

Metabolic profiling of urine and blood plasma in rat models of drug addiction on the basis of morphine, methamphetamine, and cocaine-induced conditioned place preference

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Abstract The metabolic profiles of urine and blood plasma in drug-addicted rat models based on morphine (MOR), methamphetamine (MA), and cocaine (COC)-induced conditioned place preference (CPP) were investigated. Rewarding effects induced by each drug were assessed by use of the CPP model. A mass spectrometry (MS)-based metabolomics approach was applied to urine and plasma of MOR, MA, and

COC-addicted rats. In total, 57 metabolites in plasma and 70 metabolites in urine were identified by gas chromatography–MS. The metabolomics approach revealed that amounts of some metabolites, including tricarboxylic acid cycle intermediates, significantly changed in the urine of MOR-addicted rats. This result indicated that disruption of energy metabolism is deeply relevant to MOR addiction. In addition, 3-hydroxybutyric acid, L-tryptophan, cystine, and *n*-propylamine levels were significantly changed in the plasma of MOR-addicted rats. Lactose, spermidine, and stearic acid levels were significantly changed in the urine of MA-addicted rats. Threonine, cystine, and spermidine levels were significantly increased in the plasma of COC-addicted rats. In conclusion, differences in the metabolic profiles were suggestive of different biological states of MOR, MA, and COC addiction; these may be attributed to the different actions of the drugs on the brain reward circuitry and the resulting adaptation. In addition, the results showed possibility of predict the extent of MOR addiction by metabolic profiling. This is the first study to apply metabolomics to CPP models of drug addiction, and we demonstrated that metabolomics can be a multilateral approach to investigating the mechanism of drug addiction.

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Introduction

In general, drug addiction is defined as a biological status with loss of autogenous regulation as a result of drug abuse.

It is well known to encompass several behavioral stages: intoxication, bingeing, craving, and withdrawal [1, 2].

Various studies have been conducted to reveal the underlying mechanism of drug addiction, and it has become clear that some genes and proteins are important in drug addiction [1–6]. One of the latest studies reported that microRNA is also of crucial importance in determining vulnerability to drug addiction [7, 8].

However, the neurobiological changes accompanying and the molecular mechanism leading to drug addiction are not known in detail. This is because drug addiction status involves a wide range of biological compounds, for example genes, transcripts, and enzymes (proteins), and its mechanism is attributable to extremely complicated biological functions. Li et al. addressed the limitations or bias of individual techniques and studies on drug addiction; they insisted on the importance of comprehensive understanding across different techniques and studies not only on genes but also on other biological compounds, as mentioned above [4].

Recent rapid progress in analytical technology has succeeded in enabling bioinformatics studies on genes, transcripts, proteins, and metabolites, generically known as “omics sciences” [9, 10]. The metabolome, i.e. the total set of endogenous metabolites in an organism, is the “ome” which is inevitably closest to the phenotype. As is well known, metabolites are the end products and by-products of complicated biosynthetic and catabolism pathways, and their diversity is one of the most significant aspects of the metabolome. Thus, comprehensive analysis of the metabolome, termed metabolomics, is a powerful technique not only to complement data derived from genomics, transcriptomics, and proteomics, but also to investigate the phenotypic changes caused by exogenous stimuli more predictively than other omics approaches [9–11].

It is quite important to multilaterally understand the underlying mechanism of drug addiction. Metabolomics can possibly be used to investigate the mechanism from a different perspective. There have been reports of metabolomics studies of the blood or brain metabolic profiles of morphine-treated mice and abusers [12–15]. In particular, basic study using animal models of drug addiction is indispensable for appropriate investigation of the effect of addiction on metabolic profiles, because metabolic profiles are readily affected by such external factors as diet, environmental variation, and circadian fluctuation [9, 16, 17].

There have been many studies on the involvement of brain function in drug addiction, whereas its effect on the whole body has not been studied as much, even though valuable information might be available. Hidden effects of drug addiction may be revealed by applying metabolomics to urine or blood, because disorders of both the central and peripheral nervous systems could be reflected by changes in biological specimens. If there is a possibility that metabolomics can be used to detect drug addiction, then metabolic profiling of urine and/or plasma may become a valuable diagnostic tool for assessment and treatment of drug addiction.

Therefore, we examined the effect of drug addiction on the metabolic profiles of urine and blood plasma by using rat models of drug addiction. These models were trained by chronic treatment with morphine (MOR), *d*-methamphetamine (MA), and cocaine (COC), and were assessed properly by use of the conditioned place preference (CPP) model. In order to study their metabolic profiles in detail, an established mass spectrometry-based metabolomics technique was applied to the urine and plasma of rats.

Materials and methods

Animal experiment

Twenty-four male Sprague–Dawley rats (seven weeks old, weight 281–312 g) were purchased from Charles River Japan (Yokohama, Japan). This animal study was approved by the Animal Experimental Committee of Dainippon Sumitomo Pharma. Preparation of drug-addicted rats and the procedure of the CPP model were carried out in accordance with previous reports, with slight modification [18–20]. The dose administered was 4 mg kg⁻¹ MOR, 2 mg kg⁻¹ MA, and 10 mg kg⁻¹ COC, on the basis of the weight of each rat. The sequence of the animal experiment is shown in Fig. 1, and the details of the experiment are described in the [Electronic Supplementary Material](#).

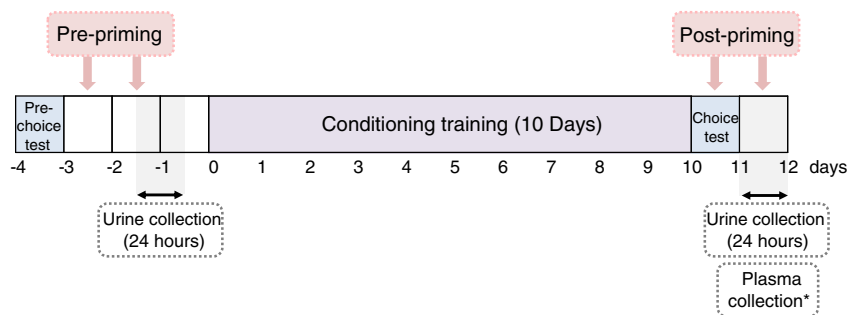
Sample collection and preparation

Urine was combined and stored under refrigeration with dry ice for 24 hours immediately after pre-priming or choice-test. Blood was drawn from the abdominal aorta under isoflurane anesthesia. After anticoagulation treatment with ethylenediamine tetraacetic acid dipotassium (EDTA-2 K) [21], blood plasma was obtained by centrifugation at 3000 rpm for 10 min at 5 °C. Urine and plasma were quickly frozen in liquid nitrogen and stored at –80 °C until analysis [22]. The extraction protocol was the same as described in previous reports [9, 11, 23–25], with slight modification; details are given in the [Electronic Supplementary Material](#).

Instrumental analysis and statistical analysis

Gas chromatography–mass spectrometric (GC–MS) analysis was performed as described elsewhere [24, 26]. The metabolites were identified by use of built-in software and an in-house metabolome library (Fukusaki laboratory). Principal component analysis (PCA) with autoscaling was conducted on normalized datasets from urine and plasma, by use of SIMCA-P+13 software (Umetrics, Umeå, Sweden). Statistical differences for potentially significant metabolites were

Fig. 1 Sequence of animal experiment and sample collection. The *asterisk* indicates blood was taken under anesthesia after urine collection



evaluated by significance test (Welch's *t*-test, significance level 0.05). The datasets were also used for projection to latent structures (PLS) regression. Detailed information of instrumental analysis, data processing, and construction of the PLS regression model and its validation are described in the [Electronic Supplementary Material](#).

Results

CPP paradigm

The results obtained from the CPP model are listed in Table 1. The CPP score obtained from rat no. 3 in the control group was an outlier (positive CPP score) among the others (negative CPP scores). The score for rat no. 20 in the cocaine group is also an outlier (negative score). Because these results suggested that the rats 3 and 20 may be affected by factors such as external stimulation, these results were omitted from subsequent use to maintain high experimental quality.

Identification of metabolites

In total, 57 metabolites in blood plasma and 70 metabolites in urine were identified by GC–MS. Table 2 lists all identified metabolites. Under the analytical condition used, some types of metabolite were not discriminated from each other (e.g. citric acid and *iso*-citric acid). In such cases, possible compound names were annotated for the same peak.

In both urine and plasma of rat no. 13 in the MOR-administered group, peak intensities of some of the identified metabolites showed highly irregular. Because this may be a result of intravital abnormality or other invisible causes, rat no. 13 was omitted from subsequent metabolomics study.

Metabolome variation in the MOR-addicted rats

Score plots of PCA based on GC–MS analysis of urine and plasma before and after MOR addiction are shown in Fig. 2. As shown in Fig. 2a, the two groups overlapped on the PCA score plot before MOR addiction. On the other hand, MOR-

addicted and control groups were clearly separated on the PCA score plot for urine samples after MOR addiction (Fig. 2b). The two groups were partially separated on the PCA score plot for plasma samples (Fig. 2c).

All identified metabolites were checked by use of the significance test. As shown in Fig. 3a, 3-hydroxybutyric acid, L-tryptophan, cystine, and *n*-propylamine levels changed significantly in the plasma of MOR-addicted rats.

Table 1 Results from the CPP model

Group	Rat no.	Box selected for drug administration	Amount of time spent in the box (s)		CPP score
			Black box	White box	
Control	1	White box	517.8	382.2	−135.6
	2	Black box	409.2	490.8	−81.6
	3	White box	364.3	535.7	171.4
	4	White box	548.0	352.0	−196.0
	5	Black box	361.0	538.0	−177.0
	6	White box	600.0	299.0	−301.0
Methamphetamine	7	White box	304.0	596.0	292.0
	8	Black box	496.5	403.4	93.1
	9	White box	393.2	506.8	113.6
	10	White box	337.3	562.7	225.4
	11	Black box	525.7	374.3	151.4
	12	White box	355.8	544.2	188.4
Morphine	13	White box	359.8	540.2	180.4
	14	Black box	625.1	274.9	350.2
	15	White box	408.6	491.4	82.8
	16	White box	402.4	497.6	95.2
	17	Black box	589.7	310.3	279.4
	18	White box	390.6	509.4	118.8
Cocaine	19	White box	369.4	530.6	161.2
	20	Black box	338.2	561.8	−223.6
	21	White box	385.5	514.5	129.0
	22	White box	430.5	469.5	39.0
	23	Black box	513.3	386.7	126.6
	24	White box	408.5	491.5	83.0

Table 2 Metabolites identified in plasma and urine by use of GC–MS

Plasma		Urine		
1,5-Anhydro-D-glucitol	L-Lysine	2-Aminoethanol	Glycolic acid	Pyruvic acid/ Oxalacetic acid
2-Aminobutyric acid	L-Methionine	2-Hydroxypyridine	Hippuric acid	Ribose
2-Hydroxybutyric acid	L-Ornithine	2-Ketoglutaric acid	Hydroxyproline	Spermidine
2-Hydroxypyridine	L-Proline	3-Hydroxybutyric acid	Hypotaurine	Stearic acid
α -Ketoglutaric acid	L-Serine	3-Hydroxyisovaleric acid	Indoxyl sulfate	Succinic acid
3-Hydroxybutyric acid	L-Threonine	4-Hydroxymandelic acid	acid Inositol	Threitol
Aconitic acid	L-Tryptophan	4-Hydroxyphenylacetic acid	Isoleucine	Uracil
Alanine	L-Tyrosine	5-Aminovaleric acid	Lactose	Uric acid
Allantoin	L-Valine	Aconitic acid	L-Asparagine	Xanthine
Citric acid/iso-Citric acid	Malic acid	Alanine	L-Aspartic acid	Xanthurenic acid
Cysteine	Mannose	Allantoin	L-Histidine	Xylitol
Cystine	n-Butylamine	Arabinose	L-Lysine	Xylose
Glucose	Nonanoic acid	Arabitol	L-Proline	
Glutamic acid	n-Propylamine	Ascorbic acid	L-Serine	
Glutamine	Oxalic acid	β -Alanine	L-Threonine	
Glyceric acid	Palmitic acid	Catechol	L-Valine	
Glycerol	Phenylalanine	Citramalic acid	Malic acid	
Glycine	Propyleneglycol	Citric acid/iso-Citric acid	Malonic acid	
Glycolic acid	Pyrophosphate	Creatinine	Maltose	
Heptadecanoic acid	Pyruvic acid/ Oxalacetic acid	D-Glucronic acid	N-Acetylglucosamine	
Hydroxyproline	Ribose	D-saccharic acid/ D-glucaric acid	N-Acetylglutamine	
Inositol	Spermidine	Erythritol	N-Acetyl-L-leucine	
Isoleucine	Stearic acid	Ethylmalonic acid	N- α -Acetyl-L-Lysine	
Ketovaline	Succinic acid	Fucose	n-Butylamine	
L-Asparagine	Uracil	Fumaric acid	Oxalic acid	
L-Aspartic acid	Urea	Glucose	Oxamic acid	
Lauric acid	Uric acid	Glutamic acid	Pantothenic acid	
L-Histidine	Xylitol	Glyceric acid	Pyroglutamic acid	
L-Leucine		Glycine	Pyrophosphate	

In urine, 2-ketoglutaric acid, fumaric acid, malic acid, and L-threonine levels were significantly increased whereas glutamic acid, isoleucine, L-valine, L-aspartic acid, oxamic acid, 2-aminoethanol, indoxyl sulfate, and creatinine levels decreased significantly (Fig. 3b). There were no significant differences between these metabolites in the urine of control and MOR groups before addiction (Fig. 3c).

Metabolome variation in the MA-addicted rats

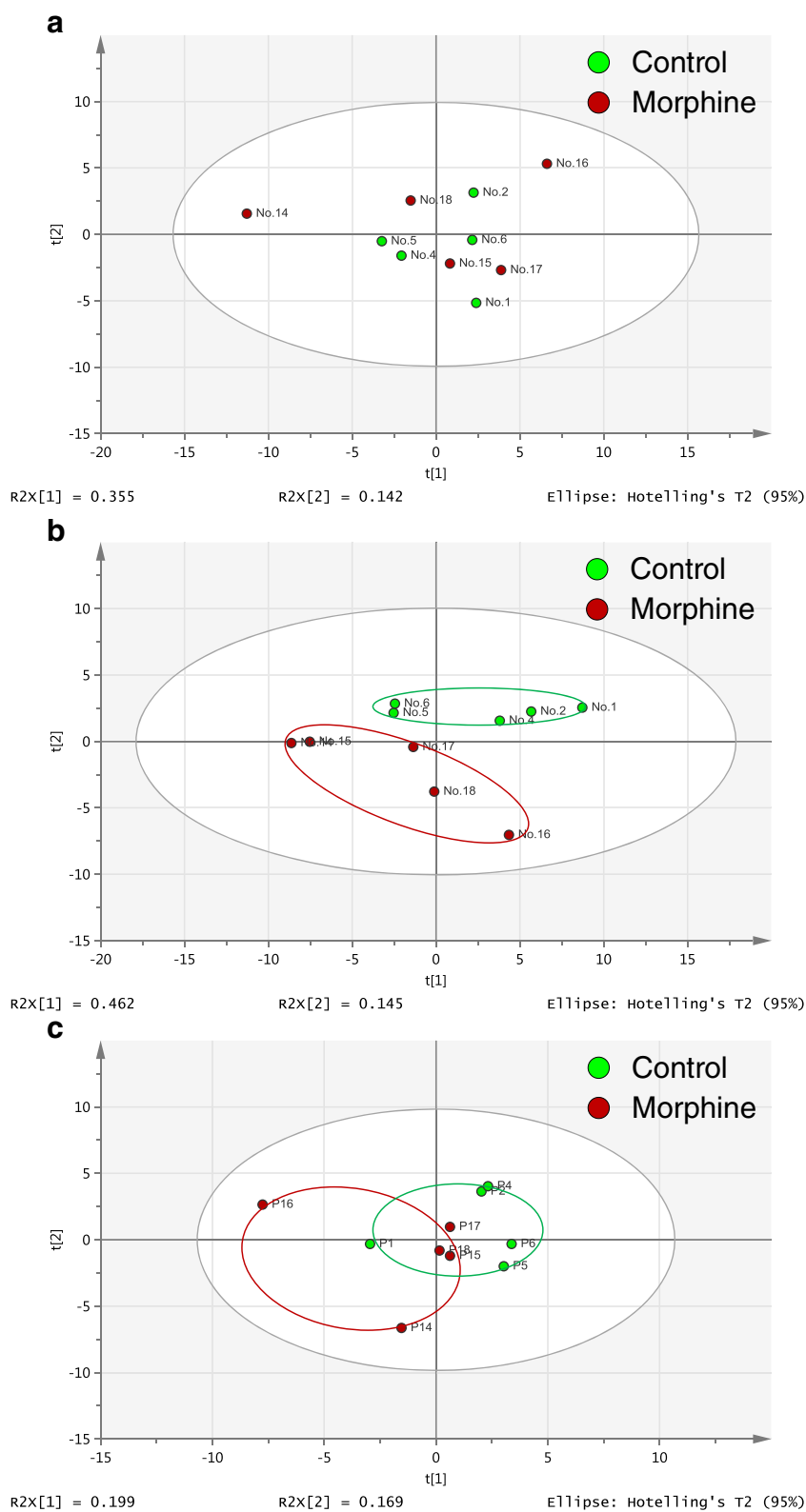
PCA score plots based on GC–MS analysis of urine and plasma before and after MA addiction are shown in Fig. 4. Results from all the rats overlapped before MA addiction, as shown in Fig. 4a. MA-addicted and control groups were not well-separated on PCA score plots for both plasma and urine after addiction (Figs. 4b and c).

The significance test for all the metabolites identified revealed significant changes in *n*-propylamine and lauric acid levels in plasma after MA addiction (Fig. 5a). In urine of the MA-addicted rats, lactose, spermidine, and stearic acid levels were significantly increased (Fig. 5b). There were no significant differences between amounts of these metabolites in the urine of control and MA groups before addiction (Fig. 5c).

Metabolome variation in the COC-addicted rats

Figure 6 shows the PCA score plots based on GC–MS analysis of urine and plasma before and after COC addiction. The results from the two groups overlapped on the PCA score plot before COC addiction (Fig. 6a). As shown in Fig. 6b, COC-addicted and control groups were not

Fig. 2 PCA score plots of urine and plasma of control and morphine groups. The control group and the morphine group are shown as *green* and *red* plots, respectively. **(a)** Urine before morphine addiction, and **(b)** urine and **(c)** plasma after morphine addiction. Each *ellipse* was given by Hotelling's T² (0.95)



separated on the PCA score plot for urine samples after COC addiction, though they were partially separated for plasma (Fig. 6c).

All identified metabolites were checked by use of the significance test. As shown in Fig. 7, L-threonine, cystine, *n*-propylamine, and spermidine levels changed significantly in

the plasma of COC-addicted rats, whereas no metabolites in urine fluctuated significantly after addiction.

Discussion

Assessment of the animal experiment

In metabolomics, careful experimental design is required if valuable results are to be obtained because metabolic profiles are subject to high temporal variability, for example circadian fluctuations, in an organism [9, 16, 17].

Therefore, animal experiments were specially designed to avoid the effects of circadian rhythms and other external stimulations, and the resulting animal models were properly evaluated by use of the CPP model, a well-established technique for assessing the rewarding effect of addiction-producing drugs [15, 27, 28]. The CPP model guaranteed that all the rats in this

metabolomics study achieved the rewarding effect: they were in a drug-addicted state. This solid animal model heightened the quality of the final results.

Post-priming treatment was performed before sample collection to avoid the effect of sudden loss of drugs. No pre or post-priming treatment effects on metabolome variation or individual differences were observed, because there was no significant difference between controls and each drug addicted-group before addiction; this was also confirmed by the overlap of results from the groups in the PCA score plots (Figs. 2a, 4a, and 6a).

Effect of MOR addiction on the metabolic profiles of urine and plasma

The concentration of L-tryptophan decreased significantly in the plasma of MOR-addicted rats. There have been reports of the relationship between MOR addiction and L-tryptophan concentration in brain or blood. Messing et al. reported an L-

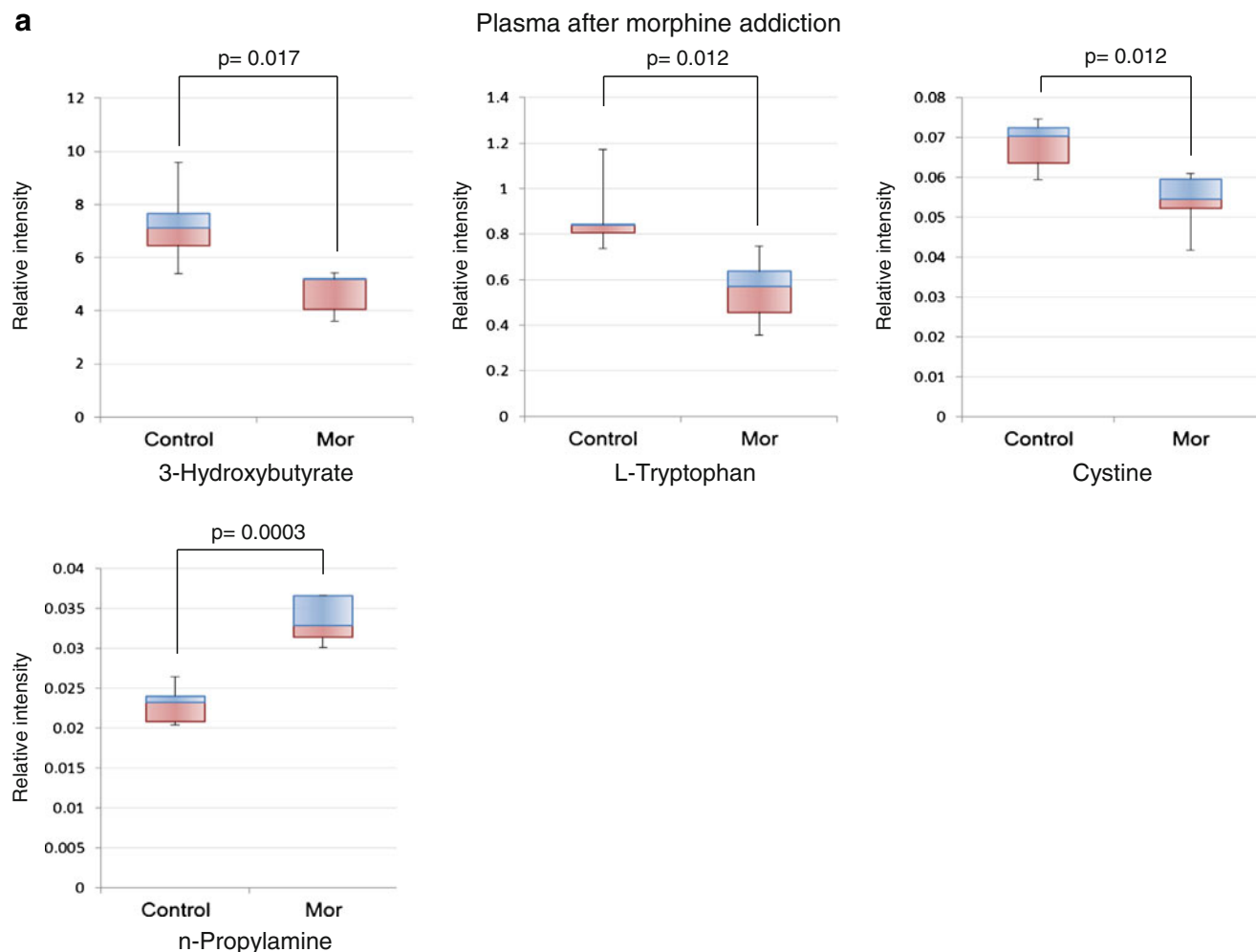


Fig. 3 (a, b) Box-and-whisker plots for significantly changed metabolites in plasma (a) and urine (b) after morphine addiction. (c) Box-and-whisker plots of the metabolites in urine before addiction. Whiskers extend to the extreme data points, and p values calculated by use of

Welch's test are shown for significantly different metabolites between controls and addicted rats. There was no significant difference for each metabolite before addiction

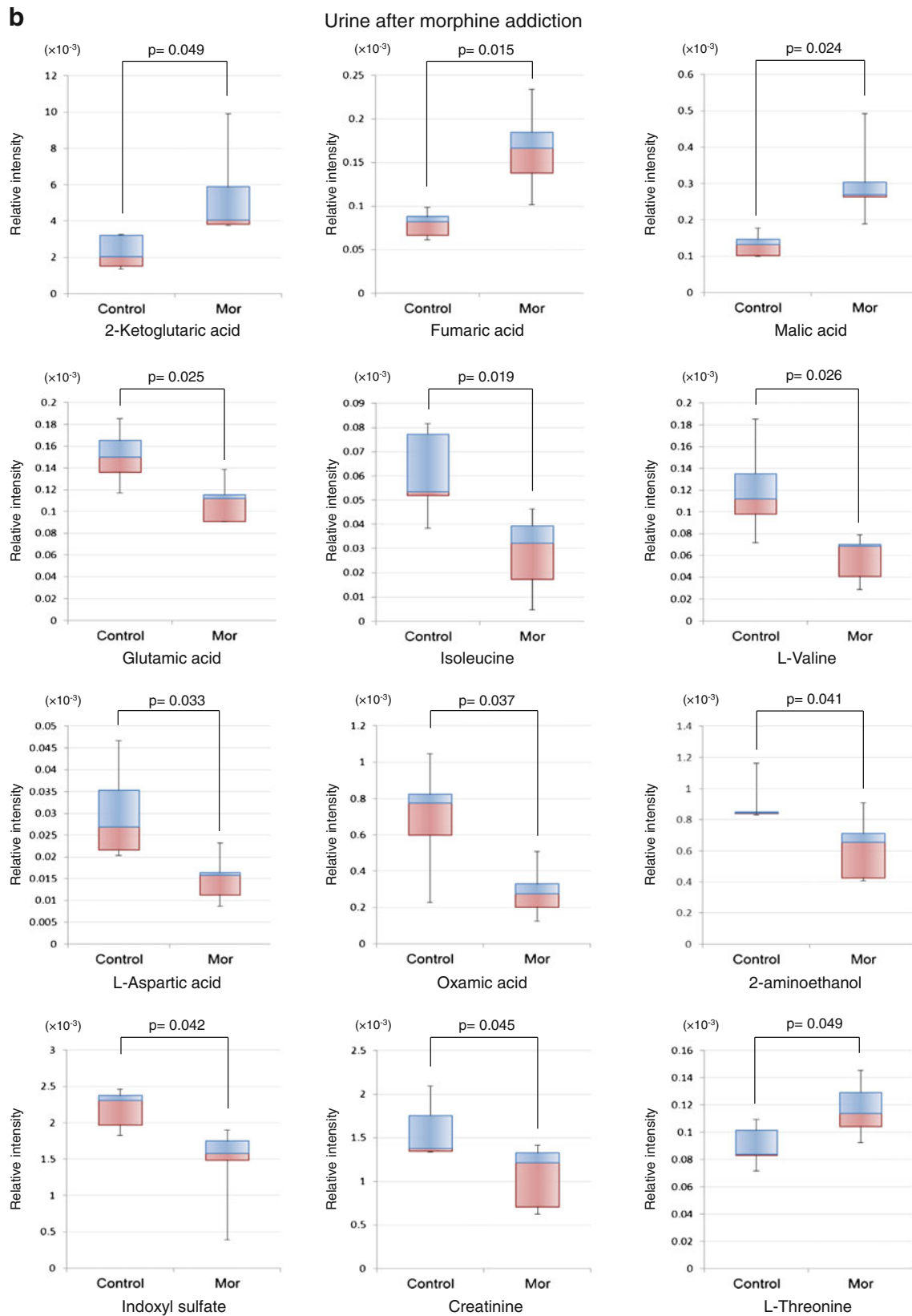


Fig. 3 (continued)

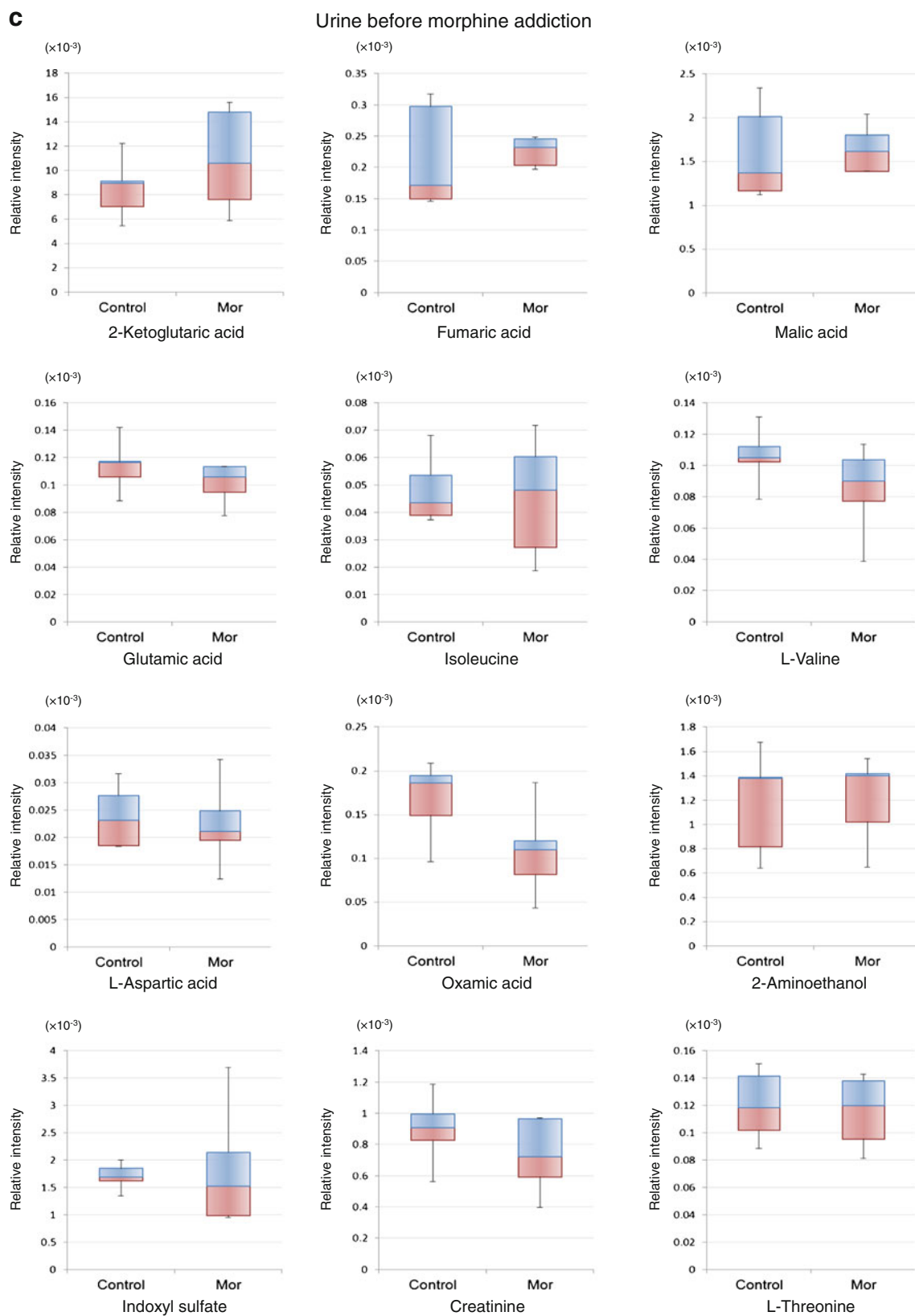
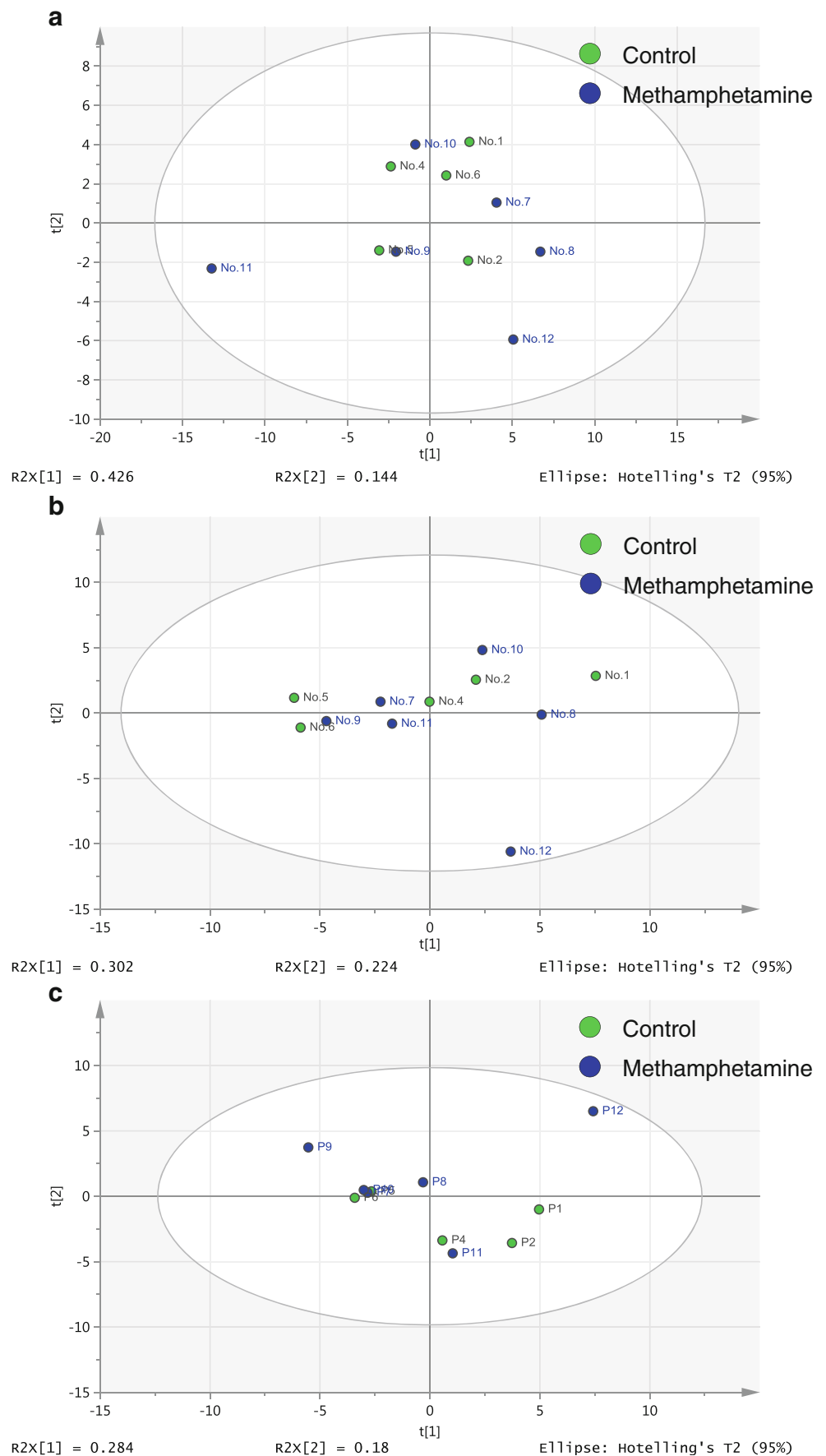


Fig. 3 (continued)

Fig. 4 PCA score plots for urine and plasma of control and methamphetamine groups. The control group and the methamphetamine group are shown in *green* and *blue* plots, respectively. **(a)** Urine before methamphetamine addiction, and **(b)** urine and **(c)** plasma after methamphetamine addiction. Each *ellipse* was given by Hotelling's T2 (0.95)



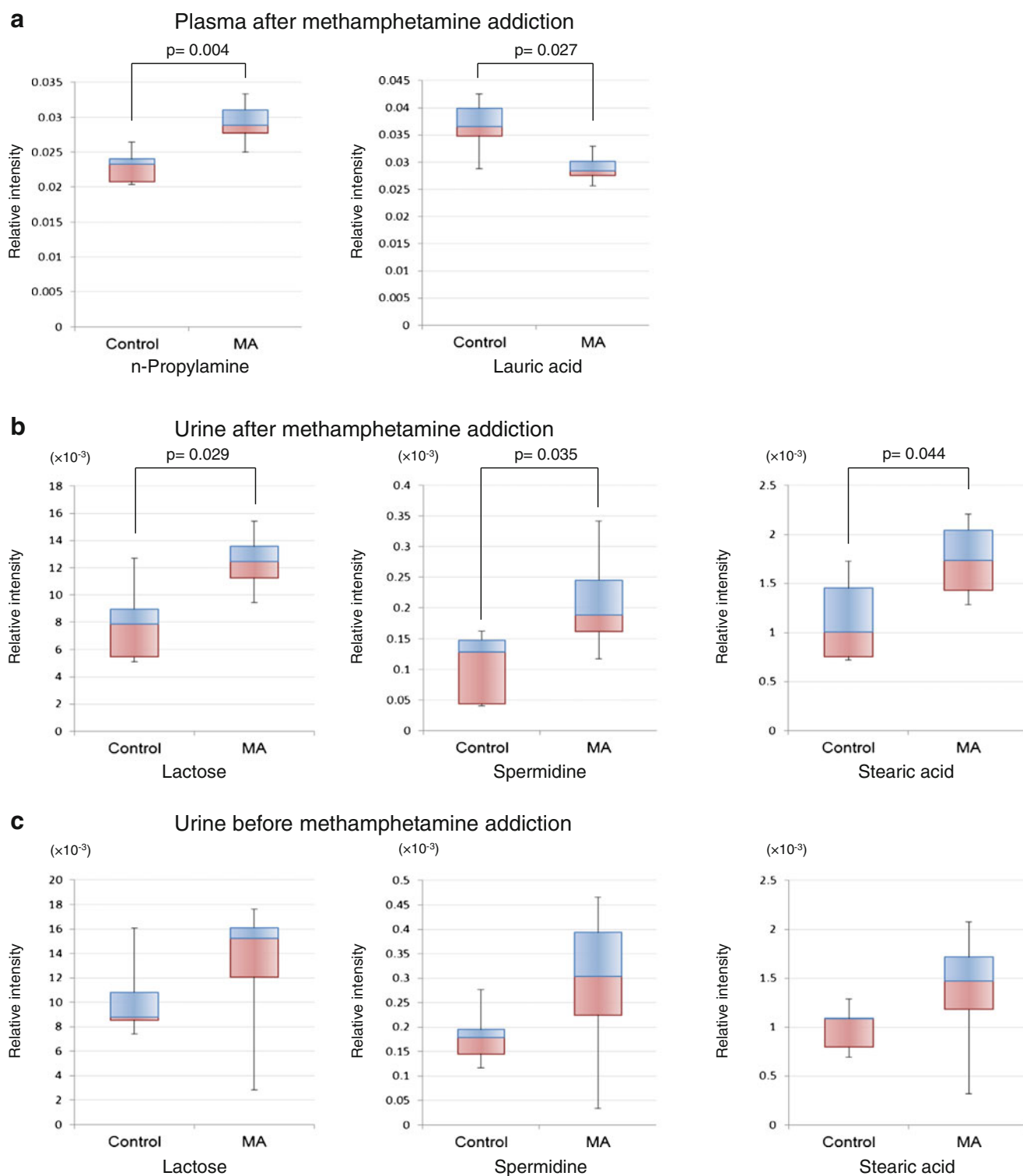


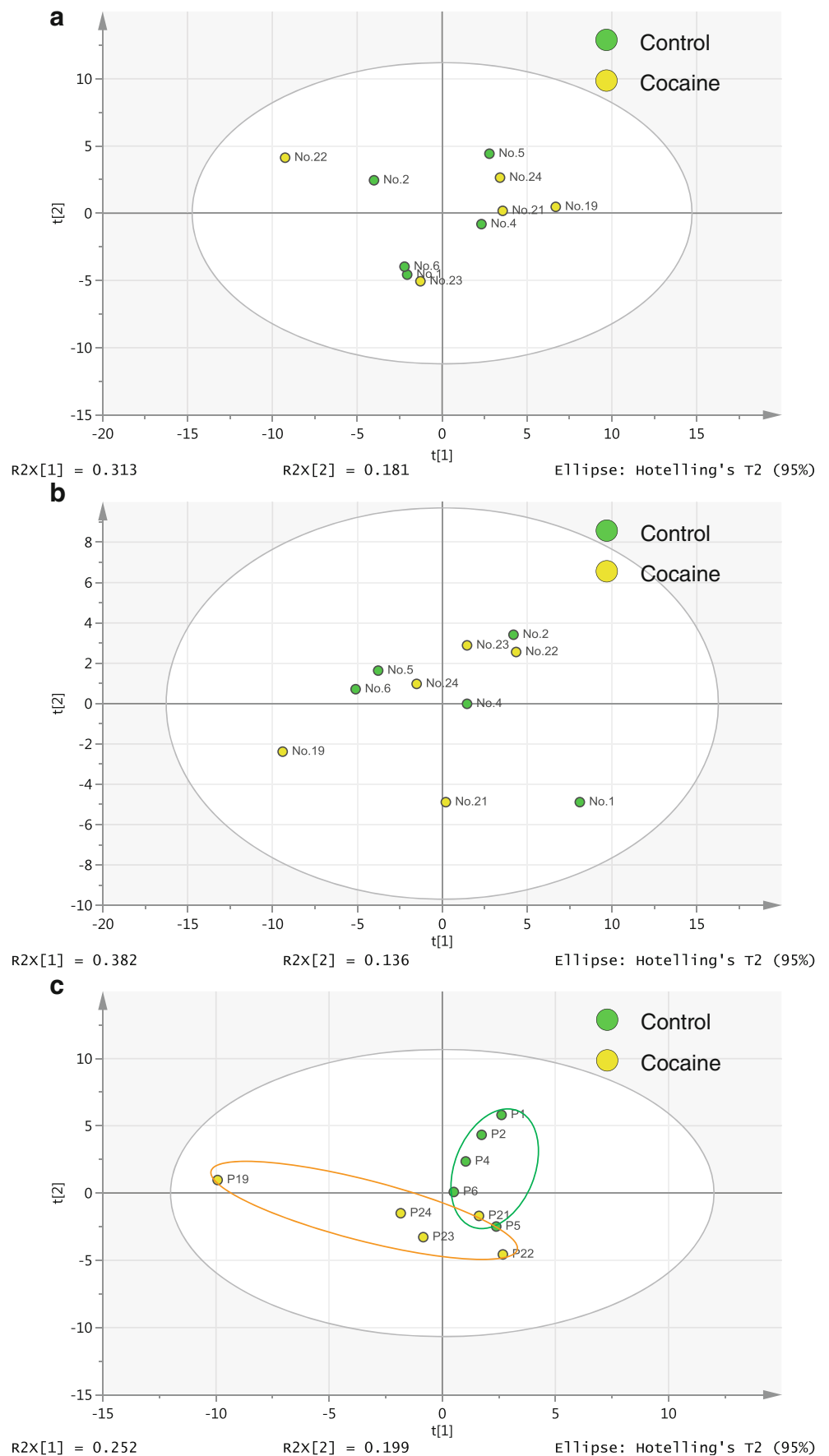
Fig. 5 (a, b) Box-and-whisker plots for significantly changed metabolites in plasma (a) and urine (b) after methamphetamine addiction. (c) Box-and-whisker plots of the metabolites in urine before addiction. Whiskers extend to the extreme data points, and p values calculated

by use of Welch's test are shown for significantly different metabolites between controls and addicted rats. There was no significant difference for each metabolite before addiction

tryptophan decrease in rat blood plasma and an increase in rat brain even after single administration of MOR at 10 mg kg^{-1} [29]. Our result is in agreement with their findings, in that the

plasma L-tryptophan level decreased in MOR-addicted rats. Larson and Takemori investigated L-tryptophan uptake from blood to brain after one pass by use of isotope-labeled L-

Fig. 6 PCA score plots for urine and plasma of control and cocaine groups. The control group and the cocaine group are shown in *green* and *yellow* plots, respectively. **(a)** Urine before cocaine addiction, and **(b)** urine and **(c)** plasma after cocaine addiction. Each *ellipse* was given by Hotelling's T₂ (0.95)



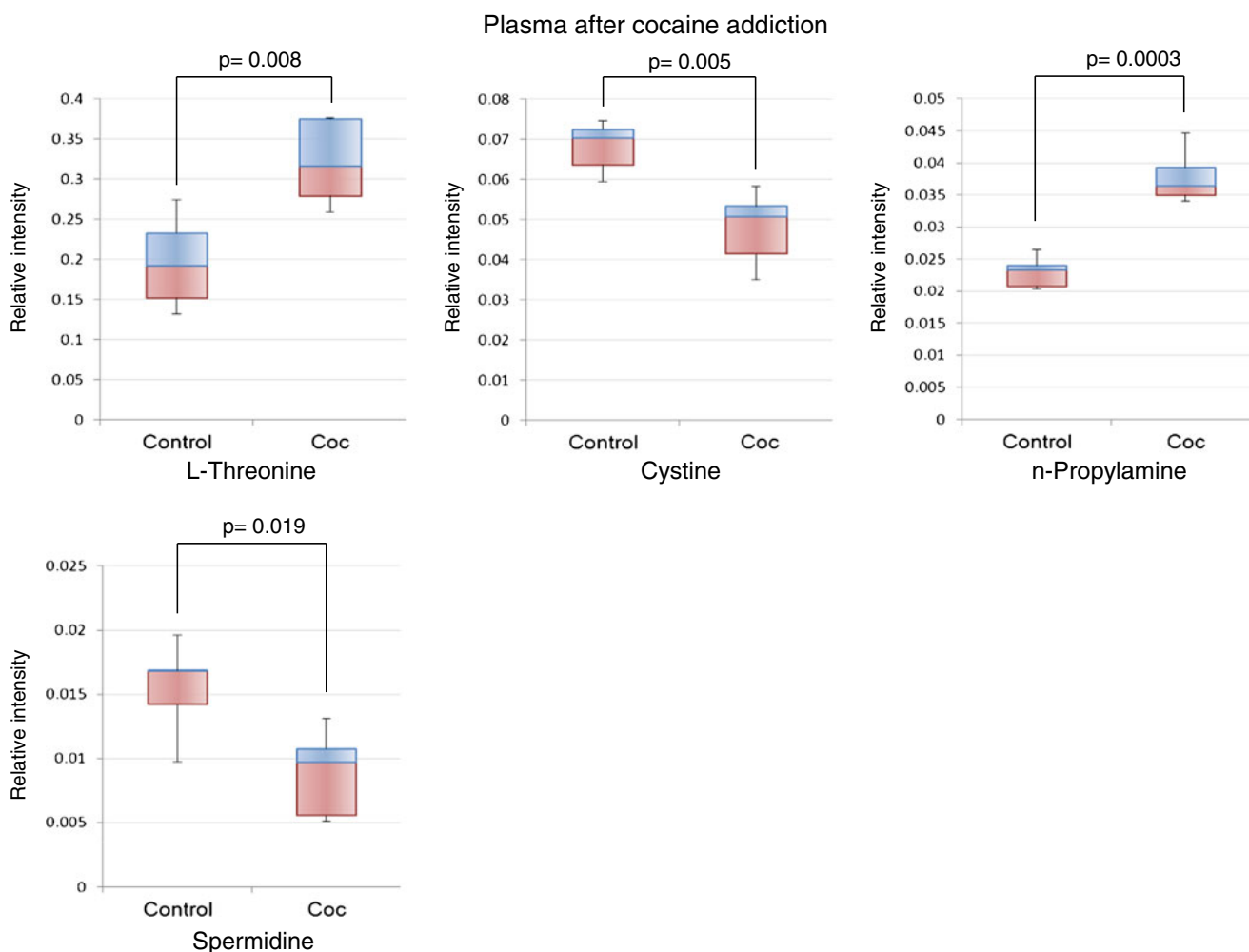


Fig. 7 Box-and-whisker plots for significantly changed metabolites in plasma after cocaine addiction. Whiskers extend to the extreme data points, and p values calculated by use of Welch's test are shown for significantly different metabolites between controls and addicted rats

tryptophan (^{14}C -L-tryptophan) [30]. They revealed that uptake of ^{14}C -L-tryptophan by the brain increased significantly 72 h after continuous subcutaneous administration of MOR. Our result may imply that MOR addiction is related to uptake of L-tryptophan from the blood by the brain.

As shown in Fig. 3b, 2-ketoglutaric acid, fumaric acid, and malic acid levels were significantly increased in the urine of MOR-addicted rats. These compounds are known intermediates of the tricarboxylic acid (TCA) cycle, and MOR addiction can affect the energy metabolism via TCA cycle disruption. Yang et al. revealed that specific proteins related to energy metabolism were significantly changed in the frontal lobe cortex of MOR-addicted rats [31], and Deng et al. reported that chronic morphine treatment causes disturbance of energy metabolism in the brain of rhesus monkeys [14]. Chen et al. also reported ATP synthesis down-regulation in the rat hippocampus after tenfold-repeated administration of MOR [5]. Although it is difficult to directly compare our results with theirs, because of the different

sample region targeted, our results also indicated that energy metabolism disruption is deeply relevant to MOR addiction. It was noteworthy that the urinary metabolomic approach could detect such disruption.

In addition, glutamic acid, which is biochemically relevant to 2-ketoglutaric acid, decreased significantly in urine, suggesting disruption of the biotransformation of glutamic acid to 2-ketoglutaric acid. Glutamic acid is also a well-known and important neurotransmitter related to MOR addiction [32], and the decrease in urinary glutamic acid suggests such a relationship.

3-Hydroxybutyric acid concentration also decreased significantly in the plasma of the MOR-addicted rats. Starvation-induced hypoglycemia depresses glycolysis and TCA cycle [33]. Low turnover of the TCA cycle collaterally induces accumulation of acetyl-CoA derived from β -oxidation of fatty acids, which generally results in hyperproduction of such ketones as 3-hydroxybutyric acid and acetone. Despite fasting, however, elevation of some TCA cycle intermediates

was observed in MOR-addicted rats, suggesting that MOR addiction may downregulate the β -oxidation pathway from fatty acids and/or ketone production from acetyl-CoA.

There were changes in other urinary metabolites, for example cystine and isoleucine, but the biological implications of these are unknown.

Effect of MA addiction on the metabolic profiles of urine and plasma

MA addiction induced some changes in the metabolic profiles of urine and plasma; *n*-propylamine and lauric acid levels changed significantly in plasma (Fig. 5a), and lactose, spermidine, and stearic acid fluctuated significantly in urine (Fig. 5b). However, the biological significance of these metabolites is unclear.

There were no observable changes in metabolites related to energy metabolism; levels of glucose and TCA intermediates, for example 2-ketoglutaric acid and malic acid, remained unchanged in urine or plasma of the MA-addicted rats. Kim et al., by use of positron emission tomography, revealed a significant decrease in cerebral glucose metabolism in the frontal white matter of abstinent MA abusers (at least four weeks) [34]. However, their result is believed to be mainly because of sudden MA extinction, and glycolysis downregulation may become observable after drug deprivation.

Interestingly, our previous study showed that some TCA intermediates, for example 2-ketoglutaric acid, fumaric acid, malic acid, and citrate, decreased in the urine of MA acute-intoxicated rats [24]. The result was quite different in this study, and implies that the biological state in MA addiction is dissimilar to that in MA acute intoxication. It may also indicate that several adaptations to chronic MA administration occurred in MA-addicted rats and resulted in no significant change in the metabolite levels.

Effect of COC addiction on the metabolic profiles of urine and plasma

As shown in Fig. 7, plasma L-threonine level increased after COC addiction. L-Threonine is known to protect against stress and strengthen immune response [35, 36]. The relationship between COC addiction and stress has been pointed out by other researchers [37, 38]. Goeders reported that self-administration of cocaine by rats increases plasma corticosterone [34], a well-known glucocorticoid relating to immune reactions and increased stress responses [39, 40]. Change in the plasma L-threonine level may reflect this relationship; no change of L-threonine level was observed in urine. The reason urinary L-threonine level did not change in conjunction with that of plasma is unclear. Because this may be a result of masking by the longer sampling time for urine, the effect of COC addiction on the metabolic profile of urine may be clarified in more detail by use of a shorter sampling time.

Also, no changes in metabolites related to energy metabolism were observed in COC-addicted rats. There have, however, been reports suggesting a relationship between COC addiction and energy metabolism [41, 42]. Volkow et al. reported that global brain glucose metabolism was significantly higher in COC abusers in an early withdrawal state (less than one week after last use of COC) than in those in a late withdrawal state (1–4 weeks after last use) and in normal subjects [42]. They also reported that frontal brain metabolic change was not observed in COC abusers in the late withdrawal state. These interesting findings suggest COC addiction may affect energy metabolism in the brain. Thus, further study is needed with consideration of dose-dependent effects or shorter sample-collection time to investigate the relationship between COC addiction and energy metabolism in more detail.

In addition, Patkar et al. reported alterations in L-tryptophan and purine metabolism in COC abusers [12], but there were no observable changes in urinary L-tryptophan and

Table 3 Variables and performance of the PLS regression model

Plasma				Urine					
Group	Number of selected variables*	Number of PLS components	Model performance		Group	Number of selected variables*	Number of PLS components	Model performance	
			R ²	Q ²				R ²	Q ²
MOR	4	1	0.950	0.599	MOR	12	1	0.509	0.015
		2	0.995	0.938			2	0.940	0.713
MA	2	1	0.665	0.340	MA	3	1	0.102	-0.100
		2	0.765	0.298			2	0.315	-0.210
COC	4	1	0.484	0.183					
		2	0.541	0.101					

* Significantly changed metabolites in drug-addicted rats

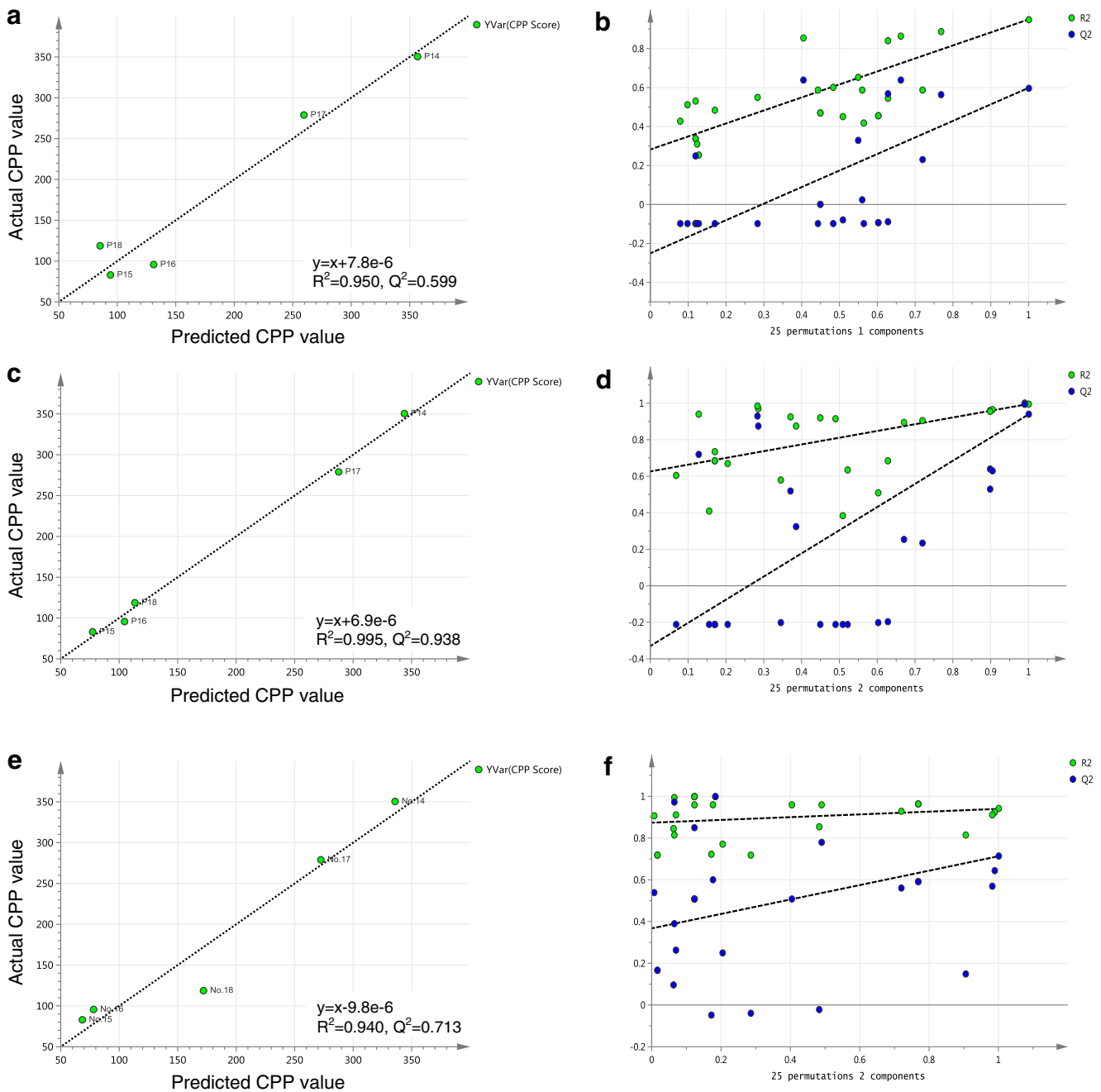


Fig. 8 (a) One-component PLS-regression line of CPP scores and (b) permutation test for plasma of morphine-addicted rats. (c) Two-component PLS-regression line of CPP scores and (d) permutation test for plasma of methamphetamine-addicted rats. (e) Two-component PLS-regression line

of CPP scores and (f) permutation test for urine of cocaine-addicted rats. The y -intercept of the regression line in permutation graphs (b), (d), and (f) shows a measure of the overfit; a near-zero slope of the line and a high y -intercept value indicates insufficiency of the model

plasma purine metabolites (xanthine and uric acid). These discrepancies can be explained as follows:

1. COC abusers in Patkar's study were abstinent for at least two weeks, and the effect of the absence of COC would be reflected in COC abusers;
2. all COC abusers in their study had a different history of drug use, and prior use of drugs could have affected the results; and

3. the species difference between rats and humans.

Possibility of prediction of CPP scores by use of the PLS-regression model

As described above, some metabolite concentrations were significantly changed in urine or plasma in the drug-addicted state, especially for MOR addiction. We further investigated

the possibility that the degree of addiction, as represented by CPP scores, can be predicted by PLS-regression. This is a trial to assess the potential of the metabolomics approach to characterize the state of drug addiction.

PLS-regression was performed with the significantly changed metabolites in each sample as independent *explanatory variables* (X). The performance of the regression model was not satisfactory for MA and COC addiction (Table 3). For MOR addiction, however, performance was good for both urine and plasma, and the model was further validated by permutation tests [43], as shown in Fig. 8. Permutation test results led to a one-component regression model ($R^2=0.950$, $Q^2=0.599$, Fig. 8a) consisting of the four significantly-changed metabolites in plasma which was acceptable as a model for possible prediction of CPP scores in MOR addiction. Although this model is not sufficient for practical use without increasing sample size or performing further validation with a new group (validation set), the result showed the model's potential to predict the degree of addiction from the metabolic profile, implying future applicability of metabolomics in drug addiction studies.

General overview and limitation of this study

MOR addiction induced changes in several metabolites in urine and plasma. In comparison with results for MOR-addicted rats, only slight changes in urine or plasma metabolic profiles were observed for MA and COC-addicted rats. The metabolic profile differences suggest that biological states were different among MOR, MA, and COC addiction. Also, the different metabolic profiles might be related to different actions on the brain reward circuitry and the resulting adaptation to chronic drug administration.

MOR inhibits GABAergic interneurons in the ventral tegmental area (VTA), and indirectly increases dopaminergic transmission [6]. MOR also activates specific opioid receptors (μ , δ , and κ) on nucleus accumbens (NAc) neurons [1, 6].

On the other hand, MA and COC only increase dopaminergic transmission in NAc, but their mechanisms of action are slightly different; unlike COC, MA exerts multiple pharmacological effects via various molecular processes [44]. MA causes neuronal storage vesicles in the cytoplasm to release neurotransmitters to the synapse [45, 46]. In addition, MA blocks the activity of monoamine transporters thereby inhibiting monoamine reuptake [47]. MA also mildly inhibits the activity of monoamine oxidase [48]. In comparison with MA's somewhat complex mechanism of action, COC is a potent blocker of plasma membrane transporters that reuptake monoamines [49].

There is, however, a limitation to this study, and further consideration is required. Urinary and/or blood plasma metabolic profiling could not measure the direct contribution to the central nervous system or peripheral regions. One of the

possible solutions to this problem may be to apply metabolomics to the brain or cerebrospinal fluid at the future study. In addition, to determine the specificity of the significantly changed metabolites, it is imperative to consider:

1. dose-dependent effects;
2. increasing the number of rats used for statistical analysis; and
3. the results obtained when other addiction-producing drugs are used.

In conclusion, we have demonstrated the possibility of describing the different biological states in drug addiction by use of metabolomics. In particular, urinary and blood plasma metabolic profiles could enable detection of the slight disruption caused by drug addiction. The metabolomics approach has the potential to predict the degree of drug addiction. Results from this study indicate that metabolomics can be a new approach to investigating drug addiction.

References

1. Camí J, Farré M (2003) Mechanisms of disease drug addiction. *N Engl J Med* 4:975–986
2. Goldstein RZ, Volkow ND (2002) Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex. *Am J Psychiatry* 159:1642–1652
3. Falcón E, McClung CA (2009) A role for the circadian genes in drug addiction. *Neuropharmacology* 56:91–96
4. Li CY, Mao X, Wei L (2008) Genes and (common) pathways underlying drug addiction. *PLoS Comput Biol* 4:28–34
5. Chen XL, Lu G, Gong YX, Zhao LG, Chen J, Chi ZQ, Yang YM, Chen Z, Li Q, Liu JG (2007) Expression changes of hippocampal energy metabolism enzymes contribute to behavioral abnormalities during chronic morphine treatment. *Cell Res* 17:689–700
6. Nestler EJ (2005) Is there a common molecular pathway for addiction? *Nat Neurosci* 8:1445–1449
7. Hollander JA, Amelio AL, Kocerha J, Bali P, Lu Q, Willoughby D, Wahlestedt C, Konkright MD, Kenny PJ (2010) Striatal microRNA controls cocaine intake through CREB signaling. *Nature* 466:197–202
8. Dreyer JL (2010) New insights into the roles of microRNA in drug addiction and neuroplasticity. *Genome Med* 2:92–98
9. Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics. *Mass Spectrom Rev* 26:51–78
10. Kaddurah-Daouk R, Krishnan KRR (2009) Metabolomics: a global biochemical approach to the study of central nervous system diseases. *Neuropsychopharmacology* 34:173–186
11. Pasikanti KK, Ho PC, Chan ECY (2008) Gas chromatography/mass spectrometry in metabolic profiling of biological fluids. *J Chromatogr B* 871:202–211
12. Patkar AA, Rozen S, Mannelli P, Matson W, Pae CU, Krishnan KR, Kaddurah-Daouk R (2009) Alterations in tryptophan and purine metabolism in cocaine addiction: a metabolomic study. *Psychopharmacology* 206:479–489
13. Mannelli P, Patkar A, Rozen S, Matson W, Krishnan R, Kaddurah-Daouk R (2009) Opioid use affects antioxidant activity and purine metabolism: preliminary results. *Hum Psychopharmacol* 24:666–675

14. Deng Y, Bu Q, Hu Z, Deng P, Yan G, Duan J, Hu C, Zhou J, Shao X, Zhao J, Li Y, Zhu R, Zhao Y, Cen X (2012) 1H-nuclear magnetic resonance-based metabolomic analysis of brain in rhesus monkeys with morphine treatment and withdrawal intervention. *J Neurosci Res* 90:2154–2162
15. Wu M, Sahbaie P, Zheng M, Lobato R, Boison D, Clark JD, Peltz AG (2013) Opiate-induced changes in brain adenosine levels and narcotic drug responses. *Neuroscience* 228:235–242
16. Hodson MP, Dear GJ, Roberts AD, Haylock CL, Ball RJ, Plumb RS, Stumpf CL, Griffin JL, Haselden JN (2007) A gender-specific discriminator in Sprague–Dawley rat urine: the deployment of a metabolic profiling strategy for biomarker discovery and identification. *Anal Biochem* 362:182–192
17. Slupsky CM, Rankin KN, Wagner J, Fu H, Chang D, Weljie AM, Saude EJ, Lix B, Adamko DJ, Shah S, Greiner R, Sykes BD, Marrie TJ (2007) Investigation of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. *Anal Chem* 79:6995–7004
18. Chen J, Liang J, Wang G, Han J, Cui C (2005) Repeated 2 Hz peripheral electrical stimulations suppress morphine-induced CPP and improve spatial memory ability in rats. *Exp Neurol* 194:550–556
19. Suzuki T, Misawa M (1995) Sertindole antagonizes morphine-, cocaine-, and methamphetamine-induced place preference in the rat. *Life Sci* 57:1277–1284
20. Åberg M, Wada D, Wall E, Izenwasser S (2007) Effect of MDMA (ecstasy) on activity and cocaine conditioned place preference in adult and adolescent rats. *Neurotoxicol Teratol* 29:37–46
21. Bando K, Kawahara R, Kunimatsu T, Sakai J, Kimura J, Funabashi H et al (2010) Influences of biofluid sample collection and handling procedures on GC–MS-based metabolomics studies. *J Biosci Bioeng* 110:491–499
22. Saude EJ, Sykes BD (2007) Urine stability for metabolomics studies: effects of preparation and storage. *Metabolomics* 3:19–27
23. Trygg JAJ, Gullberg J, Johansson AI, Jonsson P, Antti H, Marklund SL, Moritz T (2005) Extraction and GC–MS analysis of the human blood plasma metabolome. *Anal Chem* 77:8086–8094
24. Shima N, Miyawaki I, Bando K, Horie H, Zaitso K, Katagi M, Bamba T, Tsuchihashi H, Fukusaki E (2011) Influences of methamphetamine-induced acute intoxication on urinary and plasma metabolic profiles in the rat. *Toxicology* 287:29–37
25. Zhang Q, Wang G, Du Y, Zhu L, J A (2007) GC–MS analysis of the rat urine for metabolomics research. *J Chromatogr B* 854:20–25
26. Tsugawa H, Bamba T, Shinohara M, Nishiumi S, Yoshida M, Fukusaki E (2011) Practical non-targeted gas chromatography/mass spectrometry-based metabolomics platform for metabolic phenotype analysis. *J Biosci Bioeng* 112:292–298
27. Balster RL (1991) Drug abuse potential evaluation in animals. *Br J Add* 86:1549–1558
28. Tzschentke TM (1998) Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog Neurobiol* 56:613–672
29. Messing RB, Flinchbaugh C, Waymire JC (1978) Changes in brain tryptophan and tyrosine following acute and chronic morphine administration. *Neuropharmacology* 17:391–396
30. Larson AA, Takemori AE (1977) Effect of narcotics on the uptake of serotonin precursors by the rat brain. *J Pharmacol Exp Ther* 200:216–223
31. Yang L, Sun ZS, Zhu Y (2007) Proteomics analysis of rat prefrontal cortex in three phases of morphine-induced conditioned place preference. *J Proteome Res* 6:2239–2247
32. Zhu H, Rockhold RW, Ho IK (1998) The role of glutamate in physical dependence on opioids. *Jpn J Pharmacol* 76:1–14
33. Stryer L (1988) *Biochemistry*. Freeman, NY
34. Kim SJ, Lyoo IK, Hwang J, Sung YH, Lee HY, Lee DS, Jeong DU, Renshaw PF (2005) Frontal glucose hypometabolism in abstinent methamphetamine users. *Neuropsychopharmacology* 30:1383–1391
35. Farrar WL, Ferris DK, Harel-Bellan A (1989) The molecular basis of immune cytokine action. *Crit Rev Ther Drug Carrier Syst* 5:229–261
36. Wang X, Zhao T, Qiu Y, Su M, Jiang T, Zhou M, Zhao A, Jia W (2008) Metabonomics approach to understanding acute and chronic stress in rat models. *J Proteome Res* 8:2511–2518
37. Goeders NE (2002) Stress and cocaine addiction. *J Pharmacol Exp Ther* 301:785–789
38. Samyay Z, Shaham Y, Heinrichs SC (2001) The role of corticotropin-releasing factor in drug addiction. *Pharmacol Rev* 53:209–243
39. Butte JC, Kakihara R, Farnham ML, Noble EP (1973) The relationship between brain and plasma corticosterone stress response in developing rats. *Endocrinology* 92:1775–9
40. Pitman DL, Ottenweller JE, Natelson BH (1988) Plasma corticosterone levels during repeated presentation of two intensities of restraint stress: chronic stress and habitation. *Physiol Behav* 43:47–55
41. London ED, Cascella NG, Wong DF, Phillips RL, Dannals RF, Links JM, Herning R, Grayson R, Jaffe JH, Wagner HN (1990) Cocaine-induced reduction of glucose utilization in human brain. *Arch Gen Psychiatry* 47:567–574
42. Volkow ND, Fowler JS, Wolf AP, Hitzemann R, Dewey S, Bendriem B, Alpert R, Hoff A (1991) Changes in brain glucose metabolism in cocaine dependence and withdrawal. *Am J Psychiatry* 148:621–626
43. Lindgren F, Hansen B, Karcher W (1996) Model Validation by permutation tests: applications to variable selection. *J Chemom* 10:521–532
44. Barr AM, Panenka WJ, MacEwan GW, Thornton AE, Lang DJ, Honer WG, Lecomte T (2006) The need for speed: an update on methamphetamine addiction. *J Psychiatry Neurosci* 31:301–313
45. Brown JM, Hanson GR, Fleckenstein AE (2001) Regulation of the vesicular monoamine transporter-2: a novel mechanism for cocaine and other psychostimulants. *J Pharmacol Exp Ther* 296:762–767
46. Khoushouei H, Wang H, Lechleiter JD, Javitch JA, Galli A (2003) Amphetamine-induced dopamine efflux. A voltage-sensitive and intracellular Na⁺-dependent mechanism. *J Biol Chem* 278:12070–12077
47. Schmitz Y, Lee CJ, Schmauss C, Gonon F, Sulzer D (2001) Amphetamine distorts stimulation-dependent dopamine overflow: effects on D2 autoreceptors, transporters, and synaptic vesicle stores. *J Neurosci* 21:5916–5924
48. Saunders C, Ferrer JV, Shi L, Chen J, Merrill G, Lamb ME, Leeb-Lundberg LMF, Carvelli L, Javitch JA, Galli A (2000) Amphetamine-induced loss of human dopamine transporter activity: An internalization-dependent and cocaine-sensitive mechanism. *PNAS* 97:6850–6855
49. Izenwasser S (2004) The role of the dopamine transporter in cocaine abuse. *Neurotox Res* 6:379–383