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Methamphetamine (MA) Use Induces Specific Changes in LINE-1 Partial Methylation Patterns, Which Are Associated with MA-Induced Paranoia: a Multivariate and Neuronal Network Study

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Abstract

The use of psychoactive substances, including methamphetamine (MA) may cause changes in DNA methylation. The aim of this study was to examine the effects of MA use on long interspersed element-1 (LINE-1) methylation patterns in association with MA-induced paranoia. This study recruited 123 normal controls and 974 MA users, 302 with and 672 without MA-induced paranoia. The Semi-Structured Assessment for Drug Dependence and Alcoholism was used to assess demographic and substance use variables. Patterns of LINE-1 methylation were assessed in peripheral blood mononuclear cells and a combined bisulfite restriction analysis (COBRA) was used to estimate overall LINE-1 methylation (mC) while COBRA classified LINE-alleles into four patterns based on the methylation status of two CpG dinucleotides on each strand from 5′ to 3′, namely two methylated (mCmC) and two unmethylated (uCuC) CpGs and two types of partially methylated loci (mCuC that is 5′m with 3′u and uCmC that is 5′u with 3′m CpGs). MA users showed higher % mCuC and % mCuC + uCmC levels than controls. Use of solvents and opioids, but not cannabis and alcohol dependence, significantly lowered % uCmC levels, while current smoking significantly increased % uCuC levels. MA-induced paranoia was strongly associated with changes in LINE-1 partial methylation patterns (lowered % uCmC), heavy MA use, lower age at onset of MA use, and alcohol dependence. Women who took contraceptives showed significantly lower LINE-1 % mC and % mCmC and higher % uCuC levels than women without contraceptive use and men. The results show that MA-induced changes in LINE-1 partial methylation patterns are associated with MA-induced paranoia and could explain in part the pathophysiology of this type of psychosis. It is argued that MA-induced neuro-oxidative pathways may have altered LINE-1 partial methylation patterns, which in turn may regulate neuro-oxidative and immune pathways, which may increase risk to develop MA-induced paranoia.

Keywords Methamphetamine · DNA methylation · Schizophrenia · Paranoia · Immune · Inflammation · BMI · Sex

Introduction

Addiction is a neuropsychiatric and behavioral condition characterized by compulsive use of illegal or legal substances despite acknowledging their harmful effects [1]. Methamphetamine (street names: ice, meth, crystal, yaba) is a psychostimulant substance, which is readily available and highly addictive [2–4]. Use of amphetamines, including methamphetamine, has reached

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epidemic proportions and is ranked as the second most common illegal substance with an estimation of 35 million users worldwide [5]. In Thailand, an estimation of 1.7–1.9% of Thai population, aged 12–65 years, used methamphetamine during their lifetime [6, 7].

Methamphetamine (MA) administration induces a sustained release and turnover of noradrenaline and dopamine [3, 8] and may cause neuronal death in the hippocampus and frontal, prefrontal and temporal lobes, white matter hypertrophy and gliosis, glutamate neurotoxicity, and damage to neuronal dendrites [9, 10]. Such effects may explain consequent brain damage and neurocognitive impairments including episodic memory, information processing, and executive functions [11]. Moreover, MA may induce changes in the cholinergic anti-inflammatory pathway causing activated immune-inflammatory pathways and changes in gut microbiota leading to increased leaky gut, thereby further activating immune-inflammatory pathways [9]. Up to 40% of individuals who use MA may experience psychotic symptoms with positive symptoms and neurocognitive impairments being similar to those in schizophrenia [12–15]. Methamphetamine-induced paranoia (MIP) is defined as a transient condition characterized by a temporal relationship between use of MA and the consequent paranoia [12, 16]). Interestingly, MA-induced psychosis-like behaviors are employed as a model for schizophrenia [13]. Psychostimulant addiction may also cause functional changes in gene expression without changing the DNA sequence through alterations in DNA methylation [17].

DNA methylation entails the methylation of cytosine bases primarily at the 5′ position by converting cytosine to 5-methylcytosine by DNA methyltransferase (DNMTs) [18]. Most DNA methylation occurs in 5′-CpG-3′ islands, namely regions with at least 200 bp and a high percentage of CpG sites (greater than 50%) which are concentrated in promoter regions [19]. Methylated CpG sites in CpG islands of promotors may cause silencing of genes [20]. Methylation of cytosine bases of the long interspersed element-1s (LINE-1s), a group of retrotransposons, has been studied most frequently among the interspersed repetitive sequences and is known for its function in maintaining genomic integrity [19]. LINE-1 hypomethylation is accompanied by genomic instability and repression of gene expression [19, 21]. Moreover, promotor hypomethylation of LINE-1 retrotransposable elements is a prognostic marker for different human diseases including cancer [22, 23]. There is also some evidence that MA administration may alter DNA methylation patterns in animal models via effects on DNA methyltransferase 1 mRNA in the brain [24]. A recent review shows that MA abuse may be associated with epigenetic modifications including DNA methylation and that such aberration may underpin MA-induced cognitive, behavioral, and synaptic alterations [25]. Neurotoxic doses of MA increase LINE-1 expression [26] and triggers LINE-1 retrotransposition [27]. Nevertheless, there are no reports on LINE-1 methylation patterns following MA abuse and there are no data whether MA-induced psychosis is associated with changes in LINE-1 partial methylation patterns.

There is a high prevalence of other substance use disorders in individuals with MA abuse, including alcohol, cannabis, and opioid dependence or abuse [28]. Co-abuse of solvents is frequent in MA users [15, 29]. Many individuals using MA are also smokers with a low to high dependence of nicotine [14, 30] and cigarette smoking and/or other soft drugs, which are considered to be a gateway to the use of MA and other stimulants [31, 32]. Cigarette smoking is associated with site-specific DNA methylation changes, which are additionally reversible upon smoking cessation [33]. As a direct consequence of physical exposure to cigarette smoke, smokers also show changes in LINE-1s DNA methylation in the oral mucosa [34]. Likewise, previous studies reveal altered DNA methylation at the promoters of specific genes in individuals with chronic alcohol use [35, 36] or those with various kinds of inhalants exposure [37, 38]. Preclinical studies show transgenerational effects of female cannabinoid exposure on genome-wide DNA methylation [39] and on opioid-related behavioral tasks in the offspring [40, 41]. Also, inhalants may have effects on DNA methylation patterns [37, 38], although those changes need further examination [42]. Moreover, these substances are associated with MA-induced paranoia (MIP) [14, 15, 43]. Nevertheless, no studies have examined LINE-1 DNA methylation patterns in peripheral blood samples of MA users with and without co-use of these substances.

Hence, the aim of this study was to examine LINE-1 DNA methylation patterns in individuals with MA abuse with or without co-abuse of alcohol, cannabis, opioids, solvents, and nicotine and whether the changes in LINE-1 methylation are associated with MIP.

Subjects and Methods

Participants

In this study, we used data from a MIP genetic association study [14], which recruited Thai individuals with MA use who were hospitalized at Princess Mother National Institute on Drug Abuse Treatment (PMNIDAT) and from normal controls both men and women at the blood donation center, Thai Red Cross Society. All participants were 18 years old or more and of both genders. We included individuals who used MA for more than 10 instances in their lifetime and further divided the MA users in subgroups according to (a) very recent use (that is the last month prior to blood sampling); (b) recent use (< 6 months prior to blood sampling); and (c) past use, namely < 12 months and > 12 months before blood sampling was carried out. We also registered the number of instances of lifetime MA use and used two different cut-off values to denote medium (> 600 instances during last year) and heavy (>

1000 instances last year) use. Eligible participants were classified into three different groups, namely controls and MA users with and without induced paranoia.

We excluded controls and patients with a history of primary psychotic disorders, including schizophrenia, major depressive disorder, bipolar disorder, and psycho-organic disorders; neurodegenerative and neuro-inflammatory disorders, including multiple sclerosis, Parkinson's disease, and stroke; neurologic disorders including epilepsy and brain trauma; and (auto)immune disorders, including systemic lupus. We also made the diagnosis of nicotine use, namely > 100 instances of cigarette smoking in lifetime and consequently we divided the participants in two groups, namely current smokers versus past + or never smokers. The study procedure was approved by the Human Ethics Committee of the Faculty of Medicine, Chulalongkorn University (Med Chula IRB #417/57).

Measurements

Diagnostic assessments were performed using the Thai version of the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA). Demographics, substance use variables, and diagnoses were obtained using the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) Thai version [14, 44] performed by trained clinical psychologists certified for SSADDA interview. Rigorous quality control was carried out including editing, cross-editing by interviewers, and final review by interviewers and the principal investigator of the study (R.K.). The diagnosis of MIP was based on the Methamphetamine Experience Questionnaire (MEQ)–Thai version [14] by exploring paranoid experiences during MA use. This scale shows a good inter-instrument reliability (K = 0.87), while inter-rater reliability is moderate (K = 0.46) [14]. SSADDA diagnoses for substance dependence and psychiatric disorders were made using the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria [45]. In the current study, we used the SSADDA socio-demographic data, cigarette use variables (e.g., daily number of cigarettes), and MA use characteristics.

DNA Extraction, Bisulfite Modification, and COBRALINE-1s

Blood samples were sent to The Center for Excellence in Molecular Genetics of Cancer and Human Diseases, Faculty of Medicine, Chulalongkorn University, for DNA extraction. The samples were centrifuged at 1000*g* for 10 min to collect peripheral blood mononuclear cells (PBMCs) and then stored at − 80 degree Celsius (°C) before performing DNA extraction. Whole blood was extracted by (1) adding lysis buffer with 10% sodium dodecyl sulfate and proteinase K and then incubated overnight at 50 °C, (2) purifying using phenol-chloroform and centrifuging at 4 °C with 14,000*g* for 15 min, (3) precipitating the DNA pellet using 10 Molar ammonium acetate and absolute ethanol, (4) washing DNA pellet by 70% ethanol, and (5) drying DNA pellet and dissolving it by Tris-EDTA.

The combined bisulfite restriction analysis of LINE-1s (COBRALINE-1) was used for determining pattern of LINE-1s methylation. A total of 1 microgram (μg) of DNA was used in the bisulfite treatments that converted unmethylated cytosine to uracil, while methylated cytosine was not changed. The bisulfite DNA modification was performed using the EZ-DNA methylation kit and specific primers is LINE-1s-F (5′ GTTAAAGAAAGGGGTGA YGGT-3′) and LINE-1s-R (5′ AATACRCCRTTTCTTAAACC RATCTA-3′) at 95 °C denature for 15 min, 50 °C annealing for 35 cycles, and 72 °C final extension. LINE-1s were digested with *Taq*I and *Tas*I at 65 °C overnight. The digested products of bisulfite-treated LINE-1s were separated to strands with different length including 92 ($^{m}C^{u}C$), 60 ($^{u}C^{u}C$), 50 ($^{m}C^{m}C$), 42 ($^{m}C^{m}C$ and $^{u}C^{m}C$), and 32 ($^{u}C^{u}C$ and $^{u}C^{m}C$) base pairs (bp) that were measured by using polyacrylamide gel electrophoresis and stained with SYBR. We used deionized water as a negative control and HeLa, Daudu, and Jurkat as positive control.

The intensity of each band was assigned into A, B, C, D, and E (e.g., A = %92/92, B = %60/56, C = %50/48, D = %42/40, E = %32/28). The intensity of 18 bp was calculated and assigned to F = ((D + E) − (B + C))/2). Percentage of each patterns of DNA methylation was calculated by using the following formula:

$$\%(\mathrm{mC})\text{methylation} = ((\mathrm{A} + 2\mathrm{C} + \mathrm{F}) \times 100)/(2\mathrm{A} + 2\mathrm{B} + 2\mathrm{C} + 2\mathrm{F})$$
$$\%(\mathrm{mCmC})\text{hypermethylation} = ((\mathrm{C}/2) \times 100)/((\mathrm{C}/2) + \mathrm{A} + \mathrm{B} + \mathrm{F})$$
$$\%(\mathrm{uCmC})\text{partial methylation} = (\mathrm{F} \times 100)/((\mathrm{C}/2) + \mathrm{A} + \mathrm{B} + \mathrm{F})$$
$$\%(\mathrm{mCuC})\text{partial methylation} = (\mathrm{A} \times 100)/((\mathrm{C}/2) + \mathrm{A} + \mathrm{B} + \mathrm{F})$$
$$\%(\mathrm{uCuC})\text{ hypomethylation} = (\mathrm{B} \times 100)/((\mathrm{C}/2) + \mathrm{A} + \mathrm{B} + \mathrm{F})$$

Statistical Analyses

Analyses of variance (ANOVA) were used to assess differences in scale variables between diagnostic groups. Analyses of contingency tables (X^2 tests) were employed to assess associations between categorical variables. We used p correction for false discovery rate to adjust for multiple comparisons. Multivariate general linear model (GLM) analyses were employed to assess the effects of substance use (MA, alcohol, opioids, solvents, cannabis) on LINE-1 methylation patterns while adjusting for sex-hormonal state, age, and BMI. When multivariate GLM analyses were significant, tests for between-subject effects were used to check the univariate effects of the significant explanatory variables on LINE-1 methylation data. We computed model-generated estimated marginal means and consequently performed protected post hoc analyses to delineate pairwise differences between categories. We employed binary logistic regression analyses to assess the most important predictors of MA use with and without MIP. Multilayer perceptron (MLP) neural network analyses were used to discover more complex

relationships among input variables that are determined during learning processes in predicting MA use diagnosis with or without MIP. The relative number of cases assigned to the holdout (to evaluate the final network), testing (to prevent overtraining), and training (to estimate the network parameters) sets were 5, 3, and 7, respectively. One consecutive step with no further decrease in the error term was employed as stopping rule. We used a feedforward architecture model whereby the input layer contained the LINE-1 methylation data (and age, sex-hormonal state, BMI, substance use) and the output layer contained the diagnosis MIP versus controls or MA without MIP. We used one or two hidden layers with a variable number of nodes. The error and relative error were calculated as well as the (relative) importance of each of the input variables in sensitivity analyses. All statistical analyses were performed using IBM SPSS windows version 24. Tests were two-tailed and an alpha level of 0.05 indicated statistically significant results.

Results

Socio-demographic and Clinical Data

Figure 1 shows an overview of the different measurements made in the present study. Table 1 shows the socio-demographic and clinical data in controls and MA users with and without MIP. All significant differences shown in Table 1 remained significant after p correction for false discovery rate. There were no differences in age and sex and hormonal state (three groups, namely men and women with and without use of contraceptives) between the three study groups. In the MIP group, there were somewhat more subjects with MA use between 6 and 12 months. MA use the year prior to blood sampling was significantly higher in MIP subjects than in MA subjects without MIP. BMI was somewhat lower in patients with MIP as compared with non-psychotic MA patients and controls. Age at onset of MA use was significantly lower in MIP subjects than in MA subjects without MIP. There were significantly more recent smokers in MA subjects than in controls. The rate of alcohol dependence and cannabis use was significantly higher in MIP than in controls and MA individuals without paranoia. Use of opioids and solvents was significantly higher in MA abusers than in controls.

LINE-1 Methylation in Controls, MA Users, and MIP Subjects

Table 2 shows the outcome of a multivariate GLM analysis with the 6 DNA methylation data (namely general methylation, the four LINE-patterns, and the sum of both partial methylation data) as dependent variables, while adjusting for age, tobacco use (two groups, namely current versus past and never smokers), sex and hormonal state (three groups, namely men and women with and without use of contraceptives), BMI (3 groups, namely < 25, 25–30, and > 30 kg/m^2), use of opioids, solvents, cannabis, and alcohol dependence. Opioids and solvents had both significant and similar effects on LINE-1 methylation and therefore we have combined use of both substances into one group. There was a highly significant effect of diagnosis (controls, MA with and without MIP) on LINE-1 methylation patterns. Figure 2 shows the z transformations of the methylation data in the three study groups, while Table 3 shows the model-generated marginal means of these z values in the three study groups (thus after adjustment was made for background variables listed in Tale 2). Tests for between-subjects effects and protected pairwise post hoc tests showed that % uCmC was lower in MA users than in

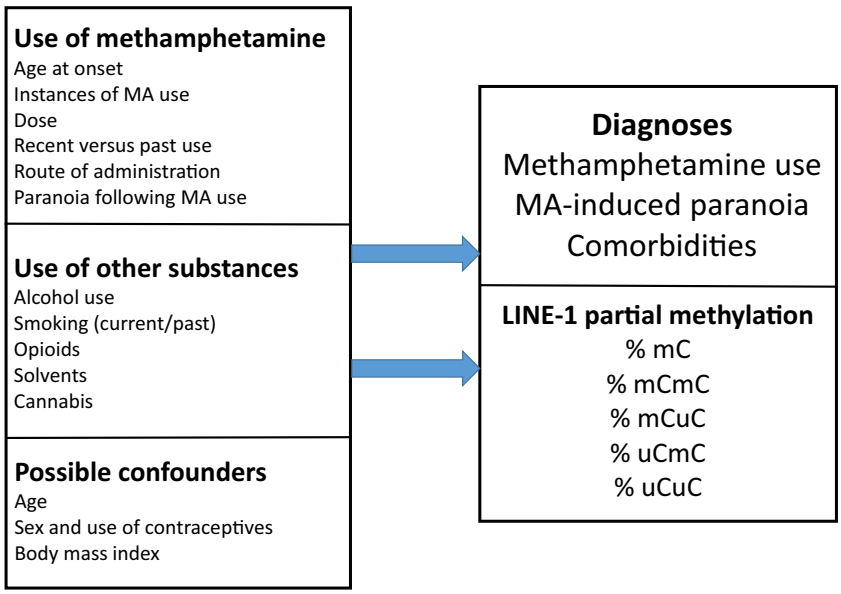

Fig. 1 Overview of the different measurements made in the present study

Table 1 Socio-demographic and clinical data in healthy control (HC) and methamphetamine (MA) abusers with and without MA-induced paranoia (MIP)

Variables	HC[A]	MA-MIP[B]	MA+MIP[C]	F/X^2	df	p*
Age (years)	27.3 (7.1)	27.1 (7.1)	26.5 (7.0)	0.96	2/1101	0.383
BMI (kg/m^2)	27.3 (2.6)[C]	27.0 (2.9)[C]	26.6 (2.8)[A,B]	3.23	2/1101	0.040
PILL/female/male	15/42/66	54/260/358	26/126/150	3.98	4	0.409
Age at onset (years)	–	19.5 (6.0)[C]	18.1 (5.9)[B]	12.60	1/980	< 0.001
Current smoking (N/Y)	105/18[B,C]	180/496[A]	64/241[A]	188.10	2	< 0.001
Alcohol dependence (Y/N)	18/105[C]	119/556[C]	108/197[A,B]	93.09	2	< 0.001
Opioid use (Y/N)	0/123[B]	23/652[A]	18/287	$Y = 0.090$	–	0.011
Cannabis use (Y/N)	4/119[C]	63/613[C]	47/258[A,B]	15.90	2	< 0.001
Solvent use (Y/N)	0/123[B]	49/626[A]	26/279	10.63	2	0.005
MA use last year (units)	–	678 (659)	1083 (1679)	16.76	1/981	< 0.001
MA use < 4 weeks/< 6 months < 1 year	–	29/538/111	10/263/32	6.75	2	0.034

Results are shown as mean (SD)

BMI body mass index, *PILL* use of hormonal contraceptives, *female* women without hormonal contraceptives

*Shown as exact p values or $p < 0.001$

[A,B,C] Significant differences between group means

controls and additionally that MIP subjects had lower % uCmC values than MA users without MIP. The levels of % mCuC were significantly higher in MA users than in controls. The sum of the percentages of both partial methylation profiles was significantly higher in MA users than in controls. Based on these findings (higher mCuC and lower uCmC in MIP) we have computed a z unit weighted composite score as z value of % mCuC (zMU)–zUM (zMU_UM). Figure 2 shows that zMU_zUM increases from controls to MA users without MIP to MA users with MIP (all at $p < 0.01$; the univariate GLM analysis is significant: $F = 10.87$, df = 2/963, $p < 0.001$ after adjusting for the same variables as shown in Table 2).

Effects of Other Drugs of Abuse on LINE-1 Methylation

Table 2 shows a significant impact of opioids and solvents on LINE-1 methylation. Tests for between-subject effects

Table 2 Association between LINE-1s methylation and methamphetamine (MA) use and MA-induced paranoia (MIP), while adjusting for background variables

Tests	Dependent variables	Explanatory variables	F	df	p*
Multivariate	All 6 methylation data	HC/MA ∀ MIP	6.71	10/1912	< 0.001
		PILL/female/male	3.29	5/956	0.006
		Smoking	5.59	10/1912	< 0.001
		BMI groups	1.93	10/1912	0.038
		Opioids or solvents	3.18	5/956	0.007
Between-subject effects	% uCmC	HC/MA ∀ MIP	6.78	2/960	0.001
	% mCuC	HC/MA ∀ MIP	13.98	2/960	< 0.001
	% uCuC + mCuC	HC/MA ∀ MIP	10.57	2/960	< 0.001
	% overall mC	PILL/female/male	23.85	2/960	< 0.001
	% mCmC	PILL/female/male	16.07	2/960	< 0.001
	% uCuC	PILL/female/male	28.53	2/960	< 0.001
	% uCuC	Smoking	6.73	1/960	0.010
	% uCmC	BMI groups	4.79	2/960	0.008
	% mCuC	BMI groups	3.00	2/960	0.050
	% uCmC	Opioids or solvents	6.89	1/960	0.008

HC/MA ∀ MIP: healthy controls, MA use with and without methamphetamine-induced paranoia. PILL/female/male: use of hormonal contraceptives/females without hormonal contraceptives/males

BMI body mass index with three groups, namely BMI < 25, BMI between 25 and 30, and BMI ∃ 30 kg/m^2

*Shown as exact p values or $p < 0.001$

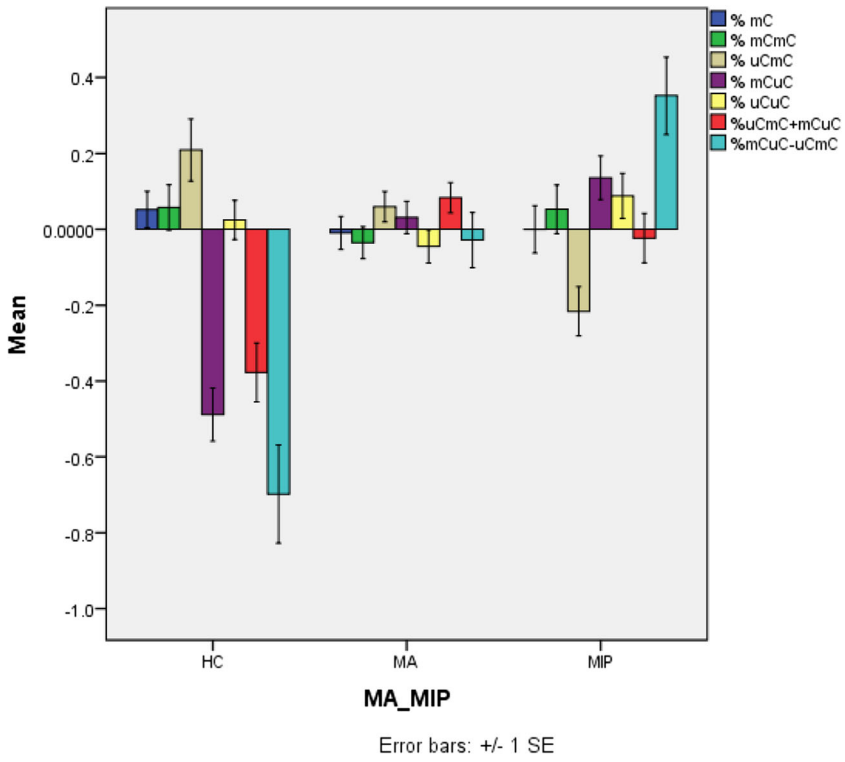

Fig. 2 *Z* transformations of the LINE-1 methylation data in three study groups, namely healthy controls (HC), MA (methamphetamine use without paranoia), and MA-induced paranoia (MIP)

showed that subjects with opioid or solvent use (mean ± SE 31.5 ± 0.2%) had significantly lower levels of % uCmC than subjects without use (32.0 ± 0.1%). Also, the zMU_zUM ratio was significantly higher in subjects with opioid or solvent use than in those without (see Fig. 2, $F = 5.57$, df = 1/964, $p = 0.018$). There were no significant effects of use of alcohol

Table 3 Model-generated estimated marginal mean values of LINE-1 methylation data in healthy controls (HC) and methamphetamine (MA) abusers with and without MA-induced paranoia (MIP)

Variables	HC[A]	MA-MIP[B]	MA+MIP[C]
% overall mC	66.2 (0.5)	66.2 (0.3)	66.2 (0.4)
% mCmc	33.1 (0.5)	32.8 (0.4)	33.1 (0.4)
% uCmC	32.0 (0.2)[C]	31.8 (0.2)[C]	31.4 (0.2)[A,B]
% mCuC	18.9 (0.3)[B,C]	20.3 (0.2)[A]	20.3 (0.2)[A]
% uCuC	22.9 (0.5)	22.2 (0.3)	22.7 (0.4)
% uCmC + % mCuC	50.9 (0.3)[B,C]	52.1 (0.2)[A]	51.9 (0.2)[A]
Variables	Women taking hormonal contraceptives[A]	Women without hormonal contraceptives[B]	Men[C]
% overall mC	64.3 (0.5)[B,C]	66.5 (0.4)[A,C]	67.7 (0.3)[A,B]
% mCmc	31.2 (0.5)[B,C]	33.4 (0.4)[A,C]	34.4 (0.4)[A,B]
% uCmC	31.7 (0.2)	31.6 (0.2)	32.0 (0.1)
% mCuC	19.9 (0.3)	20.2 (0.2)	19.7 (0.2)
% uCuC	24.6 (0.5)[B,C]	22.3 (0.4)[A,C]	20.8 (0.3)[A,B]
% uCmC + % mCuC	51.6 (0.3)	51.8 (0.2)	51.7 (0.20
Variables	BMI < 25[A]	BMI 25–30[B]	BMI ∃ 30[C]
% overall mC	66.6 (0.4)	66.4 (0.3)	65.6 (0.5)
% mCmc	33.3 (0.4)	33.4 (0.4)	32.4 (0.5)
% uCmC	32.1 (0.2)[B]	31.6 (0.2)[A]	31.6 (0.2)
% mCuC	19.5 (0.2)	19.9 (0.2)[C]	20.3 (0.3)[B]
% uCuC	22.0 (0.4)	22.7 (0.3)	23.1 (0.5)
% uCmC + % mCuC	51.6 (0.2)	51.5 (0.2)	52.0 (0.3)

BMI body mass index (in kg/m^2)

[A,B,C] Significant differences between group means

($F = 0.30$, df = 5/957, $p = 0.912$) and cannabis ($F = 0.77$, df = 5/957, $p = 0.572$) on the LINE-1 methylation patterns.

Table 2 shows a significant impact of current smoking on LINE-1 methylation. Tests for between-subject effects showed that current smokers (mean ± SE 23.0 ± 0.3%) had significantly higher levels of % uCuC hypomethylation than non-smokers (22.2 ± 0.4%). There were no significant differences in % methylation, % mCmC, % mCuC, and % uCmC between smokers and non-smokers.

Effects of Confounding and Background Variables

There were no significant effects of age on the LINE-1 methylation data ($F = 0.77$, df = 5/957, $p = 0.571$). Figure 3 shows the measurements of LINE-1 methylation patterns in female participants with and without contraceptives and men. Table 2 shows that there is a significant impact of sex and hormonal state on LINE-1 methylation, while tests for between-subject effects and protected post hoc tests performed on model-generated marginal means (Table 3) show that % mC and % mCmC hypermethylation were significantly different between the three study groups and increased from women with contraceptives to women without contraceptives to men. Also, % uCuC hypomethylation levels were significantly different between the three study groups and decreased from women with contraceptives to women without contraceptives to men. Table 2 shows that there was also a significant effect of BMI groups on LINE-1 methylation patterns. Table 3 shows that % uCmC was significantly lower in overweight people (BMI between 25 and 30 kg/m^2) than in those with a normal (20–25 kg/m^2) BMI. The % mCuC was significantly higher in subjects with a BMI > 25 and > 30 kg/m^2 than in those with a normal BMI (<25 kg/m^2).

Effects of MA Characteristics on LINE-1 Methylation

In order to delineate the effects of severity of MA dose and recent use of MA, we have entered two variables in the GLM analysis as shown Table 2, namely (a) number of instances of MA use the year previous to blood sampling and (b) groups divided as very recent use, recent use, and past use last 12 months and past use > 12 months before blood sampling. The first variable (number of instances) was entered as a scale variable and an ordinal variable using 600 or 1000 instances as cut-off value. Table 4 multivariate GLM analysis #1 shows that there were no significant effects of time and number of MA use instances (1000 instances) on LINE-1 methylation. GLM Analysis #2 shows that the differences in LINE-1 methylation patterns between MA with and without MIP remain significant after entering these two MA use characteristics and that there was a highly significant association between % uCmC and MA-induced MIP independently from the effects of the two MA use characteristics. Multivariate GLM analysis #3 shows that there was no significant association between age at onset of MA use and LINE-1 methylation profile. There were no significant effects of route of MA

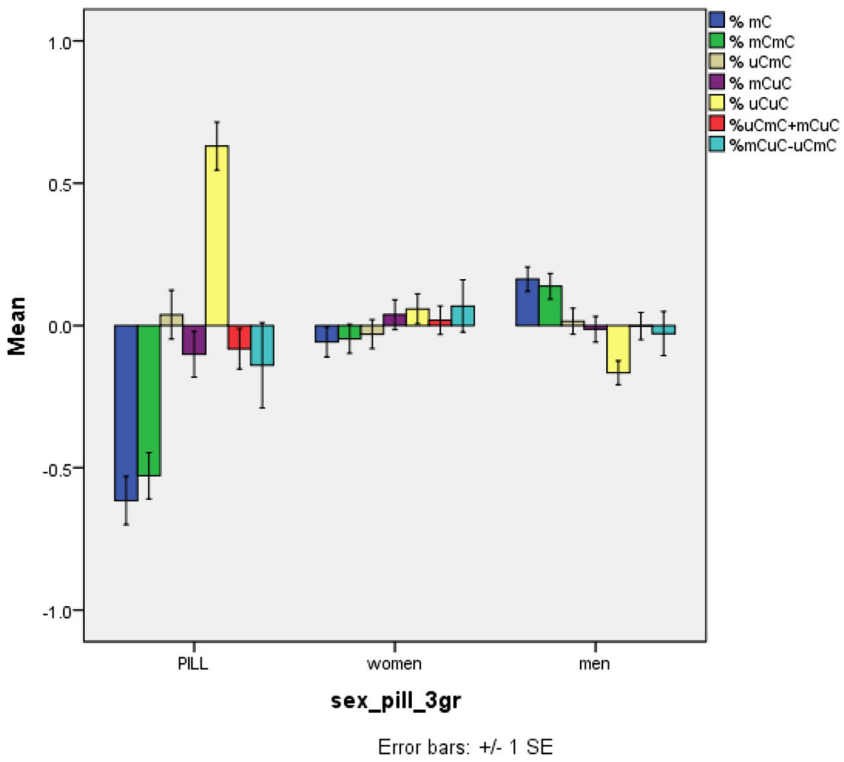

Fig. 3 *Z* transformations of the LINE-1 methylation data in three study groups, namely women taking contraceptives (PILL), women without contraceptives (women), and men

Table 4 Results of multivariate GLM analysis with LINE-1 methylation data as dependent variables and different methamphetamine (MA) use characteristics as explanatory variables

Tests	Dependent variables	Explanatory variables	*F*	df	*p**
Multivariate #1	All 6 methylation data	MA use < 4 weeks/< 6 months/< 1 year	1.18	10/1684	0.303
		MA use last year (units)	0.47	5/841	0.802
Multivariate #2	All 6 methylation data	MA use < 4 weeks/< 6 months/< 1 year	1.28	10/1682	0.234
		MA use last year (units)	0.51	5/840	0.768
		MIP	2.64	5/840	0.022
Between-subject effects	% uCmC	MIP	12.63	1/844	< 0.001
Multivariate #3	All 6 methylation data	Age at onset	1.63	5/843	0.150

*Shown as exact p values or $p < 0.001$

administration (intravenous use versus other routes) on the LINE-1 partial methylation profile (results of multivariate GLM analysis: $F = 0.16$, df = 5/1006, $p = 0.977$).

Best Prediction of MA-Induced MIP

Table 5 shows the outcome of three binary regression analyses with MA with or without MIP as dependent variables and the LINE-1 methylation data combined with other relevant predictors (as determined by Table 1 and Table 2) as explanatory variables. Regression #1 shows that increased % mCuC and lowered % uCuC together with current smoking and female sex best predicted MA use without MIP versus controls ($X^2 = 199.18$, df = 4, $p < 0.001$, Nagelkerke = 0.420); 80.9% of the subjects were correctly classified with a sensitivity of 80.3% and a specificity of 78.9%.

In Table 5, regression #2 shows that MIP (versus controls) is best predicted by increased % mCuC together with current smoking, alcohol use, and sex ($X^2 = 182.80$, df = 4, $p < 0.001$, Nagelkerker = 0.537); 82.6% of all cases were correctly classified with a sensitivity of 81.3% and a specificity of 86.0%. Figure 4 shows the results of MLP neural network analysis with MIP and controls as outcome variables and six factors and six covariates as input variables, including LINE-1 methylation data together with socio-demographic/clinical data. The MLP network was trained using two hidden layers (8 and 6 units, respectively) using hyperbolic tangent and identity as activation functions in hidden and output layers, respectively). The sum of squares error was significantly reduced in the testing set (8.006) as compared with the training set (20.857) and the percent incorrect predictions were fairly stable across the training (17.1%), testing (16.7%), and holdout (15.1%) samples. 84.9% of the cases were correctly classified in the handout set with a sensitivity of 84.9% and a specificity of 74.4%, while the AUC under the ROC curve was 0.902. The importance chart (Fig. 4) shows that % mCuC and current smoking were the most significant predictors followed at a distance by % uCmC and BMI and again at a distance use of alcohol and cannabis.

Regression #3 shows that lowered % uCmC together with heavy MA use, alcohol dependence, age at onset, and BMI are

Table 5 Results of binary logistic regression analyses with methamphetamine (MA) use with or without MA-induced psychosis (MIP) as dependent variable and LINE-1 methylation patterns, demographic and clinical data as explanatory variables

Dichotomy	Explanatory variables	Wald	df	*p**	OR	95% CI
#1. MA-MIP versus HC	% mCuC	24.16	1	< 0.001	2.07	1.55–2.77
	% uCuC	8.00	1	< 0.001	0.67	0.51–0.89
	Smoking	105.29	1	< 0.001	24.61	13.35–45.37
	Women (versus men)	9.51	1	0.002	2.21	1.33–3.67
#2. MA+MIP versus HC	% mCuC	20.75	1	< 0.001	2.37	1.65–3.40
	Smoking	82.91	1	< 0.001	25.48	13.08–49.63
	Women without hormonal contraceptives	8.27	1	0.004	2.48	1.34–4.60
	Alcohol dependence	6.69	1	0.010	2.65	1.27–5.56
#3. MA+MIP versus MA-MIP	% uCmC	14.00	1	< 0.001	0.75	0.65–0.87
	MA use last year > 600 units	20.12	1	< 0.001	2.04	1.49–2.79
	Alcohol dependence	26.07	1	< 0.001	2.40	1.71–3.35
	Age at onset MA abuse	9.52	1	0.002	0.96	0.93–0.98
	Body mass index	4.75	1	0.029	0.84	0.72–0.98

OR odd's ratio; *95% CI* 95% confidence interval, lower and upper limit

*Shown as exact p values or $p < 0.001$

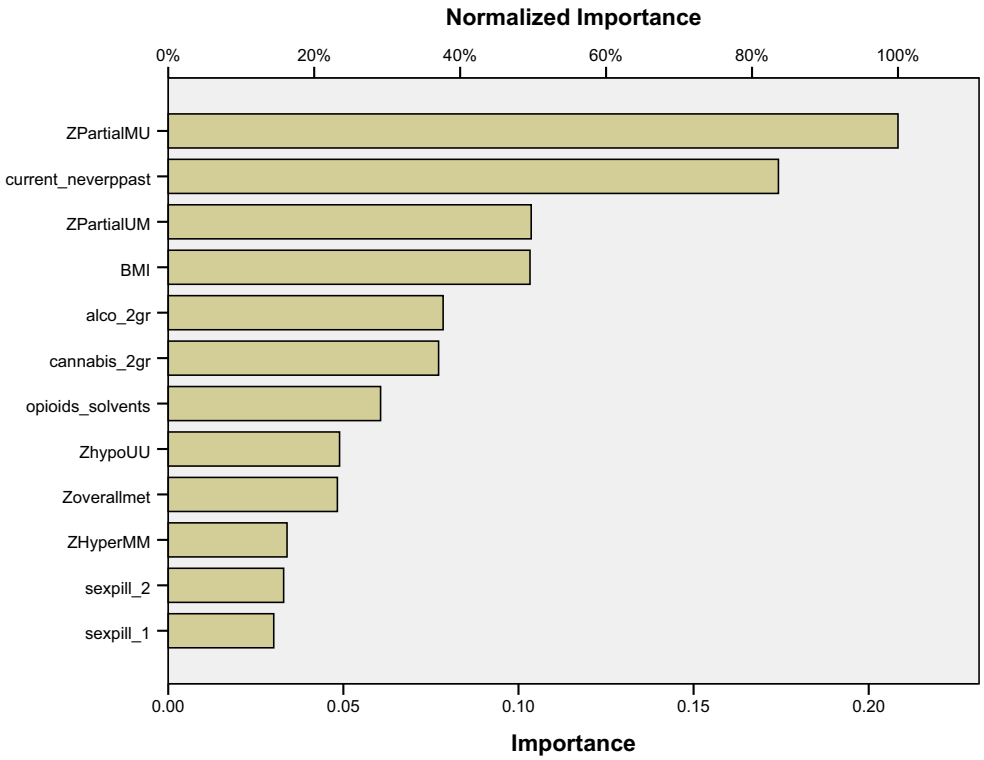

Fig. 4 Results of neural network analysis with importance of input variables separating methamphetamine-induced paranoia from controls. Input variables are as follows: zPartialMU: % mCuC; curren_neverppast: current smoking; ZPartialUM: % uCmC; BMI: body mass index; alco_2gr: alcohol dependence; cannabis_2gr: cannabis use; opioids_solvents: use of opioids and/or solvents; ZhypoUU: % uCuC; Zoveralmet: % mC; ZHyperMM: % mCmC; sexpill_2: women without contraceptives: sexpill_1: women taking contraceptives

the best predictors of MA use with MIP versus MA use without MIP ($X^2 = 81.80$, df = 5, $p < 0.001$, Nagelkerke = 0.126). Figure 5 shows the differences in age at onset of MA, BMI, severity of MA use (number of times used), and the LINE-1 data (all in z transformations) between both MA subgroups. Figure 6 shows the results (importance chart) of a neural network (MLP) analysis with MA use with MIP versus MA use without MIP as outcome variable and 10 input variables, including LINE-1 methylation data and socio-demographic/clinical data. We trained the network using two hidden layers with 7 and 5 units, respectively, and used hyperbolic tangent as the activation function in the hidden layer and identity in the output layer. The sum of squares error was significantly reduced in the testing set (37.293) as compared with the training set (75.004). The percent incorrect predictions were relatively constant among the training (31.6%), testing (29.5%), and holdout (31.4%) samples. The area under the ROC curve was 68.2%. Figure 5 (importance chart) shows that % uCmC is the dominant variable, followed at a distance by heavy use of MA (> 600 instances), alcohol dependence, age at onset of MA, and BMI.

Discussion

The first major finding of this study is that MA use is accompanied by higher % mCuC and lowered % uCmC as compared with controls. These findings indicate that MA use causes dysfunctions in LINE-1 partial methylation with a shift towards a higher % mCuC partial methylation profile. Furthermore, we could not find any associations between LINE-1 methylation and MA use characteristics including recent use and age at onset of MA use. Previously, it was reported that drugs of abuse may induce LINE-1 activity [27]. Neurotoxic doses of MA may elevate LINE-1 expression in adult rat brain [26]. Other authors found that MA induces LINE-1 retrotransposition in neuronal cell lines, which was dependent on reverse-transcriptase and activation of cAMP response element binding protein [27]. In animal models, MA administration may impact DNA methylation patterns via modulation of DNA methyltransferase 1 mRNA [24]. A recent review [25] reported that MA induces significant epigenetic modifications including DNA methylation. Nevertheless, to the best of our knowledge, our study is the first to report on LINE-1 partial methylation following MA use.

In the current study, we measured methylation levels of LINE-1 as an index of DNA methylation by using a combined bisulfite restriction analysis (COBRA) technique suited to investigate LINE-1 methylation. Moreover, COBRA "classifies LINE-1alleles into four groups depending on the methylation status of 2 CpG dinucleotides on each strand from 5′ to 3′ as detected by COBRALINE-1," whereby the methylation state of two of the CpG dinucleotides detected by COBRALINE-1 is directly associated with the methylation patterns of other CpG dinucleotides on 5′LINE-1s [34]. While most previous studies examined overall DNA or LINE-1 methylation, the

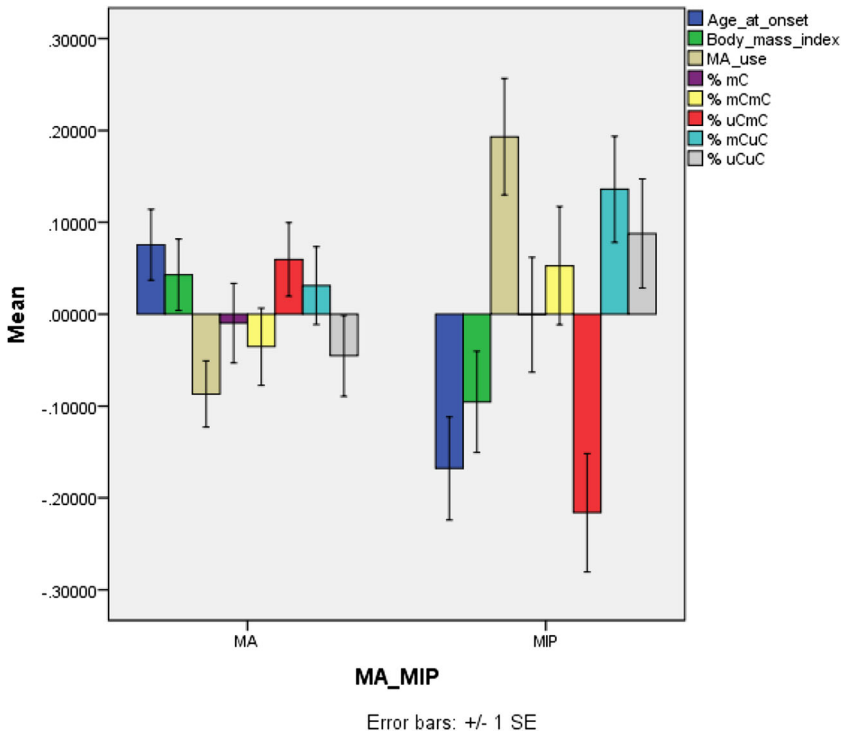

Fig. 5 Differences between methamphetamine users with MA-induced paranoia (MIP) and without MIP. Age_at_onsetMA, age at onset of MA use; MA_use, severity of MA use (number of times used)

assessment of LINE-1 methylation using COBRA with assay of the four different methylation patterns including partial methylation is superior than assays measuring global methylation [46]. For example, the uCuC hypomethylated LINE-1 loci in peripheral blood mononuclear cells differentiate oral cancer from controls more adequately than LINE-1 methylation levels [46]. Moreover, the results of the current study show that the partial methylation profiles, namely uCmC and mCuC, provide novel information about DNA methylation patterns.

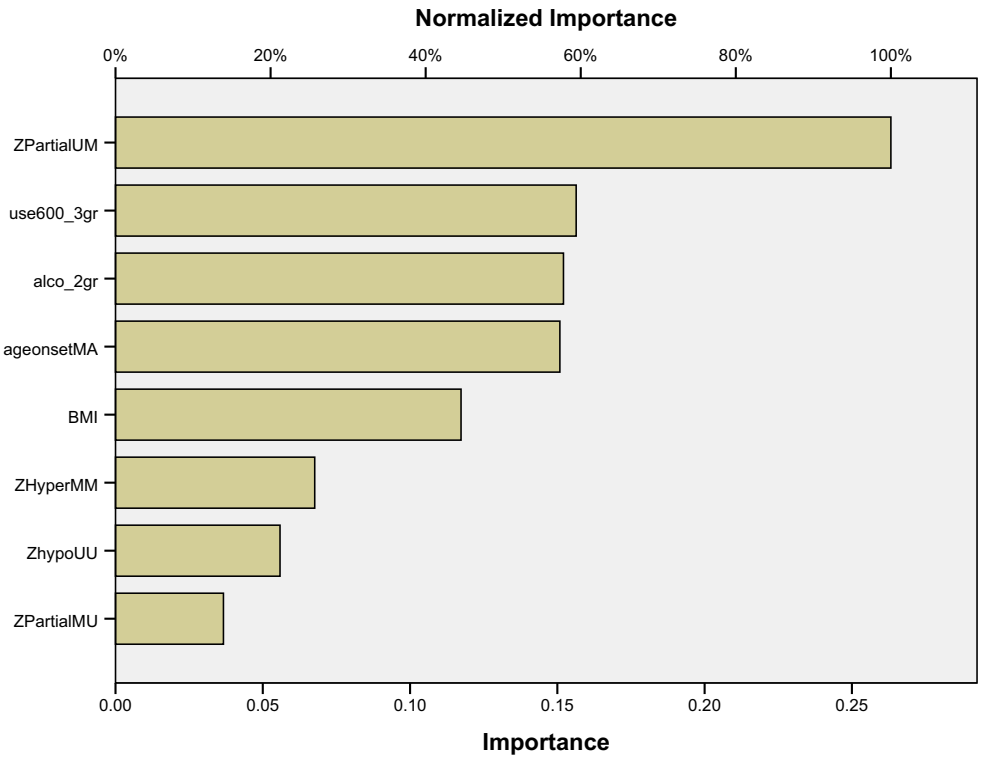

Fig. 6 Results of neural network analysis with importance of input variables separating methamphetamine-induced paranoia (MIP) from MA users without MIP. Input variables are as follows: zPartialUM: % uCmC; use600_2gr: used more than 600 times MA (versus not); alco_2gr: alcohol dependence; ageonsetMA: age at onset of MA use; BMI: body mass index; ZHyperMM: % mCmC; ZhypoUU: % uCuC; ZPartialUM: % uCmC

This study also established that other substances of abuse may affect LINE-1 methylation, including tobacco, opioids, and solvents. Firstly, we found that current smoking had a significant effect on LINE-1 methylation, with current smokers showing a significantly higher rate of hypomethylation than non-smokers. These findings extend those of Wangsri et al. who reported that current smokers had increased % mCmC and % uCuC, while % mCuC was decreased [34]. These authors concluded that smoking may change mCuC into mCmC and uCuC forms and additionally uCmC into uCuC forms. In agreement with Wangsri et al. [34], we could not find significant changes in LINE-1 mC methylation in current smokers. The differences between our study and that of the previous study may be explained by differences in LINE-1 methylation patterns among different tissues, namely mononuclear cells versus oral epithelial cells. In this respect, Nielsen et al. observed that tobacco smoking was associated with lowered LINE-1 methylation profiles in blood mononuclear cells of healthy controls [47]. Likewise, Marques-Rocha et al. reported an inverse association between current smoking and LINE-1 methylation [48].

Secondly, we also observed that opioid and solvent use was associated with lowered % uCmC methylation than subjects without opioid and solvent use. Previous studies found that white blood cells of former opiate dependent individuals currently using methadone have increased LINE-1 methylation patterns as compared with controls, suggesting that chronic opioid exposure may enhance DNA methylation [49]. Trivedi et al. showed hypermethylation of LINE-1 promotor region 4 h after morphine treatment, whereas lowered global DNA methylation occurred 24 h after morphine treatment [50]. In human MCF7 cells, methadone administration causes increased hypermethylation [51], while in TK cell6 cells, administration of solvents including styrene, benzene, hydroquinone, and trichloroethylene induces global DNA hypomethylation. Nevertheless, these results are difficult to compare with ours because we determined changes in LINE-1 partial methylation patterns, which was not measured in other studies.

In the current study, we could not find any effects of alcohol or cannabis dependence on LINE-1 methylation patterns. Wangsri et al. reported that in non-smokers the % mCuC partial methylation was significantly decreased in current alcohol users as compared with participants who never used alcohol [34]. Recently, Zhang and Gelernter reviewed 29 studies examining the association between DNA methylation and alcohol use and concluded that chronic alcohol consumption may change DNA methylation patterns, including increased global DNA methylation and hypermethylation of promoter regions of specific genes [52]. There are also a few studies which examined the impact of cannabis administration on DNA methylation profiles [53]. For example, cannabis increases CpG DNA methylation at promoter sites in human peripheral blood cells and, in the rodent, tetrahydrocannabinol alters CpG DNA methylation at promoters and intergenic regions [39, 54].

An unexpected but major finding of this study is that there was a highly significant impact of sex and hormonal state on LINE-1 methylation patterns. Thus, % mC and % mCmC hypermethylation decreased from men to women without contraceptives to women taking contraceptives, while % uCuC hypomethylation levels increased from men to women without contraceptives to women taking contraceptives to men. Only few studies have examined the associations between LINE-1 methylation and hormone levels. Wangsri et al. reported no significant differences in LINE-1 (partial) methylation patterns between men and women [34]. Increased LINE-1 methylation in males and no significant changes in association with hormone levels in women were reported by [55]. Nevertheless, in vitro results showed that in T47-Kbluc cell lines, estrogen treatment decreased LINE-1 methylation, although estrogen and dihydrotestosterone did not affect LINE-1 methylation in HUVEC, Hek293T, and MDA-kb2 cell lines [55]. These authors concluded that the natural hormonal variations in women do not impact blood cell LINE-1 methylation in part because blood cells have a lower expression of estrogen receptors. Nevertheless, the presence of progestin in contraceptive drugs may be the relevant factor affecting LINE-1 methylation as observed in our study.

Finally, we found somewhat lowered % uCmC in overweight subjects but no effects of age on LINE-1 methylation patterns. Previous studies showed that lowered DNA methylation, as assessed with by LINE-1 methylation measurements, is associated with increased risk for metabolic syndrome in obese subjects [56]. As in Wangsri et al. [34], we could not find a significant association between age and LINE-1 methylation patterns, although these authors reported borderline increased hypomethylation with increasing age in current smokers. Other studies found LINE-1 demethylation with increasing age [57] or no effects at all [55].

The third and major finding of this study is that MIP is significantly associated with a specific partial methylation profile, namely increased mCuC and decreased uCmC as compared with normal controls. Neural network analysis showed that 84.9% of all cases were correctly classified using LINE-1 methylation and clinical variables; in descending order of importance: % mCuC, current smoking (both positively), % uCmC, BMI (both inversely), and cannabis and alcohol dependence (both positively). In addition, MIP was significantly discriminated from MA use without paranoia whereby 68.6% of all cases are correctly classified using the following input variables (in descending order of importance) % mCuC (positively), age at onset of MA, BMI (both inversely), heavy use of MA (> 600 instances), alcohol dependence, and % uCuC (all three positively). Thus, the LINE-1 methylation profile associated with MIP consists of specific changes in partial methylation, namely lowered % uCmC, increased % mCuC, and additionally increased hypomethylation. Therefore, it may be hypothesized that subjects with lowered % uCmC following MA use

are those who will develop paranoia. Again, these results are difficult to compare with results obtained in schizophrenia because we assessed a more complete pattern of LINE-1 partial methylation, while previous studies only measured global methylation. A recent systematic review in schizophrenia [58] showed inconsistent findings suggesting hypermethylation and hypomethylation as well as a no changes of global methylation and methylation at specific loci.

Previous research showed that heavy use of MA and severity of dependence increase risk for MIP [14] and that MA users have an elevated risk for developing schizophrenia [59]. Smoking and alcohol use are other risk factors for MIP [14, 60, 61], while use of cannabis in adolescents is associated with later schizophrenia [62, 63]. Early onset cannabis use also increases risk for psychostimulant-related psychosis, including cocaine-induced paranoia [64]. MA dependence and long duration of MA use are associated with a low BMI [65], although there are no studies which examined the association between BMI and MA-related psychosis. Moreover, recently, we detected a significant association between a low BMI and deficit schizophrenia [66] which may be in accordance with Kretschmer's description of a leptosome phenotype in association with schizophrenia. Interestingly, a recent genome-wide association study found that BMI and schizophrenia had the most overlapping genetics on human metabolism [67].

The findings on LINE-1 methylation patterns in MIP may be relevant to the pathophysiology of schizophrenia. In this regard, schizophrenia is conceptualized as a disorder characterized by activated immune-inflammatory and nitro-oxidative pathways, which cause neuroprogression including abnormalities in neuro-circuitry, neuronal plasticity, neuronal dendrite growth, neural connectivity, membrane receptor expression, neurogenesis, and apoptosis [68–70]. Moreover, MA use may cause increased oxidative stress [71], which may affect LINE-1 hypomethylation patterns [72]. Intragenic LINE-1, in turn, regulates gene expression in cis whereby LINE-1 hypomethylation is accompanied by repression of gene expression, dysregulation of DNA repair genes (including PPP2R2B), DNA double-strand break repair, and genomic instability (including retrotransposition) [34, 73, 74]. Importantly, intragenic LINE-1 is probably involved in the regulation of immune functions and inflammation, oxidative stress processes, cell differentiation, apoptosis, and cellular response to external stimuli thereby explaining a possible link with a variety of neurodegenerative, autoimmune, and immune diseases including schizophrenia [75]. As a consequence, we may hypothesize that MA use induces changes in LINE-1 partial methylation patterns and that users with more severe changes in LINE-1 methylation (lowered % uCmC, increased % mCuC, and increased hypomethylation) are at risk to develop paranoia via altered regulation of cellular processes, immune and oxidative stress pathways. In this respect, it is important to note that MA-induced psychosis-like behaviors are considered to be a model for schizophrenia [13], which raises the question whether similar changes in partial LINE-1 methylation patterns may be observed in schizophrenia. In this respect, another brain disorder, namely autism with severe cognitive impairments, is accompanied by aberrations in LINE-1 methylation patterns [76].

One limitation is that the current study has a cross-sectional design, which does not allow to make causal inferences. In addition, it would have been more interesting if we had measured oxidative biomarkers. Strengths are that we used a new COBRA assay to measure LINE-1 methylation patterns, which revealed aberrations in partial methylation.

In summary, MA users showed higher % mCuC and % mCuC + uCmC levels than controls, while MIP is significantly associated with a specific partial methylation profile, namely increased % mCuC and decreased % uCmC as compared with normal controls. These results are important as intragenic LINE-1 regulates gene expression in cis and mediates gene expression. By inference, MA use could have profound effects on gene expression, dysregulation of DNA repair genes, DNA double-strand break repair and genomic instability. Moreover, MA-induced changes in LINE-1 partial methylation are associated with MA-induced paranoia and therefore may explain in part the pathophysiology of this type of psychosis. Future research should examine LINE-1 partial methylation patterns in schizophrenia phenotypes and should examine novel treatments targeting LINE-1 methylation in order to prevent MIP.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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