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ORIGINAL INVESTIGATION

Alterations in tryptophan and purine metabolism in cocaine addiction: a metabolomic study

Ashwin A. Patkar · Steve Rozen · Paolo Mannelli · Wayne Matson · Chi-Un Pae · K. Ranga Krishnan · Rima Kaddurah-Daouk

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Abstract
Background Mapping metabolic "signatures" can provide new insights into addictive mechanisms and potentially identify biomarkers and therapeutic targets.
Objective We examined the differences in metabolites related to the tyrosine, tryptophan, purine, and oxidative stress pathways between cocaine-dependent subjects and healthy controls. Several of these metabolites serve as biological indices underlying the mechanisms of reinforcement, toxicity, and oxidative stress.
Methods Metabolomic analysis was performed in 18 DSM-IV-diagnosed cocaine-dependent individuals with at least 2 weeks of abstinence and ten drug-free controls. Plasma concentrations of 37 known metabolites were analyzed and compared using a liquid chromatography electrochemical array platform. Multivariate analyses were used to study the relationship between severity of drug use [Addiction Severity Index (ASI) scores] and biological measures.
Results Cocaine subjects showed significantly higher levels of *n*-methylserotonin ($p<0.0017$) and guanine ($p<0.0031$) and lower concentrations of hypoxanthine ($p<0.0002$), anthranilate ($p<0.0024$), and xanthine ($p<0.012$), compared to controls. Multivariate analyses showed that a combination of *n*-methylserotonin and xanthine contributed to 73% of the variance in predicting the ASI scores ($p<0.0001$). Logistic regression showed that a model combining *n*-methylserotonin, xanthine, xanthosine, and guanine differentiated cocaine and control groups with no overlap.
Conclusions Alterations in the methylation processes in the serotonin pathways and purine metabolism seem to be associated with chronic exposure to cocaine. Given the preliminary nature and cross-sectional design of the study, the findings need to be confirmed in larger samples of cocaine-dependent subjects, preferably in a longitudinal design.

Keywords Metabolomics · Methylation · n methyl serotonin · Cocaine · Tryptophan · Addiction · Purine

A. A. Patkar · S. Rozen · P. Mannelli · C.-U. Pae · K. R. Krishnan · R. Kaddurah-Daouk
Department of Psychiatry and Behavioral Sciences,
Duke University Medical Center,
Durham, NC, USA

W. Matson
Department of Systems Biochemistry,
Bedford VA Medical Center,
Bedford, MA, USA

S. Rozen · K. R. Krishnan
Duke-NUS Graduate Medical School,
Singapore, Singapore

C.-U. Pae
The Catholic University of Korea College of Medicine,
Seoul, Republic of Korea

A. A. Patkar (✉)
Duke University Medical Center,
2218 Elder Street, Suite 127,
Durham, NC 27705, USA
e-mail: Ashwin.Patkar@duke.edu

R. Kaddurah-Daouk (✉)
Duke University Medical Center,
Rm. 3552 Blue Zone, Duke South/Clinics, Box 3903, Durham,
NC 27710, USA
e-mail: Kaddu001@mc.duke.edu

Introduction

The role of neurotransmitter systems in cocaine addiction, although recognized (Engblom et al. 2008; Oleson et al. 2009; Patkar et al. 2008), has not been comprehensively evaluated with respect to mechanisms of toxicity and addiction (Kalivas and Volkow 2005). Investigations of neurobiological actions of cocaine usually center on receptor binding and enzyme inhibition, with limited attention to events such as aberrant metabolism, oxidative stress, signaling disturbances, or membrane dysfunction. Metabolomics is a rapidly growing discipline that permits simultaneous evaluation of multiple metabolites to uncover the changes in the relevant biochemical pathways. Mapping these metabolic "signatures" can provide new insights into addictive mechanisms and potentially identify biomarkers and therapeutic targets. Sophisticated metabolomic analytical platforms and informatics tools now make it possible to define signatures for disease and pathways implicated in central nervous system disease processes, such as motor neuron disease, schizophrenia, depression, and Parkinson's disease (Bogdanov et al. 2008; Kaddurah-Daouk and Krishnan 2009; Kaddurah-Daouk et al. 2007, 2008; Kristal et al. 2007; Paige et al. 2007; Rozen et al. 2005; Yao and Reddy 2005).

To date, there have been no published data investigating cocaine addiction utilizing a metabolomic approach. We employed a targeted metabolomic approach for this study. The rationale to study the metabolites of the tryptophan/tyrosine/purine/and oxidative stress pathways is as follows: Data from animal experiments suggest that modifications in 5-HT activity may contribute to drug reinforcement (Burmeister et al. 2003; Hall et al. 2004; Przegalinski et al. 2003). Neurobiological studies in humans tend to support these findings (Buydens-Branchey et al. 1999; Jacobsen et al. 2000; Little et al. 1998). Moreover serotonergic disturbances appear to be linked to behavioral measures that may have treatment implications (Patkar et al. 2003). Interestingly, aberrant degradation in the serotonin pathway leading to increase in *n*-methylserotonin and its metabolite bufotenine, compounds with psychotomimetic properties, has been implicated in psychiatric disorders (McBride 2000; Takeda et al. 1995). Methylation of tryptamine has been important in the development of designer drugs with hallucinogenic and potentially neurotoxic effects such as *N*,*N*-dimethyltryptamine (DMT), 5-methoxy-*N*,*N*-diisopropyltryptamine ("foxy"), and *N*-methyltryptamine (Nakagawa and Kaneko 2008). Cocaine exposure has been also found to alter arylalkylamine *N*-acetyltransferase expression leading to changes in synthesis of acetyl serotonin and melatonin, metabolites that may play a role in behavioral sensitization to cocaine (Akhisaroglu et al. 2004; Uz et al. 2005). Cocaine metabolism can lead to oxidative stress through generation of reactive oxygen species and effect on the purine (adenosine/guanine/ xanthine/hypoxanthine) pathways (Kovacic 2005). Oxidative stress has been implicated in the toxic effects of cocaine on the brain, e.g., intoxication and convulsions (Sharan et al. 2003), heart (Isabelle et al. 2007), the genes (Yu et al. 1999), and the fetus (Lipton et al. 2003). There is also evidence that alterations in key neurotransmitters can both be modified by and contribute to oxidative stress (Yao and Reddy 2005).

The aims of this study were to (a) examine the differences in plasma metabolites related to tryptophan (serotonin and kynurenine), tyrosine (dopamine and catecholamines), and purine (adenosine, guanine, and xanthine) pathways between cocaine-dependent subjects and healthy controls and (b) study the relationships between the metabolic changes and clinical measures of addiction. Several of these metabolites serve as biological indices underlying the mechanisms of reinforcement and toxicity, and such data could help to better understand the effects of cocaine in humans and their clinical implications.

Methods

Subjects

Eighteen subjects were recruited from patients attending a publicly funded, intensive outpatient cocaine treatment program. The protocol was approved by the Institutional Review Board of the University, and written informed consent was obtained from subjects. The Structured Clinical Interview (SCID) for DSM-IV Axis I Disorders (First et al. 1997) was then administered to volunteers. Inclusion criteria were axis I diagnosis of cocaine dependence and at least 2 weeks of abstinence to minimize the effects of recent drug use on biological parameters. Abstinence for 2 weeks was monitored by urine drug screens three times a week for 2 weeks. Exclusion criteria were a diagnosis of schizophrenia, bipolar disorder or major depression, unstable medical disorders, pregnancy, or treatment with psychotropic medication in the previous 4 weeks. Subjects who used or abused other substances (except nicotine) were included only if their primary drug was cocaine. Urine drug screens and breath alcohol levels were obtained for all subjects.

Ten controls were recruited from those responding to local advertisements. Consent and screening procedures were similar to those followed for cocaine subjects. Control subjects were excluded if they had a history of substance abuse or dependence (except nicotine), a major psychiatric disorder, an unstable medical illness, a positive urine drug

screen, or who were taking psychotropic medications in the previous 4 weeks.

Clinical assessments

Severity of drug use in cocaine subjects was assessed using the Addiction Severity Index (ASI) (McLellan et al. 1992). The ASI is a 30–40-min structured interview assessing seven problem areas in substance-dependent persons. For each domain, a composite score that ranges from 0 (minimum) to 1 (maximum) is provided to assess the adequacy of functioning in these areas during the previous 30 days. Cigarette smoking was assessed using the Fagerstrom Test for Nicotine Dependence (FTND), a widely used and validated six-item questionnaire to assess severity of smoking (Heatherton et al. 1991). Depressive symptoms were assessed by the Beck Depression Inventory, a 21-item self report (Beck and Steer 1987).

Sample preparation and metabolomic analysis

Samples were prepared for analysis by extraction in acidified acetonitrile and analyzed using a liquid chromatography electrochemical array platform (LCECA) as previously described (Bogdanov et al. 2008; Kristal et al. 1998; Rozen et al. 2005; Shi and Palmer 2002; Vigneau-Callahan et al. 2001). Briefly, 20 cc of freshly drawn blood with anticoagulant citrate dextrose was centrifuged at 750×*g* for 7 min to remove red blood cells and stored at −80°C. Archived plasma was brought to 0°C and a 250-µl sub-aliquot of stored sample was immediately mixed with 1 ml of acetonitrile/0.4% acetic acid at −25°C and vortexed for 45–60 s, then the temperature was brought to −15°C in a cold block, and vortexed again for 30–45 s. Samples were centrifuged for 15 min at 12,000×*g* at 4°C. One milliliter of the resulting supernatant was transferred to a 2-ml screw top vial and evaporated to dryness using centrifugal evaporation. Sufficient vacuum was generated to freeze the sample during this step. The sample was reconstituted in 200 µl of mobile phase A, and 100 µl were loaded two autosampler vials, one of which was archived at −80°C. The time/temperature sequence of the extraction and reconstitution protocol preserves the easily oxidized species in an LCECA profile of plasma. Immediate mixing of plasma with −25°C acetonitrile was performed to stop any biochemical/chemical degradation. At −25°C, the plasma/acetonitrile mixture (ca. 83% acetonitrile) is liquid, and extraction has been shown to be ca. 100% efficient at −15°C (Kristal et al. 1998; Vigneau-Callahan et al. 2001). Profiles are stable in acetonitrile extract, dried extract, and mobile phase-diluted extract. During the sample preparation, pools were created from equal volumes of sub-aliquots of all samples. All assays were run in sequences that include ten samples, authentic reference standard mixtures of 80 known compounds, pools of all samples, and duplicate preparations of the same sample. Duplicates were spaced at short and long intervals through the run to reflect the performance of the total data base. Run orders of all samples in this study were randomized. The sequences minimized possible analytical artifacts during further data processing. Pools and duplicates were used to access the precision of the entire data set. Additionally, the pools were used as references for time normalization (stretching). A practical advantage of LCECA for this study is the relative freedom from maintenance events. This is important for the generation of consistent databases from large numbers of samples over extended time periods. In our prior work, we have run LCECA systems continuously for 24 h/day over 6 months without significant technical problems.

Data generation and analyses

All chromatograms in the study were background-corrected (BC) to eliminate the base line drift inherent in gradient profiles. By controlling analytical conditions, the location of any particular peak in a 16-channel 110 min chromatogram was held within ±5–30 s through the study. BC files were then sequentially time-normalized against a single pool in the middle of the study sequence. A two-step stretching protocol with a multitude of peaks was used. First, proprietary software (ESA, CEAS 512) was used to align 15–20 major peaks in the chromatogram and interpolate the positions between them. Then, an additional 20–25 smaller peaks present in most samples were selected from the derivative file, and those were realigned, keeping the major peaks in the same position. Selected peaks were aligned within ±0.5 s and non-selected peaks within ±1–1.5 s over the entire 110 min assay.

First, all responses matching the retention and EC signature of compounds in the reference standard were exported in concentration units of nanogram per millimeter. Second, all responses matching resolved peaks in the pool of all samples were exported in terms of their relative response to the pool value. The concentrations of these are subsequently estimated by the total coulombs in the peak assuming a molecular weight of 200 and a 2-electron charge transfer.

Statistical analyses

Cocaine-dependent subjects and controls were compared on clinical and demographic variables using two-tailed *t* tests, Wilcoxon rank-sum tests, and Fisher's exact tests as appropriate. The Wilcoxon rank-sum test (equivalent to the Mann–Whitney *U* test) was employed to compare the metabolite levels between the cocaine-dependent persons

and controls because the metabolite levels are not normally distributed. When multiple statistical tests are carried out simultaneously, it is necessary to take into consideration the possibility of obtaining a low p value by chance. We addressed this by computing Q values (Storey 2003) corresponding to the p values. The Q values estimate cumulative false discovery rates (FDR) in the context of multiple tests. FDR methodologies, which focus on the balance of false and true positives, address the issue of multiple testing and maintain the power of the experiment in detecting meaningful results (Karp et al. 2007). There are standard implementations for computing Q values, and they are easy to interpret. The strength of Q values is that they allow the experimenter to choose a FDR that is acceptable for subsequent studies, for example, orthogonal validation techniques. We constructed a logistic regression model to identify variables that predict classification of the cocaine and control groups. For logistic regression, we used the R function glm() (www.r-project.org). We began with a large model that included as independent variables the compounds that showed the most significant differences between the controls and the cocaine-dependent subjects. Specifically, this starting model included the variables in Table 2, plus xanthosine, tyramine, and guanosine (p values for differences between cocaine-dependent patients and controls of 0.051, 0.058, and 0.093, respectively). To find a minimal model from among these variables, we used the function stepAIC from the R package MASS (Venables and Ripley 2002) with the argument, direction="both". This function searches for statistical models with the objective of minimizing the Akaike information criterion (AIC). The AIC is designed to balance the accuracy of a model with its complexity (the number of independent variables). Thus, stepAIC searches for a model that provides a good statistical fit but that avoids over-fitting stemming from inclusion of too many independent variables. This is important, especially in situations such as the present analysis, in which there many potential independent variables compared to the number of observations (patients and controls). In these situations, by including a large number of independent variables, it is possible to derive models that are overly optimistic, in the sense that they provide a better fit to the observations in hand than they would on data from a new set of observations.

In addition, we also estimated how well the model would perform on new data: its "generalization error." We used two approaches: sevenfold cross-validation and leave-one-out bootstrapping (Hastie et al. 2001). Both approaches estimate the generalization error by repeatedly fitting models using a subset of the subjects and then using these models to predict the patient or control status of the remaining subjects. For sevenfold cross-validation, we permuted the samples 1,000 times, by reordering them while maintaining their status as patient or control. For each permutation, we divided the samples into seven groups of four. Then, for each group, we built a predictor using the other 24 samples and predicted the classes (control or subjects) for the four samples in the group. The reported error rate (<0.002) is the average over all groups and all replicates. For leave-one-out bootstrapping, we report the "0.632 estimator" defined in equation 7.51 of the publication (Hastie et al. 2001), based on 500 bootstrap replicates. Leave-one-out bootstrapping is based on multiple replicates of random resampling of the observations (subjects), with replacement. For each replicate, the model is fit to the resampled observations and then used to predict the status (control or patient) of the subjects not in the resampled set. The "0.632 estimator" includes a correction to account for the fact that models built on resampled data are based on fewer observations than a model based on all the observations and are therefore probably not as accurate. The code for cross-validation and leave-one-out bootstrapping, as well as the code used to generate Fig. 1, is available on request.

To examine the relationship between clinical measures (ASI scores) and metabolites, multivariate linear regression analyses were used to predict the outcome (ASI drug domain score) from a combination of metabolites.

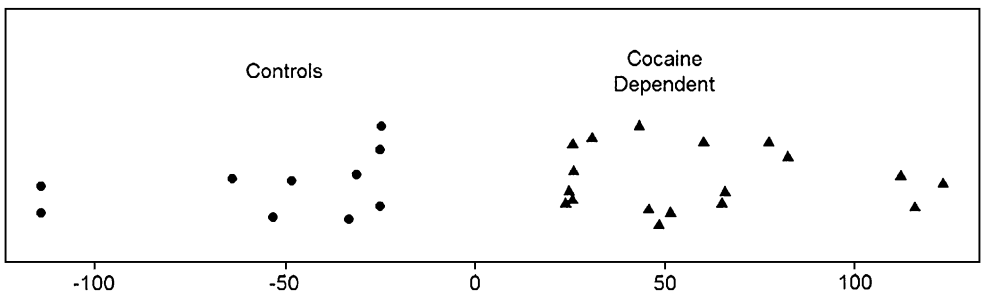

Fig. 1 Logistic regression on the four compounds *n*-methylserotonin, guanine, xanthine, and xanthosine separates healthy controls from cocaine-dependent subjects. *Circle* Control. *Triangle* Cocaine-dependent. The x axis is the untransformed response variable of the underlying linear regression on the four compounds, in other words, $\log_e(p/(1-p))$, where p is the predicted probability that a given subject is cocaine-dependent

Table 1 Baseline characteristics of the sample

Baseline characteristics	Cocaine (n=18) mean (SD) or count (%)	Controls (n=10) mean (SD) or count (%)
Age	35.0 (7.22)	35.0 (10.76)
Female	6 (33.3%)	4 (40%)
African American*	16 (88.9%)	3 (30%)
Cigarette smokers**	15 (83.3%)	3 (30%)
Mean cigarettes/day***	10.3 (6.96)	3.5 (5.80)
Addiction Severity Index****		
Alcohol	0.09 (0.05)	0
Drug	0.12 (0.08)	0
Employment	0.74 (0.14)	0
Family	0.13(0.06)	0
Legal	0.05 (0.03)	0
Medical	0.18 (0.08)	0
Psychological	0.14 (0.12)	0
Urine positive for THC	3 (16.7%)	0

*p=0.003, Fisher's exact test

**p=0.01, Fisher's exact test

***p=0.02, Wilcoxon rank-sum test

****p<0.0002 for each component of the Addiction Severity Index, Wilcoxon rank-sum test

We arrived at multiple linear regression models by starting with a model that contained as independent variables the compounds and compound ratios with nominally significant p values between the cocaine-dependent subjects and controls, which are shown in Table 2. We then proceeded by backward elimination of model variables with p values >0.05. We generated models in this way for the group of all 18 patients and for the 16 African-American patients. We used the R lm function for these models.

Results

Sample characteristics

Data are presented on 18 cocaine-dependent subjects and ten controls who had participated in study. Table 1 summarizes the clinical characteristics of the sample.

The subjects and controls did not differ significantly in gender or age. However, there was a greater proportion of African-American individuals in the cocaine-dependent

Table 2 Distribution of metabolites between patients with cocaine dependence and the controls

Metabolites	Cocaine (n=18)	Controls (n=10)	P value	Q value
Hypoxanthine	1410 (407)	3520 (2370)	0.000173	0.00718
n-Methylserotonin	0.26 (0.15)	0.09 (0.05)	0.00168	0.0302
Anthranilate	6.34 (4.16)	11.98 (5.02)	0.00233	0.0302
Guanine	2.56 (1.17)	1.26 (1.12)	0.00304	0.0302
Hypoxanthine over xanthine	3.29 (1.00)	5.01 (1.98)	0.00364	0.0302
Xanthine over xanthosine	667 (632)	1190 (646)	0.00723	0.0499
Xanthine	448 (136)	675 (276)	0.0116	0.0687
4-Hydroxyphenylacetate	172 (234)	51 (21)	0.0135	0.0699

Values represent means and SD. Statistical analysis was done with Wilcoxon rank-sum tests. The distribution of all other investigated metabolites and metabolite ratios, xanthosine, tyramine, guanosine, norepinephrine, 2-hydroxyphenylacetic acid, 3-hydroxyanthranilic acid, *n*-acetylserotonin, oxidized glutathione, L-dopa, tryptophol, 3-hydroxykynurenine, cystine, tryptophan/kynurenine, reduced glutathione/oxidized glutathione, 5-hydroxytrptophan, vanillylmandelic acid, tryptophan/4-hydroxyphenyllactic acid, gamma tocopherol, homogentisic acid, methionine, serotonin, methoxy-hydroxyphenly glycol, homovanillic acid/5-hydroxyindoleacetic acid, reduced glutathione, 4-hydroxybenzoic acid, tryptophan/tyrosine, tryptophan, 4-hydroxyphenyllactic acid, 5-hydroxyindoleacetic acid, kynurenine, 3-o-methyldopa, tyrosine, alpha tocopherol, 2,4-dihydroxyphenylacetic acid, homovanillic acid, and uric acid were not significantly different between patients with cocaine dependence and the controls (p>0.05 uncorrected for multiple hypothesis testing).

group compared to controls. Nearly 85% of subjects fulfilled all seven DSM-IV criteria of cocaine dependence. The remaining 15% fulfilled three to six DSM-IV criteria for cocaine dependence (minimum of three criteria required for diagnosis). Subjects were abstinent from cocaine for 19.8±5.8 days prior to the blood draw as confirmed by self-report and urine drug screens. No patient or control had received any psychotropic medications for at least 4 weeks.

Reflecting the patient population in clinical settings, a significant proportion of the cocaine-dependent subjects displayed additional substance abuse diagnoses. Eighty-three percent of cocaine-dependent patients smoked cigarettes, on average about ten cigarettes/day, and three subjects had tetrahydrocannabinol (THC)-positive urine, indicating use of marijuana in the previous 6 weeks. None of the subjects were positive for alcohol on breathalyzer test.

Metabolic difference between cocaine-dependent subjects and controls

We analyzed the blood plasma of individuals with cocaine dependence and healthy controls using electrochemical detection, which consists of high-performance liquid chromatography separation followed by with coulometric array detection. This technique separates compounds in two dimensions: by hydrophobicity and oxidation potential. Chromatographic data were reduced to tabular form by initial signal processing, which included aligning the chromatograms in the temporal dimension, followed by the use of peak heights as estimators of compound concentration. We obtained relative concentrations for 37 metabolites derived from the tryptophan/tyrosine/serotonin and purine (adenine/guanine/hypoxanthine/xanthine/xanthosine) pathways.

We found that cocaine-dependent subjects showed significantly higher levels of *n*-methylserotonin ($p<0.0017$, $Q=.03$) and guanine ($p<0.003$, $Q=0.03$) and lower concentrations of hypoxanthine ($p<0.0002$, $Q=0.007$), anthranilate ($p<0.003$, $Q=.03$) and a trend effect for xanthine ($p<0.012$, $Q=.07$), compared to controls (Table 2). The hypoxanthine/xanthine ratio was lower, while the xanthine/xanthosine ratio was higher in cocaine subjects compared to controls. The Q values reported here are estimates of cumulative FDRs (Storey 2003), and we report them to account for multiple hypotheses testing, that is, the comparison of multiple compounds between patients and controls.

Because three cocaine-dependent subjects had THC in urine, we compared the three THC-positive cocaine subjects with the remaining 15 cocaine subjects without any THC in the urine. We observed differences between several compound levels that were nominally significant, that is, had $p<0.05$ when each statistical test was considered in isolation and without taking multiple hypotheses testing into consideration. However, analysis accounting for multiple hypothesis testing showed that no compound had a Q value <0.27.

Two compounds that were nominally significantly different in this comparison were also significantly different between patients and controls: hypoxanthine and guanine. These differences did not contribute to the differences between patients and controls, however. Hypoxanthine was higher in the THC-positive patients than in THC-negative patients, even though hypoxanthine was lower in patients compared to controls. Guanine was lower in THC-positive patients than in THC-negative patients, even though guanine was higher in patients compared to controls.

There was also a larger proportion of cigarette smokers among patients than controls. However, there is no evidence that this contributed to the differences in compound levels between patients and controls. Cigarette smokers ($n=15$) and non-smokers ($n=3$) among the cocaine group showed nominally significant differences in several compounds, although after considering multiple hypothesis testing, no compound had a Q value <0.65. Among the compounds with nominally significant differences between smokers and non-smokers, only anthranilate was significantly different in patients compared to controls. Among patients, anthranilate was higher in smokers than non-smokers, even though anthranilate was lower in patients compared to controls.

Logistic regression model to separate cases from controls

We next analyzed these data to determine if the metabolites were capable of distinguishing cocaine-dependent individuals from controls. We developed a logistic regression model by using the AIC, as discussed in "Methods," to guide simplification of a starting model that contained the metabolites that were different between the two groups at $p<0.1$. We determined that a regression model with four compounds, *n*-methylserotonin (coefficient=190, SE=2.46, $p<10^{-15}$), guanine (coefficient=21.7, SE=0.283, $p<10^{-15}$), xanthine (coefficient=0.148, SE=0.0021, $p<10^{-15}$), and xanthosine (coefficient=41.3, SE=0.560, $p<10^{-15}$), separated the 18 cocaine-dependent subjects from the ten controls with no overlap between the two groups. Figure 1 summarizes this result.

We used two techniques, detailed in "Methods," to estimate what the error rate of this model would be on new subjects, in other words, its generalization error rate: The mean sevenfold cross-validation estimate is <0.002 and the leave-one-out bootstrap estimate is <0.019 (Hastie et al. 2001). Consistent with these low error estimates, the AIC value for this model is 10, which provides additional evidence that the model is accurate and not likely to be

overfit. We also investigated whether demographic variables were contributing to the differences between the two groups. Including age, sex, race, and their interactions as explanatory variables does not improve the model, and simplifying from the model that includes age, sex, race, and their interactions yields the four-compound model above.

Relationship of metabolites to severity of drug use

We performed multivariate linear regressions to determine the contribution of metabolites that were different between cases and controls toward predicting severity of drug use. Because ASI composite scores include multiple domains, some of which may not be directly related to drug use (e.g., employment and legal scores), we only included ASI drug scores as a measure of severity of cocaine use. We found that a model using *n*-methylserotonin ($p=1.3\times10^{-5}$) and xanthine ($p=.03$) as independent variables accounted for 73% of the variance in ASI drug score. Table 3 summarizes this model. *n*-Methylserotonin in a simple linear regression accounted for 62% of the variance in ASI drug score.

We repeated this analysis for the 16 African-American patients and found that a model with the independent variables *n*-methylserotonin ($p<7\times10^{-6}$), hypoxanthine/xanthine ($p<0.008$), anthranilate ($p<0.018$), xanthine ($p<0.025$), and 4 hydroxyphenylacetate ($p<0.039$) explained 88% of the variance in ASI drug score. *n*-Methylserotonin in a simple linear regression accounted for 61% of the variance in ASI-drug score in the African Americans.

Discussion

To the best of our knowledge, this is the first study of cocaine addiction in humans utilizing the metabolomics approach. There were three key findings from this study: First, cocaine-dependent subjects showed significantly higher concentrations of *n*-methylserotonin and lower concentrations of anthranilate compared to controls, suggesting alterations in the tryptophan metabolism. Second, higher concentrations of guanine and lower concentrations of hypoxanthine and xanthine were seen in cocaine-dependent subjects compared to controls, suggesting changes in purine metabolism. Finally, higher *n*-methylserotonin and, to a lesser extent, lower xanthine were associated with higher ASI drug scores, a clinical measure of severity of cocaine use.

Alterations in tryptophan metabolism

Figure 2 summarizes the key aspects of metabolic pathways of tryptophan and the changes observed in cocaine subjects versus controls. The strongest and most consistent finding was the association of elevated *n*-methylserotonin with cocaine exposure.

It was also strongly predictive of severity of cocaine use and, together with xanthine, explained approximately 73% of variance in severity of cocaine use. It is worth noting that this elevation was observed after a minimum of 2 weeks of documented abstinence from cocaine, indicating that elevated levels of *n*-methylserotonin may be less related to acute effects of cocaine and more likely to be associated with chronic cocaine exposure.

We found no significant changes in the tryptophan, serotonin, or 5-HIAA levels, suggesting that the biosynthesis of serotonin from tryptophan or its inactivation through oxidative deamination into 5HIAA was unaffected. However, it appears that aberrant methylation of serotonin to produce elevated levels of *n*-methylserotonin may be occurring following chronic cocaine exposure. An enzyme that *n*-methylates serotonin by transferring a methyl group from *S*-adenosylmethionine to the amino group of serotonin has been found in rabbit lung and parotid gland of the *Bufo marinus* toad (Axelrod 1962). The enzyme also *N*-methylates a variety of other compounds such as dopamine and tryptamine and is inhibited by the antipsychotic drug chlorpromazine, a dopamine antagonist. Whether this enzyme may play a role in the formation of *n*-methylserotonin in cocaine-dependent individuals merits further investigation. It is also possible that the pathway that transforms serotonin into melatonin may be affected. This process involves acetylation of the amine group by *N*-acetyl transferase, leading to *N*-acetyl-serotonin and subsequent methylation of the OH group by 5-hydroxyindole-*O*-methyltransferase to obtain acetyl-5-methoxytryptamine or melatonin. There was a trend toward increased levels of *N*-acetylserotonin in cocaine subjects compared to controls, and it is possible that the activity of the enzymes involved in this pathway may be also altered.

While the precise effects of *n*-methylserotonin are not known, it is interesting to note that it is a precursor of *N*, *N*-dimethyl-5-hydroxytryptamine (bufotenine), a hallucinogenic dimethylated indolethylamine formed from serotonin (Chilton et al. 1979). Bufotenine is structurally similar to the hallucinogens psilocybin and DMT and has approximately the same affinity for 5HT2A and 5HT2C receptors

Table 3 Multiple regression analysis for predictors of severity of cocaine use

Cocaine use	Variable	Model coefficient	*t*	*p* value	*R*
ASI-Drug	*n*-Methylserotonin	0.53	6.4	1.3×10^{-5}	0.85

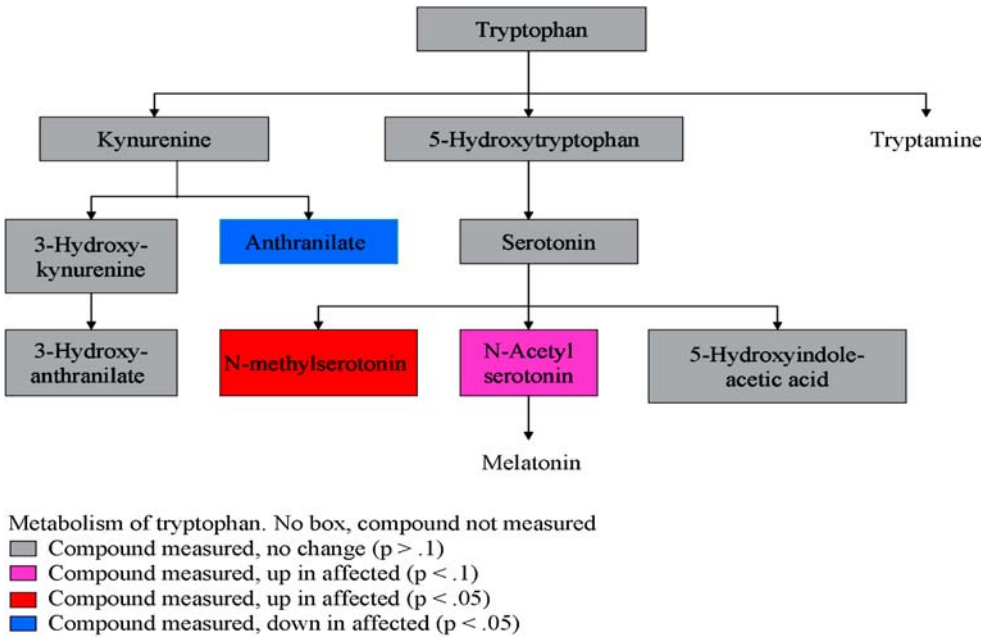

Fig. 2 Metabolism of tryptophan. *No box* Compound not measured

as lysergic acid diethylamide (Karch 2006). In several species of toads, bufotenine and *n*-methylserotonin are concentrated in brain, blood, skin tissues, venom, and urine (Takeda et al. 1995). There have been case reports of people abusing bufotenine (ingesting toad venom or smoking dried toad skin) for its psychotomimetic effects (Hitt and Ettinger 1986). The US Drug enforcement Administration has classified bufotenine as a schedule I controlled substance, indicating very high abusable properties. The elevated levels of *n*-methylserotonin, which has 5HT2 A/C agonist properties in cocaine patients, is consistent with findings from neuroendocrine challenge studies in cocaine patients that employed metachlorophenylpiperazine, a 5HT2C/A partial agonist (Buydens-Branchey et al. 1997; Patkar et al. 2006). The clinical significance of the elevated *n*-methylserotonin in cocaine-dependent patients deserves further exploration, both in terms of a biological marker of cocaine use as well as its potential contribution to the psychotomimetic and reinforcing effects of cocaine.

Alterations in purine metabolism

Purines have been found to have key roles in neuromodulation and neurotransmission, cellular growth and signaling, and energy metabolism. Studies in stimulant addiction have been limited and primarily involved animal models. Knockout mice experiments have shown that the lack of adenosine 2A receptors diminishes the addictive-reinforcing efficacy of cocaine (Chen et al. 2000). In animals withdrawn from chronic treatment with either morphine or cocaine, there are persistent increases in extracellular adenosine in the ventral tegmental region, a brain region that is intimately involved in the rewarding effects of these drugs, leading to speculations that interactions between adenosine and dopamine receptors systems may underlie behavioral sensitization to stimulants (Burnstock 2009; Tsai 2005).

Figure 3 summarizes the purine metabolic pathway and the changes observed in cocaine subjects versus controls.

In cocaine-dependent subjects, the xanthine and hypoxanthine levels and hypoxanthine/xanthine ratios were lower, while guanine levels were higher compared to controls. While the findings do indicate alterations in purine metabolism, it appears that some metabolic pathways (e.g., adenine and inosine to xanthine) showed reduced catabolism, while others (e.g., xanthine to guanine and guanine to guanosine) showed enhanced catabolism. Such pattern of accumulation of certain purine metabolites and elimination of others is typical of chronic disease states, such as diabetes, gout, schizophrenia, epilepsy, and chronic kidney disease (Kristal et al. 1999; Lara et al. 2006; Puig et al. 1994; Vianna et al. 2002). There are suggestions that the purine metabolites may be involved in oxidative-stress-related toxicity of cocaine to the brain and heart (Kovacic 2005). While we did not find significant changes in oxidative-stress-related metabolites (e.g., glutathione), this could be because we did not study acute effects of cocaine.

Strengths and limitations

The strengths of the study were a well-characterized sample with DSM-IV diagnosis of cocaine dependence and exclusion of other comorbid psychiatric disorders and concomitant medications. The high-performance liquid

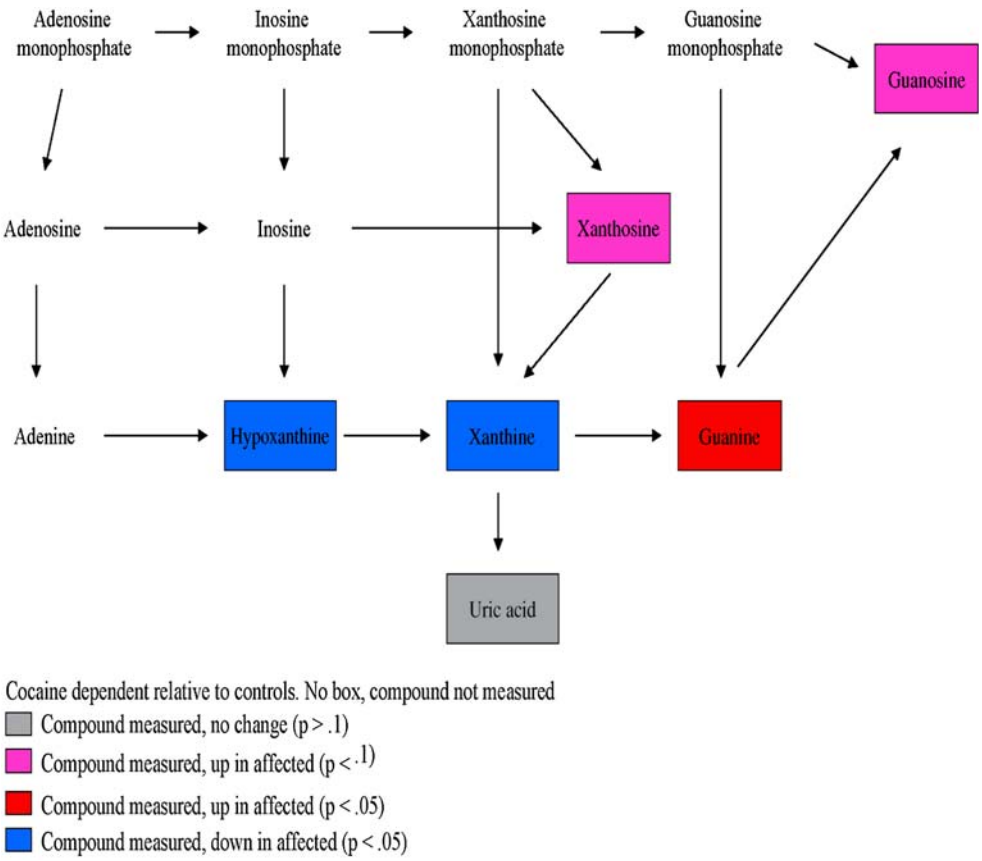

Fig. 3 Cocaine-dependent relative to controls. *No box* Compound not measured

chromatography assay has been validated with high sensitivity and quantitative precision and employed in other disease states (Bogdanov et al. 2008; Kristal et al. 2007). However, certain limitations of the study deserve comment. First, the number of metabolites that were studied was large relative to the number of study participants, raising the possibility of chance findings due to multiple comparisons. However, analysis of cumulative FDRs (Q values) indicates that differences in compound level between patients and controls are robust after accounting for multiple hypothesis testing. Another limitation is that, reflecting the "real-world" clinical setting, cocaine-dependent individuals had a history of using nicotine, marijuana, and alcohol. Whether these substances influence *n*-methylserotonin levels in humans is not known. While we monitored for abstinence from illicit drugs and alcohol for two weeks, statistical analysis of the data for effects of nicotine and THC was limited by the sample size. Therefore, we cannot exclude the possibility that prior use of these substances could have affected the findings. Moreover, there is a possibility that the changes in metabolites could have resulted from sudden abstinence in cocaine-dependent individuals. Third, dietary intake was not controlled and could have potentially affected the results. Fourth, the cases and controls were not matched on ethnic background; however, the logistic regression models suggest that cocaine dependence can be better modeled as a function of metabolite levels than as a function of ethnic background. Additionally, data derived from other studies using the LCECA platform shows that levels of *n*-methylserotonin are very low in healthy controls irrespective of ethnic background or gender. This suggests that observations made here with respect to *n*-methylserotonin are not related to the fact that majority of cocaine users were black males. Finally, *n*-methylserotonin levels explain, in a statistical sense, much of the ASI drug score in both the set of all cocaine-dependent patients and in the subset of African-American patients. Fifth, peripheral changes in metabolites in blood may not necessarily reflect comparable changes in the brain.

Conclusions and future directions

The accumulation of *n*-methylserotonin in subjects with chronic cocaine exposure indicates disturbances in methylation processes in the serotonin metabolic pathways. However, no causality can be inferred, given the cross-sectional nature of the study. Given the psychotomimetic effects of this compound and its metabolite bufotenine, further studies utilizing animal models and neuroimaging may clarify the role of this compound in cocaine addiction. The alterations in purine metabolites need to be further explored in replication experiments and in studies that also

determine changes in purine receptors and redox pathways. Finally, whether any of the metabolites can serve as biological markers in cocaine addiction need to be studied in prospective longitudinal studies. Metabolomics may prove to be an important approach for human studies related to addictive properties and neuropsychiatric effects of cocaine.

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