D-Amino Acid Aberrations in Cerebrospinal Fluid and Plasma of Smokers

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The glutamatergic neurotransmission system and the N-methyl-D-aspartate receptor (NMDAR) have been implicated in smoking and alcohol consumption behavior. Preclinical studies have demonstrated that nicotine and ethanol influence NMDAR functionality, which may have a role in tendencies to consume these substances. Nonetheless, little is known about concentrations of NMDAR coagonists in the cerebrospinal fluid (CSF) and plasma of individuals who smoke or consume alcohol. Glycine and L- and D-stereoisomers of alanine, serine, and proline were therefore measured using ultra-high-performance liquid chromatography-tandem mass spectrometry in 403 healthy subjects. Nicotine and alcohol consumption were quantified using questionnaires. Possible differences in NMDAR coagonist concentrations in plasma and CSF were investigated using ANCOVA with age, body mass index, and storage duration as covariates. The significance threshold was Bonferroni corrected ($\alpha = 0.00625$). Compared with non-smokers, smokers displayed lower levels of D-proline in plasma ($p = 0.0027$, Cohen's $d = -0.41$) and D-proline in CSF ($p = 0.0026$, Cohen's $d = -0.43$). D-Serine in CSF was higher in smokers than in non-smokers ($p = 0.0052$, Cohen's $d = 0.41$). After subdividing participants based on smoking quantity, dose-dependent decreases were demonstrated in smokers for D-proline in plasma ($F = 5.65$, $p = 0.0039$) and D-proline in CSF ($F = 5.20$, $p = 0.0060$). No differences in NMDAR coagonist levels between alcohol consumption groups were detected. To our knowledge, this is the first report to implicate D-amino acids in smoking behavior of humans. Whether such concentration differences lie at the root of or result from smoking habits may be addressed in prospective studies.

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INTRODUCTION

In Europe, the prevalence of smoking was ~28.6% in 2005 (WHO, 2007, 2012). Smoking decreases life expectancy by 12–20 years and is one of the major mortality risk factors in the world: more than 5 million people worldwide die of the consequences of tobacco consumption yearly (WHO, 2012).

Although genetic variants in genes encoding neuronal nicotinic acetylcholine receptor subunits (eg, *CHRNA3* and *CHRNA5*) are associated with smoking quantity, the explained variances by such single-nucleotide polymorphisms are low (Tobacco and Genetics Consortium, 2010; Liu *et al*, 2010). This suggests that other pathways, such as the glutamatergic neurotransmission system, have a role in smoking behavior. Glutamate is the prime excitatory neurotransmitter in the central nervous system (CNS) and binds to several receptors, including the ionotropic N-methyl-D-aspartate receptor (NMDAR). Animal studies have reported that nicotine increases glutamate concentrations in the ventral tegmental area (VTA) (Fu *et al*, 2000; Schilstrom *et al*, 2000) and exerts an excitatory effect on the NMDAR in dopaminergic neurons in the VTA

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(Fu *et al*, 2000; Grillner and Svensson, 2000; Mansvelder and McGehee, 2000), the nucleus accumbens (Schilström *et al*, 1998), and the central nucleus of the amygdala (Kenny *et al*, 2009). In addition, nicotine self-administration in rats upregulates the expression of NMDAR subunits in the VTA and amygdala (Kenny *et al*, 2009). *In vivo* blockade of the NMDAR by NMDAR antagonists diminishes nicotine-induced dopamine release (Schilström *et al*, 1998) and nicotine self-administration (Kenny *et al*, 2009), but promotes tolerance to nicotine administration in rats (Shoaib *et al*, 1994; Shoaib and Stolerman, 1992). NMDAR antagonism could therefore constitute a target in the treatment of nicotine dependence, although to date this has not been successful in humans (Liechti and Markou, 2008).

The other substance frequently associated with burden of disease in Western society is alcohol. The World Health Organization estimated that alcohol is currently the world's third largest risk factor for burden of disease (WHO, 2009). In contrast to nicotine, ethanol has been reported to block the NMDAR (Hoffman *et al*, 1989; Little, 1991; Lovinger *et al*, 1989; Weight *et al*, 1991). Chronic alcohol administration increases the number of NMDARs, thus playing a role in alcohol dependence and withdrawal seizures (Grant *et al*, 1990; Gulya *et al*, 1991; Lovinger *et al*, 1989). Moreover, NMDAR antagonists (eg, MK-801 and ketamine) cause ethanol-like effects in animals and humans (Hundt *et al*, 1998; Kotlinska and Liljequist, 1997; Krystal *et al*, 1998).

Glycine, D-serine, D-alanine, and L-proline are coagonists at the NMDAR (Henneberger *et al*, 2010; Henzi *et al*, 1992; Kleckner and Dingledine, 1988; Martin *et al*, 1992; Matsui *et al*, 1995; McBain *et al*, 1989; Mothet *et al*, 2000; Nong *et al*, 2003; Panatier *et al*, 2006; Sakata *et al*, 1999; Snyder and Kim, 2000). In particular, D-serine and glycine were demonstrated to strongly impact synaptic and extrasynaptic NMDAR signaling, whereas manipulation of both these coagonists has yielded a more nuanced understanding of NMDAR functionality (Papouin *et al*, 2012). To date, neither in plasma nor cerebrospinal fluid (CSF) have NMDAR coagonists been investigated for their role in smoking or alcohol consumption tendencies. Elucidating possible substance use-associated abnormalities in NMDAR coagonist levels may deepen the understanding of NMDAR involvement in substance use and susceptibility to nicotine and alcohol dependence. Moreover, the success rates of pharmacotherapies in the treatment of nicotine (Eisenberg *et al*, 2008) and alcohol (Rosner *et al*, 2008) dependence are limited and therefore novel agents may contribute to treatment optimization. Elucidating NMDAR coagonist functionality in subjects who consume these substances will potentially set the stage for further research into pharmacological modulations of NMDAR gating in pre-clinical settings and human consumers.

As the NMDAR is involved in smoking and alcohol use, we hypothesized that consumers of nicotine and alcohol display altered levels of NMDAR coagonists in CSF and plasma. Given the demonstrated increase in glutamate concentrations in the rat brain and upregulation of the NMDAR after nicotine administration (Grillner and Svensson, 2000; Mansvelder and McGehee, 2002; Schilström *et al*, 2000), we postulated that coagonist levels are elevated

in smokers. As ethanol blocks the NMDAR (Hoffman *et al*, 1989; Little, 1991; Lovinger *et al*, 1989; Weight *et al*, 1991), we expected NMDAR coagonists in alcohol consumers to be decreased. Enantiomers of the above-mentioned coagonists were included in the current study as synthesis and degradation of stereoisomers are likely to be interdependent. We thus measured concentrations of glycine (that is not chiral) and the enantiomers of alanine, serine, and proline in a unique study population with CSF and plasma available for 403 subjects. We then compared these levels across categories of nicotine and alcohol consumption to detect possible concentration differences associated with the number of cigarettes or alcoholic beverages consumed.

MATERIALS AND METHODS

Subjects

Subject recruitment has been described in detail previously (Luykx *et al*, 2013). In brief, from August 2008 to November 2011, 403 subjects were recruited at outpatient preoperative screening services in and around Utrecht, the Netherlands. At these services, subjects are advised by the anesthesiologist to start fasting at least 6 h preoperatively and refrain from smoking and alcoholic beverages at least 24 h before the procedure (no biochemical verification). This enabled us to study sustained effects—ie, effects persisting for at least 24 h—of smoking and alcohol on amino-acid concentrations. We included patients (i) undergoing spinal anesthesia for minor elective surgical procedures, (ii) ranging between 18 and 60 years of age, and (iii) with four grandparents born in the Netherlands or other North-Western European countries (Belgium, Germany, the UK, France, and Denmark). Each candidate participant received a personal telephone interview to exclude subjects with psychotic or major neurological disorders (stroke, brain tumors, neurodegenerative diseases) and to record any self-reported history of other psychiatric illness or any use of psychotropic medication. Informed consent was obtained from the participants and the Ethics Committee of the UMCU and all local ethics committees approved the study.

CSF and Plasma Collection and Chemical Analyses

Whole blood was collected in EDTA tubes for plasma extraction. Plasma was extracted by centrifuging whole blood at ambient temperature for 10 min at 2500 g, after which plasma was stored at -80°C . The standardized operating procedures adopted to collect 6 ml of CSF from each subject were described previously (Luykx *et al*, 2012). Chemical analyses of the L- and D-isomers of alanine, serine, proline, and glycine (no D-isomer) were conducted using ultra-high-performance liquid chromatography-tandem mass spectrometry according to a validated method (Visser *et al*, 2011).

Questionnaires

During a 2-week period after the elective procedure, subjects filled out online questionnaires about their health.

The following questions regarding current smoking and alcohol consumption habits were asked:

- Do you smoke? If so, how many cigarettes do you smoke per day?
- Do you drink alcohol? If so, how many units of alcohol do you drink per day?

Smokers were asked to choose between the following divisions of number of cigarettes smoked per day: <1; 1–10; 11–20; 21–30; and >30. Alcohol consumption was quantified using the following cutoffs: <1; 1–3; 4–6; and >6 alcoholic beverages.

We chose web-based symptom questionnaires as these have been validated as reliable assessment tools in a range of epidemiological studies (Ekman *et al*, 2006; Gosling *et al*, 2004) and may decrease socially desirable responses compared with face-to-face interviews or questionnaires that are filled out in clinical settings (Buchanan and Smith, 1999; Joinson, 1999).

Statistical Analyses

Regarding smoking habits, the participants were divided into two groups: non-smokers (no current smoking) and smokers (any number of cigarettes smoked daily). Alcohol users were divided into mild alcohol consumers (<1 alcohol unit/day) and moderate alcohol consumers (≥ 1 alcohol units/day) as only 11.2% of the study population proved abstinent.

Outliers were defined as subjects having at least one measurement with 3 or more standard deviations (SDs) from the mean and were excluded from further analyses. As no generally accepted covariates for amino-acid measurements are available, we comprehensively assessed possible covariates during the study period. The following variables were tested: age, sex, the rostrocaudal concentration gradient (reflected by the participants' height), lumbar puncture level (binary, ie, lumbar vertebrate levels ≤ 3 vs > 3 as estimated by the anesthesiologist), time elapsed before storage (continuous, in hours), time of the day of lumbar puncture (continuous, rounded to the half hour), storage duration until chemical analyses (continuous, in months), body mass index (BMI (continuous), and amount of CSF drawn (continuous, as in 8% of the cases > 7 or < 5 ml of CSF were drawn). Relevant covariates were defined as variables that correlated with more than one amino acid at a Spearman's ρ p -value < 0.05 . If covariates were collinear ($r > 0.5$), the covariate with most missing data was excluded from the model. Only for height and weight, data were missing (for 25 out of 403 subjects, ie, 6.2%) and replaced by the mean per sex (183.4 cm and 86.8 kg for men; 170.5 cm and 74.6 kg for women). Using ANOVA and χ^2 tests possible smoking and alcohol consumption-dependent differences were assessed.

Normality of the distributions was verified with a Kolmogorov–Smirnov (K-S) test and defined as a two-tailed asymptotic p -value ≥ 0.05 . Non-normally distributed amino acids were logarithm (log) transformed. Homogeneity of variances (defined as a Levene's test p -value > 0.05) and homogeneity of regression slopes (by visual inspection of the scatterplots between the amino acids and the

covariates) between groups were verified. To unravel possible interaction effects of alcohol and smoking behavior on amino-acid levels, we applied a generalized linear model and tested possible interaction effects of the above-mentioned dichotomous smoking and alcohol traits on amino-acid concentrations (Bonferroni-corrected type III Wald χ^2 $\alpha = 0.05/8 = 0.00625$).

A one-way ANCOVA was conducted correcting for all relevant covariates (defined above). Significance was Bonferroni corrected ($\alpha = 0.05/8 = 0.00625$, as four NMDAR coagonists were tested for both nicotine and alcohol consumption, whereas stereoisomers in CSF and plasma were highly correlated, Supplementary 1). Stratification by sex was not performed due to the relatively small number of female participants ($N = 99$). In the event Bonferroni-corrected significance was attained, we tested whether concentration differences were substance dose dependent. To that end, covariates that correlated at $p < 0.05$ with that amino acid were determined and ANCOVA correcting for these covariates (or ANOVA in the event no covariate correlated with that amino acid) was conducted for the following substance consumption categories: <1 consumption a day (category 1); 1–10 cigarettes or 1–3 alcoholic beverages a day (category 2); and > 10 cigarettes or > 3 alcoholic beverages a day (category 3). Given the small numbers of subjects smoking > 20 cigarettes a day or consuming > 4 alcoholic beverages daily, this subdivision resulted in the most equal numbers of subjects per category. All statistical analyses were conducted using SPSS version 20 (SPSS, Chicago, IL).

RESULTS

Subject and NMDAR Coagonist Characteristics

Information about smoking and alcohol consumption habits in addition to NMDAR coagonist levels in either CSF or plasma were available for 403 subjects. Exclusion of outliers (55 subjects) brought the study population to 348 subjects (249 men and 99 women). Characteristics of these 348 subjects ($N = 325$ for whom CSF was available; $N = 307$ for whom plasma was available) are summarized in Table 1. The only significant differences in subject characteristics across substance use categories were found for psychiatric comorbidity and psychotropic medication (both increased in smokers vs non-smokers; χ^2 p -values of 0.030 and 0.018, respectively). Six NMDAR coagonists were normally distributed (L-serine and D-serine in CSF; L-alanine, glycine, D-serine, and L-proline in plasma; Supplementary 2). The other NMDAR coagonists were log transformed, resulting in a formally normal distribution by K-S testing for all concentrations except L-proline in CSF (that approximated normality upon visual inspection, Supplementary 2). Supplementary 3 presents the mean NMDAR coagonist concentrations parsed by substance use category and body fluid.

NMDAR Coagonist Concentrations in Smokers and Non-Smokers

The covariates age, BMI, and storage duration were the only covariates that correlated with > 1 amino acid. These

Table 1 Subject Characteristics

Parameter	Total	Non-smokers	Smokers	Mild alcohol users	Moderate alcohol users
Subjects (N)	348	251	97	228	120
Age (years)	39.5 (10.9)	40.5 (10.5)	36.7 (11.5)	40.0 (10.5)	38.6 (11.7)
Sex (% males)	71.6	71.3	72.2	65.8	82.5
BMI	25.8 (3.66)	25.9 (3.49)	25.6 (4.09)	26.6 (5.88)	25.7 (3.28)
Storage duration (months)	7.8 (4.8)	7.6 (4.5)	8.1 (5.5)	7.9 (4.8)	7.4 (4.8)
CSF quantity (ml)	5.4 (1.1)	5.4 (1.0)	5.3 (1.1)	5.4 (1.1)	5.5 (1.0)
Arthroscopy (%)	66.1	66.9	63.9	63.6	70.8
Psychiatric comorbidity (%)	4.3	2.8	8.2	3.9	5.0
Psychotropic medication (%)	4.6	2.4	10.3	5.3	3.3
Other medication (%)	14.7	16.7	9.28	13.6	16.7

Means (SD) are given unless stated otherwise. The only significant differences between substance use categories were found for smoking: psychiatric comorbidity and psychotropic medication (in italics, non-smokers vs smokers respective χ^2 p -values of 0.030 and 0.018).

Table 2 Concentrations (in $\mu\text{mol/l}$) of all Measured Amino-Acid Enantiomers in Non-Smokers vs Smokers

	Mean $\pm$ SD Non-smokers	Mean $\pm$ SD Smokers	P-value	F-ratio	Cohen's d
L-Alanine CSF	31.5 $\pm$ 7.20	30.4 $\pm$ 6.63	0.76		
D-Alanine CSF	0.16 $\pm$ 0.08	0.15 $\pm$ 0.09	0.47		
Glycine CSF	6.21 $\pm$ 2.04	6.37 $\pm$ 1.69	0.08		
L-Serine CSF	23.4 $\pm$ 3.55	24.06 $\pm$ 3.74	0.28		
D-Serine CSF	1.23 $\pm$ 0.21	1.31 $\pm$ 0.23	0.0052	7.91	0.41
D-Proline CSF	0.01 $\pm$ 0.007	0.008 $\pm$ 0.005	0.0026	9.22	-0.43
L-Proline CSF	0.69 $\pm$ 0.35	0.74 $\pm$ 0.34	0.04		
L-Alanine plasma	337 $\pm$ 71.2	327 $\pm$ 77.5	0.21		
D-Alanine plasma	0.86 $\pm$ 0.47	0.76 $\pm$ 0.41	0.021		
Glycine plasma	164 $\pm$ 45.3	172 $\pm$ 46.7	0.061		
L-Serine plasma	84.2 $\pm$ 15.4	86.8 $\pm$ 16.1	0.41		
D-Serine plasma	1.10 $\pm$ 0.26	1.08 $\pm$ 0.22	0.21		
L-Proline plasma	172 $\pm$ 41.0	182 $\pm$ 49.2	0.24		
D-Proline plasma	0.32 $\pm$ 0.16	0.26 $\pm$ 0.14	0.0027	9.61	-0.41

Significant findings are in bold ($p < 0.00625$). F-ratios and effect sizes are reported when the ANCOVA P -value is significant (covariates: age, BMI, and storage duration); nominally significant results ($p < 0.05$) are in italics.

showed correlations ($p < 0.05$) with several amino acids (Supplementary 4). For these covariates no data were missing. No interaction effects between alcohol and smoking habits on amino-acid levels were detected.

After correction for these three covariates, all D-enantiomers in plasma were lower in smokers than in non-smokers, but for plasma only D-proline reached Bonferroni-corrected significance: the D-proline concentration in plasma was lower in smokers than in non-smokers ($F_{1,302} = 9.61$, $p = 0.0027$, Cohen's $d = -0.41$; Table 2 and Figure 1a). The plasma D-alanine difference between smokers and non-smokers was nominally significant ($p = 0.021$) and plasma D-serine was only slightly decreased in smokers ($p = 0.21$).

D-Proline in CSF was also lower in smokers than in non-smokers ($F_{1,320} = 9.22$, $p = 0.0026$, Cohen's $d = -0.43$; Figure 1b), whereas D-serine in CSF was higher in smokers than in non-smokers ($F_{1,320} = 7.91$, $p = 0.0052$, Cohen's $d = 0.41$; Figure 1c).

We then assessed whether the concentration differences were substance dose dependent for the Bonferroni-corrected significant results. Although for D-serine in CSF no smoking dose-dependent effect was detected, we found significant differences between the three categories for D-proline (Table 3):

D-Proline in plasma (covariates: age and BMI): $F_{2,302} = 5.65$, $p = 0.0039$ (Figure 2a).

D-Proline in CSF (covariate: age): $F_{2,321} = 5.20$, $p = 0.0060$ (Figure 2b).

Finally, as psychiatric comorbidity and psychotropic medication significantly differed between smokers and non-smokers, we tested whether these variables were correlated with any of the above-mentioned amino acids. D-Serine in plasma correlated with psychiatric comorbidity (Spearman's $\rho = 0.16$, $p = 0.005$), but correcting for this variable did not change the results (data not shown). Variances and regression slopes were homogeneous between groups for all reported differences.

NMDAR Coagonist Concentrations in Mild and Moderate Alcohol Consumers

No differences in NMDAR coagonist levels between mild (< 1 unit/day) and moderate alcohol consumers (≥ 1 units/day) were detected (Supplementary 5).

Amino-Acid Reference Values

As reference values for most D- and L-amino acids in CSF and plasma are currently lacking or based on limited study populations, we provide these values before removal of any outliers to give an impression of their naturally occurring variation (Supplementary 6).

DISCUSSION

Here, we demonstrate an increase in CSF D-serine and a decrease in CSF and plasma D-proline in smokers compared with non-smokers ($N = 348$). The D-proline concentration differences proved substance dose-dependent. No differences

Figure 1 D-Amino-acid concentration differences between smokers and non-smokers: interquartile ranges (boxes) with medians (lines in boxes), whiskers (10–90 percentiles), and dots (values falling outside the 10–90 percentiles). (a) D-Proline in plasma in smokers vs non-smokers. (b) D-Proline in CSF in smokers vs non-smokers. (c) D-Serine in CSF in smokers vs non-smokers.

Table 3 Per Smoking Category, Concentrations ($\mu\text{mol/l}$) of the Amino Acids that Differed Significantly Between Smokers and Non-Smokers and ANCOVA Summary Statistics are Given

	< 1 cigarette/day (N = 243)	1–10 cigarettes/day (N = 31)	> 10 cigarettes/day (N = 51)	F-Statistic	P-Value
D-Proline in Plasma	0.32 ± 0.16	0.29 ± 0.16	0.24 ± 0.10	5.65	0.0039
D-Proline in CSF	0.010 ± 0.007	0.008 ± 0.006	0.007 ± 0.005	5.20	0.0060

in NMDAR coagonists between mild and moderate alcohol consumers were found.

The current study being the first to comprehensively compare NMDAR-coagonist levels in the CSF and plasma of smoking and non-smoking subjects, our results cannot be directly compared with previous findings. To our knowledge, only in smoking pregnant women and smoking schizophrenia patients and controls have amino-acid levels been compared with non-smokers. None of these two studies detected smoking behavior-dependent differences in

plasma amino-acid levels (Jauniaux *et al*, 1999; Clelland *et al*, 2011). However, instead of parsing the results by enantiomers, only total amino-acid concentrations were investigated in those studies. As the primary contribution to total amino-acid concentrations comes from L-enantiomers, such previous findings combined with the data presented here suggest that smoking may be associated only with D-enantiomers of amino acids.

A possible mechanism whereby smoking leads to D-amino acid aberrations relates to the direct effect of nicotine on the

Figure 2 D-Proline in plasma (a) and D-proline in CSF (b) concentrations significantly differ by smoking quantity category (1 = < 1 cigarette; 2 = 1–10 cigarettes; 3 = > 10 cigarettes per day): interquartile ranges (boxes) with medians (lines in boxes), whiskers (10–90 percentiles), and dots (values falling outside the 10–90 percentiles).

NMDAR. Nicotine-dependent activation of nicotinic acetylcholine receptors enhances glutamatergic transmission (Mansvelder and McGehee, 2000). Glutamate can then activate the NMDAR, leading to long-term potentiation (Mansvelder and McGehee, 2000). Conceivably, long-term potentiation may thereafter result in D-serine upregulation in the CNS as D-serine is the most important ligand at the synaptic NMDAR glycine-binding site (Papouin *et al*, 2012). Such increased D-serine consequential to nicotine effects in the CNS is in line with the high CSF D-serine concentrations we observed in smokers. Relatively high CSF D-serine in smokers is further consistent with a burgeoning body of evidence demonstrating lack of efficacy of D-cycloserine in reducing tobacco consumption (Kamboj *et al*, 2012; Nesic *et al*, 2011; Santa Ana *et al*, 2009; Yoon *et al*, 2013). Further potentiating D-serine-dependent NMDAR neurotransmission by D-cycloserine administration in smokers would likely be unsuccessful for this indication.

To our knowledge, no significant differences in NMDAR coagonist levels between mild and moderate alcohol consumers have been reported, although increased glutamate and proline levels in plasma of alcohol-dependent patients with positive alcohol breath tests have been reported (Walter *et al*, 2008). This suggests that alcohol consumption shortly before sampling affects glutamate and proline levels. The subjects included in the current study had fasted before lumbar puncture and were instructed to be abstinent for at least 24 h before lumbar puncture. Possibly, such previously reported effects of alcohol on proline concentrations are short lived. Higher proline levels in plasma in subjects with alcohol abuse or dependence compared with healthy controls have been described (Clelland *et al*, 2011). Our study population differed in that here analyses were performed on subjects from the general population— not a patient–control cohort.

A limitation of this study is that no formal assessments of alcohol and nicotine dependence or other measures relevant to smoking habits (eg, vulnerability to relapse and smoking initiation) were conducted. Therefore, the effects of such

diagnoses and behavioral characteristics on D-amino acid levels cannot be construed from our data. In addition, the data we provide are cross-sectional and therefore inferences about causality cannot be made. Furthermore, the limited number of study participants precluded us from running linear regression as few smokers fell in high consumption categories. Although designs quantitatively assessing smoking habits and measuring NMDAR coagonists in the plasma of a substantial number of participants are feasible, CSF would be a more cumbersome target. On a similar note, this study was underpowered to stratify analyses by sex given the small number of female subjects. Finally, whether nicotine-dependent influences on NMDAR coagonist levels are sustained over time cannot be deduced from our data, as measurements were not performed at different time points. However, as participants were advised to refrain from smoking at least 24 h preoperatively, it is possible that D-amino acid aberrations in smokers are not short lived.

In conclusion, the differences in D-amino acids in CSF and plasma that we detected support their involvement in smoking behavior. Animal studies may determine whether nicotine-dependent activation of the NMDAR is mediated by D-serine and D-proline. Future longitudinal designs in humans that incorporate measurements of D-amino acid levels before and after substance consumption may clarify whether differences in such levels are state or trait dependent. Moreover, given previously highlighted sex-dependent differences in smoking behavior (Townsend *et al*, 1994), upcoming experiments will hopefully attain sufficient power to stratify by sex. Furthermore, formally diagnosing nicotine dependence and assessments of additional smoking behavior measures (eg, vulnerability to relapse and smoking initiation) in such projects may tease apart associations of substance use phenotypes with D-amino acid aberrations.

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)