the 103 new subjects. There was a significant genotype \times drug effect on scores of elation (rs36017: drug \times

genotype (two-way ANCOVA): $p=0.033$) at rs36017, but not at rs1861647 in the 103 new subjects. Thus, these SNPs influenced response to D-amphetamine in both in the initial 56 subjects (Dlugos et al. 2007) and in the new 103 subjects. As expected, the analysis of the combined sample (Tables 3 and 4) was most significant.

SLC6A2 haplotypes and the POMS scales elation and vigor

Association analyses with the POMS scales elation and vigor were also carried out with *SLC6A2* haplotypes. D' values between the 11 polymorphisms in our sample are shown in Fig. 4 (Haploview software version 4.1). LD criteria were not significantly different from those given in the HapMap project. Based on our data and the CEU HapMap data, three haplotype blocks were assessed using Haploview. The first block was formed from rs2397771 and rs3785143, the second was formed from rs36017, rs10521329, and rs3785155, and the third haplotype block was formed from the two loci rs1861647 and rs5569 (Fig. 4). Haplotype blocks 2 and 3 included the two SNPs that showed significant associations with elation and vigor (Table 3). Haplotype pairs for these blocks (blocks 2 and 3)

were estimated for each individual using PLINK. Correlation analyses for the 200-mg dose were performed since this was the only dose that showed significant associations in the single SNP analyses. Empirical p values were calculated and 5,000 permutations were specified and performed for each haplotype to calculate empirical p values adjusted for testing more than one haplotype.

Four reconstructed three-SNP haplotypes from rs36017, rs10521329, and rs3785155 (GAA, CAG, CCG, GCG) and three reconstructed two-SNP haplotypes from rs1861647 and rs5569 (AT, AC, GC) were assessed for association analyses with elation and vigor at the 20-mg dose (Table 4). Haplotype CCG from rs36017, rs10521329, and rs3785155 was significantly associated with stronger feelings of vigor ($p<0.05$), but not elation after 20 mg D-amphetamine. Additionally, a significant association was found between haplotype GC from rs1861647 and rs5569 and vigor ($p<0.05$). The association between haplotype GC from rs1861647 and rs5569 and elation did not remain significant after adjusting for testing more than one haplotype. Haplotype GC was correlated with lower levels of elation and vigor after 20 mg D-amphetamine.

Table 3 Association of POMS scores vigor and elation after amphetamine with *SLC6A2* SNPs and significantly associated haplotypes

ID	Vigor				Elation ^a			
	Drug × genotype ^b		Post hoc (20mg) ^c		Drug × genotype ^b		Post hoc (20mg) ^c	
	<i>F</i> value (<i>df</i>)	<i>p</i> value	<i>F</i> value (<i>df</i>)	<i>p</i> value	<i>F</i> value (<i>df</i>)	<i>p</i> value	<i>F</i> value (<i>df</i>)	<i>p</i> value
SNP								
rs2397771 (C/G) ^d	0.19 (4)	0.942	–	–	0.92 (4)	0.453	–	–
rs3785143 (C/T) ^e	1.78 (2)	0.171	–	–	1.03 (2)	0.359	–	–
rs192303 (C/G)	0.46 (4)	0.534	–	–	1.55 (4)	0.187	–	–
rs36024 (C/T)	0.76 (4)	0.529	–	–	0.85 (4)	0.494	–	–
rs36021 (A/T)	0.43 (4)	0.790	–	–	0.11 (4)	0.979	–	–
rs3785152 (C/T)	2.97 (2)	0.053	–	–	1.04 (2)	0.355	–	–
rs36017 (C/G) ^f	2.53 (4)	0.041	2.16 (2)	0.009	1.67 (4)	0.154	3.40 (2)	0.036
rs10521329 (A/C)	1.33 (2)	0.266	–	–	0.44 (2)	0.643	–	–
rs3785155 (A/G) ^g	0.11 (2)	0.895	–	–	1.82 (2)	0.164	–	–
rs1861647 (A/G)	2.08 (4)	0.006	3.71 (2)	0.027	1.76 (4)	0.137	3.26 (2)	0.041
rs5569 (C/T) ^h	2.11 (4)	0.079	–	–	1.73 (4)	0.143	–	–

^a Elation: gender included as between-subject covariate in statistical analyses

^b Drug by genotype effects: two-way ANOVA/ANCOVA

^c Post hoc analysis: one-way ANOVA/ANCOVA

^d Lee et al. (2005): association with panic disorder without agoraphobia, $p<0.05$

^e Kim et al. 2008, Brookes et al. 2006, Xu et al. 2008: Association with ADHD, $p<0.05$

^f Dlugos et al. (2007): association with D-amphetamine response on POMS scales positive mood and elation, $p<0.05$

^g Kollins et al. (2008): association with performance task phenotypes in ADHD children and families, $p<0.05$

^h Yang et al. (2004): association with methamphetamine response, $p<0.05$; exonic polymorphism (G1287A)

SLC6A2 gene polymorphisms and physiological measures

Association analyses were performed between peak change scores of heart rate and systolic and diastolic blood pressure at the different doses and the investigated SNPs (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. Physiological measures were not initially hypothesized to be associated with genetic variants at the beginning of the study but were assessed to control for physiological and adverse reactions to the drug.

There were significant associations between *SLC6A2* gene variants and physiological measures. Genotype C/G of rs2397771 was associated with higher diastolic blood pressure after 10 mg D-amphetamine (drug \times genotype):

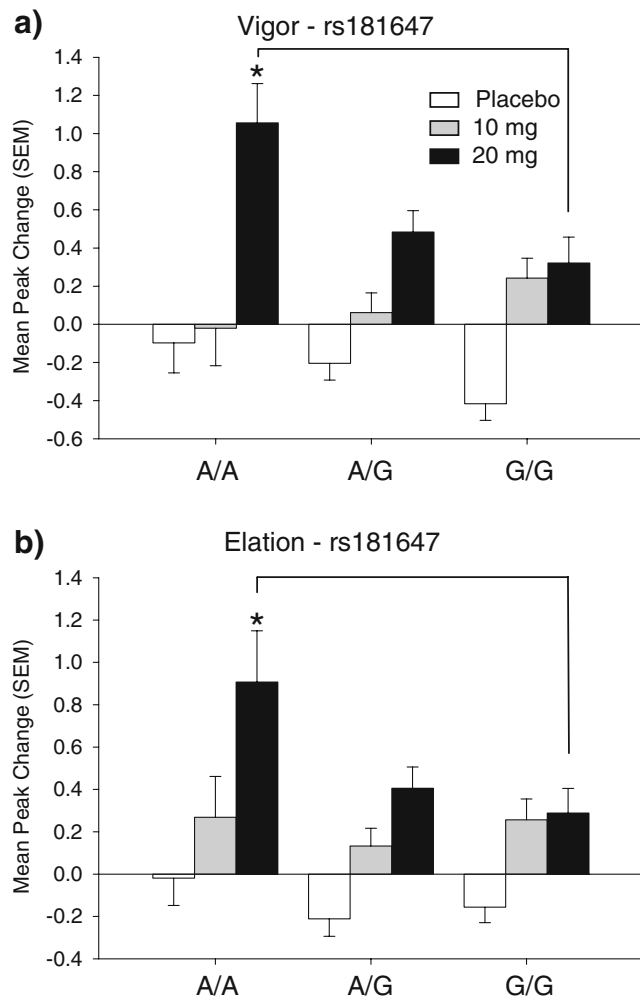


Fig. 2 Mean \pm SEM peak change scores on vigor and elation between the three genotype groups at rs181647 (A/A, $N=19$; A/G, $N=76$; G/G, $N=63$) after placebo and after 10 and 20 mg of D-amphetamine. * $p<0.05$, significant two-way ANOVA/ANCOVA, ** $p<0.05$, post hoc multiple comparisons between genotypes with Bonferroni correction

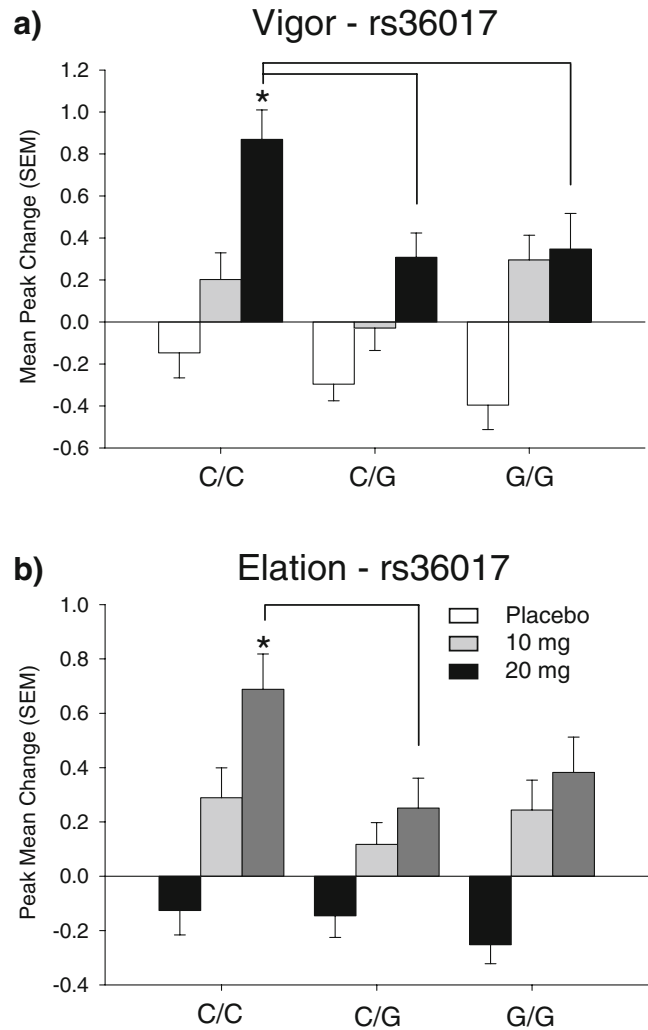


Fig. 3 Mean \pm SEM peak change scores on elation and vigor between the three genotype groups at rs36017 (C/C, $N=47$; C/G, $N=71$; G/G, $N=41$) after placebo and after 10 and 20 mg of D-amphetamine. * $p<0.05$, significant two-way ANOVA/ANCOVA, ** $p<0.05$, post hoc multiple comparisons between genotypes with Bonferroni correction

$p=0.009$, post hoc analysis [10 mg, one-way ANCOVA]: $p=0.022$). Genotype groups at rs36021 significantly differed on baseline diastolic blood pressure, which was confined to the 20-mg D-amphetamine session (drug \times genotype). This observation was not significant in post hoc analyses (one-way ANOVA). There was no significant genotype \times drug interaction between the investigated physiological measures and the other polymorphisms. Genotype groups did not differ at baseline physiological measures.

Discussion

These results confirm and extend our previous findings (Dlugos et al. 2007). As reported previously, D-amphet-

Table 4 Association between the POMS scales elation and vigor and two *SLC6A2* haplotypes: the three-SNP haplotype from rs36017, rs10521329, and rs3785155 and the two-SNP haplotype from rs1861647 and rs5569

Haplotypes	Beta ^a	r^{2b}	STAT ^c	Empirical p value ^d	Corrected empirical p value ^e
Vigor					
Haplotype block 2: rs36017, rs10521329, and rs3785155					
GAA ($F^f=0.102$)	0.008	0.000	0.040	0.9764	1
GAG ($F=0.0807$)	0.397	0.022	1.917	0.0554	0.181
CCG ($F=0.485$)	-0.276	0.040	-2.602	0.011	0.0346
GCG ($F=0.332$)	0.219	0.019	1.785	0.0756	0.2364
Haplotype block 3: rs1861647 and rs5569					
AT ($F=0.301$)	0.213	0.018	1.684	0.0998	0.2064
AC ($F=0.059$)	0.384	0.018	1.723	0.0884	0.194
GC ($F=0.640$)	-0.308	0.040	-2.578	0.0102	0.0255
Elation					
Haplotype block 2: rs36017, rs10521329, and rs3785155					
GAA ($F=0.102$)	0.041	0.000	0.232	0.817	0.9948
GAG ($F=0.0807$)	0.343	0.020	1.841	0.0664	0.2134
CCG ($F=0.485$)	-0.158	0.016	-1.637	0.1004	0.3017
GCG ($F=0.332$)	0.070	0.002	-0.634	0.5337	0.9062
Haplotype block 3: rs1861647 and rs5569					
AT ($F=0.301$)	0.154	0.011	1.356	0.1766	0.3533
AC ($F=0.059$)	0.341	0.018	1.707	0.0891	0.1954
GC ($F=0.640$)	-0.242	0.030	-2.244	0.0270	0.0657

All tests are for the 20-mg dose

^aRegression coefficient

^bProportion of phenotypic variability explained by haplotype

^cWald test (based on t distribution)

^dPermutated p value

^ePermutated p value adjusted for testing multiple haplotypes (5,000 permutations)

^fFrequency

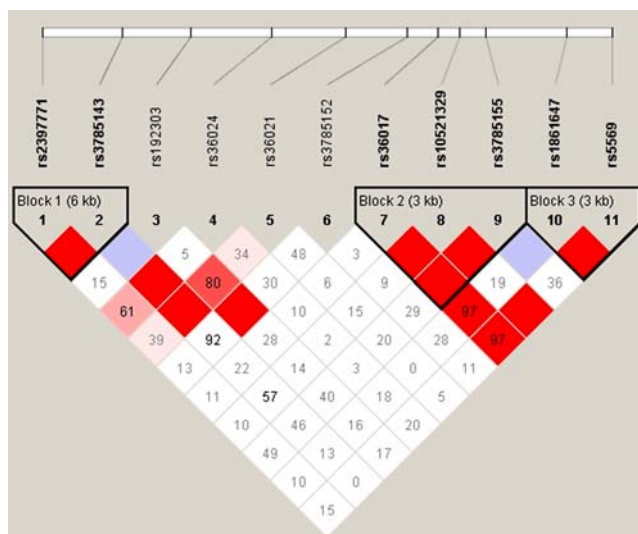


Fig. 4 LD analyses: D' values of SNPs along the *SLC6A2* gene, illustrating three haplotype blocks. D' values were calculated by Haploview version 4.0

amine (20 mg) produced significantly higher ratings of vigor and elation in subjects with genotypes C/C at rs36017 and A/A at rs1861647 than individuals with other genotypes. The primary findings were extended in the haplotype analysis (Tables 3 and 4). Haplotype CCG from rs36017, rs10521329, and rs3785155 was significantly related to feelings of vigor and haplotype GC from rs1861647 and rs5569 was significantly related to lower feelings of vigor (Table 4). The SNPs were associated at the 20-mg dose only. At the lower dose, D-amphetamine did not significantly increase elation and vigor scores (Fig. 2). Thus, the higher 20-mg dose might be necessary to detect significant genotype-dependent differences in amphetamine response. These findings add to a growing body of evidence that genetic variations in the *SLC6A2* influence responses to amphetamine (Cheng and Wooten 1982; Gainetdinov et al. 1999; Taylor and Ho 1978).

Several lines of evidence indicate that individuals who experience stronger or more positive mood alterations after

ingestion of amphetamine may be more susceptible to using the drug repeatedly and to developing a substance abuse disorder. 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' retrospective reports indicate that their initial subjective responses to psychostimulants and opioids predicted their future use (Haertzen et al. 1983). Based on the current data, we would predict that people with genotype C/C at rs36017 or with genotype A/A at rs1861647 would experience more pronounced increases in feelings of vigor and elation after taking D-amphetamine and would, therefore, be more inclined to use amphetamine again in the future.

The exact mechanisms whereby the identified SNPs and haplotypes affect subjective response to amphetamine are not known. It is unclear whether the highly linked associated polymorphisms are in LD with a functional variant that has not been identified yet or whether these polymorphisms directly influence mRNA processing, stability, or splicing. However, the associated polymorphism rs36017 is located in transcription factor binding sites (TRANSFAC program, MatInspector, Genomatix Software, <http://www.genomatix.de/index.html>). Thus, compared to subjects with the G allele at locus rs36017, subjects with the C allele have the additional transcription factor binding site aryl hydrocarbon–Arnt heterodimers, fixed core (family/matrix, V\$AHRR/AHRARNT.02) and lost the transcription factor binding site signal transducer and activator of transcription 3 (V\$STAT3.02). These differences in additional transcription factor binding sites may alter transcription rates of the *SLC6A2* gene. Subjects with the G allele and the additional transcription factor binding site signal transducer and activator of transcription 3 (V\$STAT3.02) might respond less to amphetamine due to higher *SLC6A2* transcription rates and consequently higher *SLC6A2* protein levels. These in silico observations require confirmation by in vitro *SLC6A2* gene expression studies.

The present findings add to a growing literature on the genetic influences on responses to amphetamine. Our laboratory and others have identified several candidate genes that are associated with amphetamine response. For example, Comings et al. 1997 reported that genetic variation in the *CNR1* gene was associated with intravenous use of cocaine and amphetamine. We (Lott et al. 2005, 2006) have reported associations between acute responses to D-amphetamine and polymorphisms in the dopamine (*SLC6A3*) and serotonin (*SLC6A4*) transporter genes in healthy volunteers. In one case, a gene involved in intracellular signaling (i.e., casein kinase I epsilon) was found to be related to acute amphetamine responses in both

mice (Palmer et al. 2005) and humans (Veenstra-VanderWeele et al. 2006). The present findings with *SLC6A2* extend the growing literature on genetic determinants of responses to amphetamine. With further studies of this kind, it may be possible to distinguish different aspects of the response to amphetamine that are under the control of partially overlapping sets of genes.

Apart from differences in responses to D-amphetamine, the *SLC6A2* genotypes were also related to lifetime marijuana use and current caffeine use. Although the explanation for these associations is unknown, it is unlikely that these differences are related to our primary findings. First, separate correlation analyses showed that current and lifetime substance abuse were not related to responses to D-amphetamine in the entire sample. Additionally, we excluded any participants with high use of caffeine, cigarettes, or alcohol or any history of substance abuse. Finally, genotype groups of the polymorphisms rs36017 and rs1861647 that were found to be associated with D-amphetamine response did not differ on any of the demographic outcome measures.

It is known that altered norepinephrine transporter function contributes to hypertension, heart failure, and cardiomyopathy (Böhm et al. 1998; Esler et al. 1981; Merlet et al. 1999). Two of the investigated polymorphisms were associated with physiological measures in the present study. Genotype C/G of rs2397771 was associated with higher diastolic blood pressure after 10 mg D-amphetamine. Interestingly, this is the same SNP that has been found to be associated with agoraphobia in patients with panic disorder. However, our finding reflects a pattern of inheritance consistent with overdominance. Overdominance is a relatively rare phenomenon where heterozygous individuals are different from either homozygote groups, raising the possibility that our finding reflects a false positive. We also found that the rs36021 genotype groups differed significantly on baseline diastolic blood pressure (two-way ANOVA). However, this finding appeared to stem from unexplained precapsule differences, and post hoc analyses were not significant (one-way ANCOVA). These apparent correlations were of marginal significance and might not reflect a real association.

Our study has limitations. Our sample was relatively small, given the relatively small contribution that an individual SNP is likely to contribute to a complex phenotype like the subjective response to a drug. It is unknown whether rs36017 and rs1861647 themselves alter the regulation of *SLC6A2* or whether they are instead in LD with some other functional variant. Furthermore, we cannot explain why we failed to detect effects with the genetic variant rs5569, which was found to be associated with drug response in another study (Yang et al. 2004). Our findings need confirmation in larger samples, including association analyses of additional genes, gene–gene interactions, and

SLC6A2 polymorphisms–gene interactions to detect possible differences in drug response. For example, with larger samples, it may be possible to study the effects of the rare exonic variant rs1805067 (Gly478Ser), which has been shown to have a functional effect by reducing norepinephrine reuptake (Runkel et al. 2000). Finally, imaging studies would be necessary and helpful to further elucidate how and in which brain regions the investigated genetic variations modulate response to amphetamine.

In summary, the present findings confirm and extend a previous observation that genetic variations of the *SLC6A2* are related to quantitative D-amphetamine response in healthy human volunteers. In this highly controlled study involving double-blind administration of two doses of D-amphetamine and placebo to carefully screened volunteers, we replicated a previous finding with *SLC6A2* and we extended this to more complex haplotypes. Studies such as these are important because acute differences in responses to a drug may contribute to variability in risk for abuse and dependence on a drug. Moreover, the findings contribute to our understanding of the neurobiological mechanisms by which stimulant drugs such as amphetamine affect the brain and behavior.

Acknowledgements We gratefully thank Dr. Andrew Skol, Dr. David Goldman, Dr. Colin Hodgkinson, and Pei-Hong Shen for their invaluable input and technical support. We also thank Ms. Margo Meverden and Ms. Patricia Kriegel for their skillful technical assistance. This work was supported by DA021336, DA02812, and MO RR00055.

Ethical standards The experiments comply with the current laws of the United States where they were performed.

References

- American Psychiatric Association (1994) Diagnostic and statistical manual of mental disorders, 4th edn. American Psychiatric Press, Washington
- Biederman J, Spencer T (1999) Attention-deficit/hyperactivity disorder (ADHD) as a noradrenergic disorder. *Biol Psychiatry* 46:1234–1242
- Böhm M, Castellano M, Flesch M, Maack C, Moll M, Paul M, Schiffer F, Zolk O (1998) Chamber-specific alterations of norepinephrine uptake sites in cardiac hypertrophy. *Hypertension* 32:831–837
- Brauer LH, de Wit H (1996) Subjective responses to D-amphetamine alone and after pimozone pretreatment in normal healthy volunteers. *Biol Psychiatry* 39:26–32
- Brookes K, Xu X, Chen W, Zhou K, Neale B, Lowe N (2006) The analysis of 51 genes in DSM-IV combined type attention deficit hyperactivity disorder: association signals in DRD4, DAT1 and 16 other genes. *Mol Psychiatry* 11:934–953
- Cheng CH, Wooten GF (1982) Dopamine turnover estimated by simultaneous LCEC assay of dopamine and dopamine metabolites. *J Pharmacol Methods* 8:123–133
- Comings DE, Muhleman D, Gade R, Johnson P, Verde R, Saucier G, MacMurray G (1997) Cannabinoid receptor gene (CNR1) association with i.v. drug use. *Mol Psychiatry* 2:161–168
- Crabbe JC, Jarvik LF, Liston EH, Jenden DJ (1983) Behavioral responses to amphetamine in identical twins. *Acta Genet Med Gemellol (Roma)* 32:139–149
- de Wit H, Uhlenhuth EH, Johanson CE (1986) Individual differences in the reinforcing and subjective effects of amphetamine and diazepam. *Drug Alcohol Depend* 196:341–360
- Dlugos A, Freitag C, Hohoff C, McDonald J, Cook EH, Deckert J, de Wit H (2007) Norepinephrine transporter gene variation modulates acute response to D-amphetamine. *Biol Psychiatry* 61:1296–1305
- Esler M, Jackman G, Bobik A, Leonard P, Kelleher D, Skews H, Jennings G, Korner P (1981) Norepinephrine kinetics in essential hypertension. Defective neuronal uptake of norepinephrine in some patients. *Hypertension* 3:149–156
- Fergusson DM, Horwood LJ, Lynskey MT, Madden PA (2003) Early reactions to cannabis predict later dependence. *Arch Gen Psychiatry* 60:1033–1039
- Gainetdinov RR, Wetsel WC, Jones SR, Levin ED, Jaber M, Caron MG (1999) Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science* 283:397–401
- Greenhill LL, Pliszka S, Dulcan MK, Bernet W, Arnold V, Beitchman J (2002) Practice parameter for the use of stimulant medications in the treatment of children, adolescents, and adults. *J Am Acad Child Adolesc Psychiatry* 41:26–49
- Haertzen CA, Kocher TR, Miyasato K (1983) Reinforcements from the first drug experience can predict later drug habits and/or addiction: results with coffee, cigarettes, alcohol, barbiturates, minor and major tranquilizers, stimulants, marijuana, hallucinogens, heroin, opiates and cocaine. *Drug Alcohol Depend* 11:147–165
- Heim C, Nemeroff CB (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* 49:1023–1039
- Hodgkinson CA, Yuan Q, Xu K, Shen PH, Heinz E, Lobos EA, Binder EB, Cubells J, Ehlers CL, Gelernter J, Mann J, Riley B, Roy A, Tabakoff B, Todd RD, Zhou Z, Goldman D (2008) Addictions biology: haplotype-based analysis for 130 candidate genes on a single array. *Alcohol Alcohol* 43:505–515
- Hohoff C, McDonald JM, Baune BT, Cook EH, Deckert J, de Wit H (2005) Interindividual variation in anxiety response to amphetamine: possible role for adenosine A2A receptor gene variants. *Am J Med Genet B Neuropsychiatr Genet* 139B:42–44
- Horn AS (1973) Structure activity relations for the inhibition of 5-HT uptake into rat hypothalamic homogenates by serotonin and tryptamine analogues. *J Neurochem* 21:883–888
- Inoue K, Itoh K, Yoshida K, Shimizu T, Suzuki T (2004) Positive association between T-182C polymorphism in the norepinephrine transporter gene and susceptibility to major depressive disorder in a Japanese population. *Neuropsychobiology* 50:301–304
- Johanson CE, Uhlenhuth EH (1980) Drug preference and mood in humans: diazepam. *Psychopharmacology (Berl)* 71:269–273
- Kim H, Mittal DP, Ladarola MJ, Dionne RA (2006) Genetic predictors for acute experimental cold and heat pain sensitivity in humans. *J Med Genet* 43:e40
- Kim JW, Biederman J, McGrath CL, Doyle AE, Mick E, Fagermess J, Purcell S, Smoller JW, Sklar P, Faraone SV (2008) Further evidence of association between two NET single-nucleotide polymorphisms with ADHD. *Mol Psychiatry* 13:624–630
- Kirley A, Lowe N, Hawi Z, Mullins C, Daly G, Waldman I, McCarron M, O'Donnell D, Fitzgerald M, Gill M (2003) Association of the 480 bp DAT1 allele with methylphenidate response in a sample

- of Irish children with ADHD. *Am J Med Genet B Neuropsychiatr Genet* 121B:50–54
- Kollins SH, Anastopoulos AD, Lachiewicz AM, Fitzgerald D, Morrissey-Kane E, Garrett ME, Keatts SL, Ashley-Koch AE (2008) SNPs in dopamine D2 receptor gene (DRD2) and norepinephrine transporter gene (NET) are associated with continuous performance task (CPT) phenotypes in ADHD children and their families. *Am J Med Genet B Neuropsychiatr Genet* 147B:1580–1588
- Lee YJ, Hohoff C, Domschke K, Sand P, Kuhlenbäumer G, Schirmacher A, Freitag CM, Meyer J, Stöber G, Franke P, Nöthen MM, Fritze J, Fimmers R, Garritsen HS, Stögbauer F, Deckert J (2005) Norepinephrine transporter (NET) promoter and 5'-UTR polymorphisms: association analysis in panic disorder. *Neurosci Lett* 377:40–43
- Leonard BE (1997) The role of noradrenaline in depression: a review. *J Psychopharmacol* 11:39–47
- Lott DC, Kim S, Cook EH Jr, de Wit H (2005) Dopamine transporter gene associated with diminished subjective response to amphetamine. *Neuropsychopharmacology* 30:602–609
- Lott DC, Kim SJ, Cook EH, de Wit H (2006) Serotonin transporter genotype and acute subjective response to amphetamine. *Am J Addict* 15:327–335
- Martin WR, Sloan JW, Sapira JD, Jasinski DR (1971) Physiologic, subjective, and behavioral effects of amphetamine, methamphetamine, ephedrine, phenmetrazine, and methylphenidate in man. *Clin Pharmacol Ther* 12:245–258
- McNair D, Lorr M, Droppleman DL (1971) Profile of mood states. Educational and Industrial Testing Service, San Diego
- Merlet P, Pouillart F, Dubois-Randé JL, Delhayé N, Fumey R, Castaigne A, Syrota A (1999) Sympathetic nerve alterations assessed with 123I-MIBG in the failing human heart. *J Nucl Med* 40:224–231
- Nurnberger JI Jr, Gershon ES, Simmons S, Ebert M, Kessler LR, Dibble ED, Jimerson SS, Brown GM, Gold P, Jimerson DC, Guroff JJ, Storch FI (1982) Behavioral, biochemical and neuroendocrine responses to amphetamine in normal twins and “well-state” bipolar patients. *Psychoneuroendocrinology* 7:163–176
- Palmer AA, Verbitsky M, Suresh R, Kamens HM, Reed CL, Li N, Burkhart-Kasch S, McKinnon CS, Belknap JK, Gilliam TC, Phillips TJ (2005) Gene expression differences in mice divergently selected for methamphetamine sensitivity. *Mamm Genome* 16:291–305
- Piazza PV, Deminière JM, Le Moal M, Simon H (1989) Factors that predict individual vulnerability to amphetamine self-administration. *Science* 245:511–513
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155:945–959
- Raiteri M, Del Carmine R, Bertollini A, Levi G (1977) Effect of sympathomimetic amines on the synaptosomal transport of noradrenaline, dopamine and 5-hydroxytryptamine. *Eur J Pharmacol* 41:133–143
- Ressler KJ, Nemeroff CB (1999) Role of norepinephrine in the pathophysiology and treatment of mood disorders. *Biol Psychiatry* 46:1219–1233
- Ricca V, Mannucci E, Paionni A, Di Bernardo M, Cellini M, Cabras PL, Rotella CM (1999) Venlafaxine versus fluoxetine in the treatment of atypical anorectic outpatients: a preliminary study. *Eat Weight Disord* 4:10–14
- Runkel F, Brüß M, Nöthen MM, Stöber G, Propping P, Bönisch H (2000) Pharmacological properties of naturally occurring variants of the human norepinephrine transporter. *Pharmacogenetics* 10:397–405
- Spencer TJ, Abikoff HB, Connor DF, Biederman J, Pliszka SR, Boellner S, Read SC, Pratt R (2006) Efficacy and safety of mixed amphetamine salts extended release (adderall XR) in the management of oppositional defiant disorder with or without comorbid attention-deficit/hyperactivity disorder in school-aged children and adolescents: a 4-week, multicenter, randomized, double-blind, parallel-group, placebo-controlled, forced-dose-escalation study. *Clin Ther* 28:402–418
- Taylor D, Ho BT (1978) Comparison of inhibition of monoamine uptake by cocaine, methylphenidate and amphetamine. *Res Commun Chem Pathol Pharmacol* 21:67–75
- Veenstra-VanderWeele J, Qaadir A, Palmer AA, Cook EH Jr, de Wit H (2006) Association between the casein kinase 1 epsilon gene region and subjective response to D-amphetamine. *Neuropsychopharmacology* 31:1056–1063
- Verhoeff NP, Christensen BK, Hussey D, Lee M, Papatheodorou G, Kopala L, Rui Q, Zipursky RB, Kapur S (2003) Effects of catecholamine depletion on D2 receptor binding, mood, and attentiveness in humans: a replication study. *Pharmacol Biochem Behav* 74:425–432
- White TL, Justice AJ, de Wit H (2002) Differential subjective effects of D-amphetamine by gender, hormone levels and menstrual cycle phase. *Pharmacol Biochem Behav* 73:729–741
- Williamson S, Gossop M, Powis B, Griffiths P, Fountain J, Sstrang J (1997) Adverse effects of stimulant drugs in a community sample of drug users. *Drug Alcohol Depend* 44:87–94
- Xu X, Hawi Z, Brookes KJ, Anney R, Bellgrove M, Franke B, Barry E, Chen W, Kuntsi J, Banaschewski T, Buitelaar J, Ebstein R, Fitzgerald M, Miranda A, Oades RD, Roeyers H, Rothenberger A, Sergeant J, Sonuga-Barke E, Steinhausen HC, Faraone SV, Gill M, Asherson P (2008) Replication of a rare protective allele in the noradrenaline transporter gene and ADHD. *Am J Med Genet B Neuropsychiatr Genet* 147B:1564–1567
- Yang L, Wang YF, Li J, Faraone SV (2004) Association of norepinephrine transporter gene with methylphenidate response. *J Am Acad Child Adolesc Psychiatry* 43:1154–1158