DNA Methylation Regulates Cocaine-Induced Behavioral Sensitization in Mice

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The behavioral sensitization produced by repeated cocaine treatment represents the neural adaptations underlying some of the features of addiction in humans. Cocaine administrations induce neural adaptations through regulation of gene expression. Several studies suggest that epigenetic modifications, including DNA methylation, are the critical regulators of gene expression in the adult central nervous system. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and consequent promoter region hypermethylation is associated with transcriptional silencing. In this study a potential role for DNA methylation in a cocaine-induced behavioral sensitization model in mice was explored. We report that acute cocaine treatment caused an upregulation of *DNMT3A* and *DNMT3B* gene expression in the nucleus accumbens (NAc). Using methylated DNA immunoprecipitation, DNA bisulfite modification, and chromatin immunoprecipitation assays, we observed that cocaine treatment resulted in DNA hypermethylation and increased binding of methyl CpG binding protein 2 (MeCP2) at the *protein phosphatase-1 catalytic subunit* (*PP1c*) promoter. These changes are associated with transcriptional downregulation of *PP1c* in NAc. In contrast, acute and repeated cocaine administrations induced hypomethylation and decreased binding of MeCP2 at the *fosB* promoter, and these are associated with transcriptional upregulation of *fosB* in NAc. We also found that pharmacological inhibition of DNMT by zebularine treatment decreased cocaine-induced DNA hypermethylation at the *PP1c* promoter and attenuated *PP1c* mRNA downregulation in NAc. Finally, zebularine and cocaine co-treatment delayed the development of cocaine-induced behavioral sensitization. Together, these results suggest that dynamic changes of DNA methylation may be an important gene regulation mechanism underlying cocaine-induced behavioral sensitization.

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INTRODUCTION

Repeated administration of psychostimulants, such as cocaine or amphetamine, induces an enhanced behavioral response to subsequent drug exposure, a phenomenon known as psychomotor or behavioral sensitization that can persist for months (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). Psychostimulant-induced behavioral sensitization in rodents provides a model for addictive behaviors such as those associated with craving and relapse, as well as for psychotic complications of psychostimulant abuse (Koob and Bloom, 1988; Robinson and Berridge, 1993).

Persistent behavioral sensitization after repeated psychostimulant treatment indicates that drug-induced short- and long-term changes in gene expression may be involved (Nestler and Aghajanian, 1997). Recently, new concepts have emerged about chromatin modification and gene regulation in dividing and non-dividing cells. Chromatin-related modifications of gene expression patterns have been shown to be important for the long-lasting effects of psychostimulants on the brain (Kumar *et al*, 2005; Brami-Cherrier *et al*, 2005; Renthal *et al*, 2007; Renthal *et al*, 2008; Renthal and Nestler, 2008; Shen *et al*, 2008; Stipanovich *et al*, 2008; Renthal *et al*, 2009).

Over the past few years much attention has been paid to the role of histone acetylation and phosphorylation on chromatin modification in psychostimulant-induced neuroadaptations; however, there are no data concerning DNA methylation. The most common form of DNA alteration is the addition of a methyl group to the 5 position of cytosine, catalyzed by DNA methyltransferases (DNMTs) (Bestor, 2000). Cytosine methylation takes place almost exclusively at a cytosine located $5'$ to guanine: cytosine-phospho-guanine (CpG) (Singal and Ginder, 1999). The CpG dinucleotides are unevenly distributed: in mammalian genomes they are clustered in so-called CpG islands—short regions of 0.5 to 4 kb in length having a rich (60–70%) cytosine–guanine content. Over 50% of all dinucleotides in

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these islands are CpG, compared with the rest of the genome where the CpG content is $\leqslant 20\%$ (Bird, 2002). About 50% of CpG islands are located in the promoter regions and around the transcription start sites and are unmethylated in normal cells. The change from unmethylated to methylated CpG islands is recognized as an essential contributor to transcriptional silencing (Robertson and Wolffe, 2000; Jones and Baylin, 2002).

In mammalian cells, two classes of DNMTs catalyze DNA methylation. DNA methyltransferase-1 (DNMT1) is essential for maintaining DNA methylation patterns in proliferating cells and is also involved in establishing new DNA methylation patterns (de novo methylation) (Bestor, 2000; Goll and Bestor, 2005; Siedlecki and Zielenkiewicz, 2006). Members of the second class of methyltransferases, DNMT3A and DNMT3B, are also required for de novo methylation.

In this study, we investigated whether cocaine treatment changes DNMT expression in the nucleus accumbens (NAc), whether DNA methylation regulates protein phosphatase-1 catalytic subunit (PP1c) and fosB gene transcription in the NAc after acute and repeated cocaine treatment, and whether a pharmacological inhibition of DNMT alters the development of behavioral sensitization in mice.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the EU guidelines (directive 86/609/EEC) on the ethical use of animals using the experimental protocol approved by the Faculty of Medicine of the University of Tartu. Male C57BL/6 mice (weight 25–30 g) were obtained from Scanbur BK (Karlslunde, Sweden) and were maintained in temperature- and humidity-controlled rooms with 12 h light–dark cycle (light from 0700 to 1900 h). Animals were allowed access to rodent chow and water ad libitum. They were allowed to acclimate to laboratory conditions and were handled at least 4 days before use in behavioral experiments (which were conducted during the light phase of the light–dark cycle).

Behavioral Procedures

Horizontal locomotor activity was assessed in standard polypropylene cages $(15 \times 25\,\text{cm})$ uniformly illuminated with dim lighting. A light-sensitive video camera, connected to a computer, was mounted about 1.5 m above the observation cage and locomotor activity of eight animals in time was monitored and analyzed using VideoMot2 software (TSE Systems, Bad Homburg, Germany).

For experiments investigating the effect of acute and repeated cocaine treatment, animals were randomly assigned to the following treatment groups: (1) 'SAL', mice were treated for 7 days with sterile saline 0.1 ml per 10 g body weight intraperitoneally (i.p.); (2) acute cocaine 'AC', mice were treated for 6 days with saline and on the seventh day with cocaine hydrochloride (15 mg/kg, i.p., Oriola Oy, Espoo, Finland); (3) repeated cocaine 'RC', mice were treated for 7 days with cocaine (15 mg/kg, i.p.). On the first and seventh treatment days, locomotor activity was recorded for 60 min after the last injection. On the second

through to the sixth treatment days, mice were injected with the drugs and placed in the test cages for 60 min without locomotion recording.

For experiments investigating the effect of inhibition of DNMTs, intracerebroventricular (i.c.v.) infusions of zebularine (300 ng per 0.5 µl; Tocris Bioscience, Bristol, UK) or saline (0.5 µl) were performed 20 min before cocaine (15 mg/kg i.p.) or saline (0.1 ml per 10 g body weight, i.p.) treatment. Zebularine and cocaine were freshly prepared in sterile saline. Mice were randomly assigned to the following treatment groups: (1) saline (i.c.v.) + saline (i.p.) 'S + S'; (2) zebularine (i.c.v.) + saline (i.p.) 'Z + S'; (3) saline (i.c.v.) + cocaine (i.p.) 'S + C'; and (4) zebularine (i.c.v.) + cocaine (i.p.) 'Z + C'. Locomotor activity was recorded for 60 min after i.p. injection, daily for 7 days.

Intracerebroventricular Cannula Implantation

For cannula implantation, mice were anesthetized with Hypnorm (VetaPharma, Leeds, UK) and Dormicum (Roche, Basel, Switzerland). During surgery each mouse was implanted with a 26-gauge bilateral stainless steel guide cannula (Plastics One, Roankoke, VA, USA) from which the injector extended 0.5 mm to end in the ventricle. Stereotaxic coordinates used were as follows: antero-posterior, -0.4 mm from bregma, -1.25 mm lateral from the midline, and -2.0 mm from bregma measured from the tip of the cannula guide (Paxinos and Franklin, 2001). Animals were habituated to dummy cannula removal and given 4 days of recovery and handling before the start of the experimental procedure. At the beginning of the study, target coordinates and proper cannula placement were verified by slowly injecting 1 µl of methylene blue dye into the cannulas of the first four mice while they were under anesthesia. After waiting 5 min for the dye to circulate through the ventricles, the animals were killed. The brains were removed, sliced at the point of cannula entry, and target coordinates were verified.

Tissue Isolation

Dissection of the NAc was performed using a round-shape puncher. The NAc and hippocampus were immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}\text{C}$ until RNA or DNA extracts were prepared.

Measuring mRNA Levels by Real-Time PCR

Total RNA was extracted from the NAc using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Oligo-dT first-strand cDNA was synthesized from 0.4 µg of total RNA using First-Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). Real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System equipped with ABI PRISM 7000 SDS Software (Applied Biosystems, Bedford, MA, USA). Primers for mouse PP1c were from SABiosciences (In Vitro Sweden AB, Stockholm, Sweden) and primers designed for mouse DNMT1, DNMT3A, DNMT3B, and fosB were as listed in Table 1. PCR amplification was performed in a total reaction volume of 25 µl in three parallels. The reaction mixture consisted of 1 µl first-strand cDNA diluted template, 12.5 µl $2 \times$ Master SYBR Green

Table 1 Primers Used in DNA Methylation and Gene Expression Assays

Target gene	Primer sequence (5′–3′)
mRNA RT-PCR primers	
fosB mRNA	ACAGATCGACTTCAGGCGGA
	GTTTGTGGGCCACCAGGAC
DNMT1 mRNA	CCCATGCATAGGTTCACTTCCTTC
	TGGCTTCGTCGTAACTCTCTACCT
DNMT3A mRNA	GCCGAATTGTGTCTTGGTGGATGACA
	CCTGGTGGAATGCACTGCAGAAGGA
DNMT3B mRNA	TCAGAAGGCTGGAGACCTCCCTCTT
	TTCAGTGACCAGTCCTCAGACACGAA
Glyceraldehyde 3-phosphate	CTGAGTATGTCGTGGAGTCTACTGG
dehydrogenase (GAPDH)	GTCATATTTCTCGTGGTTCACACC
β-Actin	TCTCCATGTCGTCCCAGTTG
	ATGGTGGGAATGGGTCAGAAG
Methylation-specific RT-PCR primers	
fosB methylated	TGTTAATTTTAGTTTTCGGGATAGC
	TACGTCAAAAAAAATCCCTCG
fosB unmethylated	ATTACATCAAAAAAAATCCCTCACT
	TTAATTTTAGTTTTTGGGATAGTGT
PP1c methylated	ACGAAAAAAACAAAATAACCGC
	TTTTATGGGTTCGTAAAGAAGTTTC
PP1c unmethylated	ACCACAAAAAAAACAAAATAACCAC
	TTTATGGGTTTGTAAAGAAGTTTTG

RT-PCR Master Mix (Applied Biosystems), 10.5 µl H_2O, and 1 µl gene-specific 10 µM PCR primer pair stock. Amplification specificity was controlled by a melting curve analysis and a gel electrophoresis of the PCR product. Serial dilutions (fivefold) from one wild-type sample total RNA were analyzed for each target gene and allowed to construct linear standard curves from which the concentrations of the test sample and efficiency of PCR reaction were calculated. Results were normalized to *β-actin* or *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) using the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001).

Methylated DNA Immunoprecipitation Assay

Methylated DNA immunoprecipitation (MeDIP) was performed using EpiQuik Tissue Methylated DNA Immunoprecipitation Kit (Epigentek Group, Brooklyn, NY, USA). Genomic DNA was extracted from frozen mouse NAc, sonicated into fragments ranging in size from 200 to 1000 bp, and divided into immunoprecipitated (IP) and input (IN) portions. IP DNA incubated with anti-5-methylcytosine monoclonal antibody to bind methylated DNA, and the negative control was normal mouse IgG from the EpiQuik MeDIP Kit. Methylated DNA was subjected to quantitative real-time PCR using primers from SABiosciences for mouse *PP1c* and *fosB*. To evaluate the relative enrichment of target sequences after MeDIP, we calculated the ratios of the signals in the IP DNA *vs* IN DNA. The resulting values were standardized against the unmethylated control sequence GADPH, and the results of control group (SAL or S + S) were given the value of 1 and fold changes calculated.

Methylation-Specific Real-Time PCR Analysis

DNA was isolated from mouse NAc using QIAmp DNA Mini Kit (QIAGEN) and processed for bisulfite modification using Epitect Bisulfite Kit (QIAGEN). Quantitative real-time PCR was used to determine the DNA methylation status of the *PP1c* and *fosB* genes. Methylation-specific PCR primers were designed using Methprimer software (www.urogene. org/methprimer). Methylation-specific and unmethylated PCR primers were designed to target putative CpG islands detected *in silico* in promoter or non-promoter regions of the *PP1c* and *fosB* genes. Primer sequences are listed in Table 1. PCR reactions were performed as described above. Ct values were chosen within the linear range and the comparative Ct method was used to calculate differences in methylation between samples.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP of genomic DNA associated with a methyl CpG binding protein 2 (MeCP2) was carried out according to the manufacturer's protocol (Millipore, Billerica, MA, USA). Mouse NAc was minced and cross-linked in 1% formaldehyde (10 µl/mg) for 15 min at 37 °C. Adding glycine stopped the cross-linking reaction and the tissue was washed twice in ice-cold PBS containing a protease inhibitor cocktail. The minced, fixed tissue was homogenized in SDS lysis buffer and the homogenate was sonicated to produce 200–1000 bp genomic fragments. Following this, the resulting homogenate was centrifuged for 15 min at 13 000 g and the supernatant was used for ChIP assay. Immunoprecipitations were carried out at 4 °C overnight with 5 µg of rabbit polyclonal MeCP2 (Abcam, Cambridge, UK), negative control was anti-Rat IgG (Vector Laboratories, Burlingame, CA, USA) and positive control was anti-RNA Polymerase II from the EZ-ChIP Kit. A portion of the sonicated DNA was left untreated to serve as input control. Immune complexes were collected with protein A beads and, according to the manufacturer's protocol, sequentially washed two times with low salt buffer, high salt buffer, LiCl immune complex buffer, and TE buffer. Immunoprecipitated DNA was subjected to quantitative real-time PCR using ChIP primers from SABiosciences for mouse *PP1c* and *fosB*.

Western Immunoblotting

The NAc was lysed in 10 vol (w/vol) of lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X 100, and protease inhibitors), homogenized with ultrasound homogenizer, incubated 1 h at + 4 °C, and centrifuged (13 000 g for 10 min at 4 °C). The protein concentration was determined using RCDC Protein Assay (Bio-Rad, Hercules, CA, USA). The supernatants (50 µg of protein) were resolved by electrophoresis on 8% SDS–polyacrylamide gels. The membranes were blocked with 0.1% (w/w) Tween 20/TBS (T-TBS) containing 5% (w/w) non-fat dried milk at room temperature for 1 h. After blocking, the membranes were incubated overnight with the anti-PP1γ1 polyclonal

antibody (1 : 1000; Millipore) followed by incubation with biotinylated anti-rabbit IgM secondary antibody (1 : 1000) for 2 h and ABC solution for 30 min. Mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA) was used to normalize PP1c immunoreactivity on the same blot and PP1c/β-actin optical density (OD) ratio was calculated.

Statistical Analysis

Behavioral data of all experiments were analyzed using ANOVA for repeated measures, followed by Bonferroni post-test, as appropriate. Expression level of genes, MeDIP, and ChIP data were analyzed using one-way ANOVA with Bonferroni post-test. Differences in methylation-specific real-time PCR data were analyzed by two-way ANOVA with Bonferroni post-test. GraphPad Prism software was used for statistical analysis of the data. All data were expressed as mean ± SEM, and significance was set at $p < 0.05$.

RESULTS

Cocaine-Induced Upregulation of *DNMT3A* and *DNMT3B* mRNA Levels in NAc and Hippocampus

First, the effect of acute and repeated cocaine (15 mg/kg/day) on adult mice was evaluated. Acute treatment with cocaine (AC) significantly increased the locomotor activity (Figure 1; two-way ANOVA repeated measures, interaction $F_{2, 35} = 7.43$, $p = 0.002$, treatment effect $F_{2, 35} = 18.9$, $p < 0.0001$, days effect $F_{1, 35} = 28.81$, $p < 0.0001$; Bonferroni post-test, $p < 0.01$). After a daily injection of cocaine for 7 days (RC), all of the mice displayed a significantly enhanced locomotion in response to the same dose of cocaine on day 7 compared with day 1 ($p < 0.001$), indicating a behavioral sensitization. The saline-treated mice (SAL) did not show any sensitization across test sessions ($p > 0.05$).
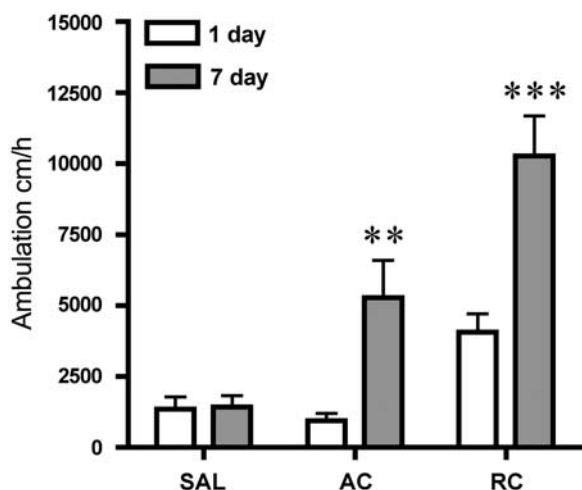

Figure 1 Cocaine-induced behavioral sensitization. Mice were treated daily for 7 days and ambulation was recorded for 1 h immediately after treatment. Treatment groups: SAL, mice were treated with saline; AC, mice were treated with saline for 6 days and with cocaine (15.0 mg/kg i.p.) on the seventh day; RC, mice were treated daily for 7 days with cocaine. Two-way ANOVA with repeated measures, followed by Bonferroni post-test, **$p < 0.01$ AC first *vs* seventh day, ***$p < 0.001$ RC first *vs* seventh day, $n = 11$. Error bars indicate SEM.

It was recently reported that *DNMT3A* and *DNMT3B* gene expression was upregulated in the adult rat hippocampus following contextual fear conditioning, suggesting that DNMTs are dynamically regulated in the adult nervous system (Miller and Sweatt, 2007). Therefore, we investigated whether or not DNMTs mRNA levels are altered by acute and repeated cocaine treatment. We assayed mRNA levels of *DNMT1*, *DNMT3A*, and *DNMT3B* at 1.5 and 24 h after acute and repeated cocaine treatment in the NAc (Figure 2a–c). A single administration of cocaine displayed an increase in *DNMT3A* mRNA level 1.5 h after treatment, relative to animals treated with saline control (*DNMT3A*; one-way ANOVA, Bonferroni post-test, $p < 0.01$ SAL *vs* AC, $n = 12$) and *DNMT3A* and *DNMT3B* mRNA levels at 24 h after treatment (*DNMT3A*; one-way ANOVA, Bonferroni post-test, $p < 0.001$ SAL *vs* AC, $n = 12$; *DNMT3B*, $p < 0.05$ SAL *vs* AC, $n = 12$). However, the repeated treatment with cocaine did not change significantly mRNA levels of *DNMT3A* and *DNMT3B* genes compared with saline control. Both acute and repeated cocaine treatment did not alter the mRNA level of *DNMT1* gene.

In a parallel study, the effect of acute and repeated cocaine treatment on *DNMT1*, *DNMT3A*, and *DNMT3B* mRNA levels in the hippocampus at 1.5 and 24 h after treatment was determined (Supplementary Figure S1 A-C). We found that the acute and repeated cocaine treatment increased *DNMT3A* mRNA levels 1.5 h after treatment (one-way ANOVA, Bonferroni post-test, $p < 0.001$ SAL *vs* AC or RC, $n = 7$) and *DNMT3B* expression level was increased in the hippocampus only after acute cocaine treatment (one-way ANOVA, Bonferroni post-test, $p < 0.001$ SAL *vs* AC, $n = 7$). There were no changes in *DNMT3A* and *DNMT3B* mRNA levels 24 h after cocaine treatment compared with the saline control. Both acute and repeated cocaine treatment did not alter the mRNA level of *DNMT1* gene in the hippocampus. Thus, the time course analysis demonstrated that *DNMT3A* and *DNMT3B* expression is dynamically regulated in the adult NAc and hippocampus in response to cocaine treatment.

Altered Patterns of Gene Expression after Cocaine Treatment

Increased expression of *DNMT3A* and *DNMT3B* might cause hypermethylation of certain gene promoters associated with CpG islands and consequently downregulate the expression of these genes. Therefore, a search was conducted for genes that show a diminished expression following cocaine treatment. Several genes were tested, such as *brain-derived neurotrophic factor* (*BDNF*), *PP1c*, *fosB*, and *adenosine A_{2A} receptor* ($A_{2A}R$), which participate in cocaine-induced neuroadaptations in the NAc and *in silico* analysis revealed CpG islands located within promoter regions.

Using quantitative real-time PCR we found that *PP1c* mRNA levels in the NAc were not altered 1.5 h after both acute and repeated cocaine treatment (Figure 3a). However, 24 h after treatment in both acute and repeated cocaine groups, the *PP1c* mRNA levels were significantly decreased compared with the saline control (one-way ANOVA, Bonferroni post-test, $p < 0.05$ AC or RC *vs* SAL, $n = 12$). To further confirm *PP1c* mRNA downregulation in the NAc,
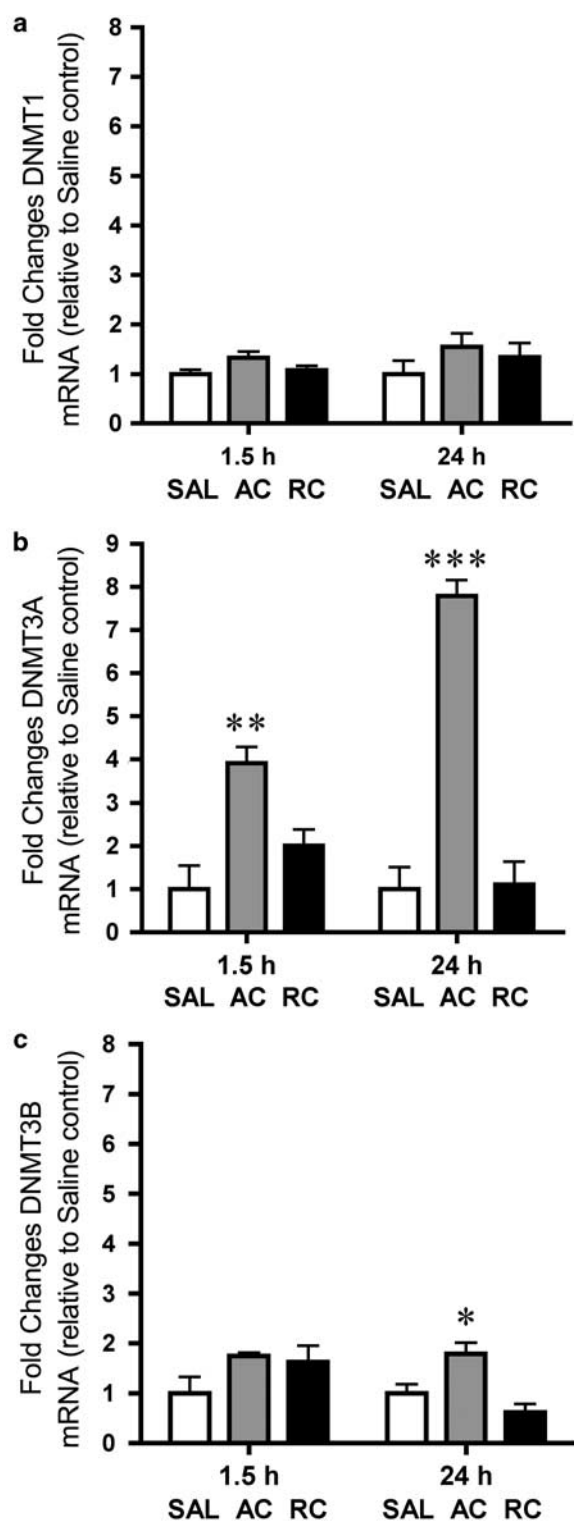
Figure 2 Acute cocaine treatment was associated with an upregulation of *DNMT3A* and *DNMT3B* mRNA levels in the NAc, using a quantitative real-time PCR. (a–c) Cocaine effect on *DNMT1*, *DNMT3A*, and *DNMT3B* mRNA levels at 1.5 and 24 h after treatment in the NAc. One-way ANOVA, followed by Bonferroni post-test, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ compared with saline (SAL) group, $n = 11$. Error bars indicate SEM.

western blot analysis was performed. We found that both acute and repeated cocaine treatment resulted in a significantly lower PP1c protein level 24 h after treatment

(Figure 3b; one-way ANOVA, Bonferroni post-test, $p < 0.05$ AC or RC *vs* SAL, $n = 9$).

As expected, it was found that acute and repeated cocaine treatment resulted in upregulation of *fosB* mRNA level 1.5 h after treatment (Figure 3c; one-way ANOVA, Bonferroni post-test, $p < 0.001$ AC or RC *vs* SAL, $n = 12$). However, there were no significant changes 24 h after acute and repeated cocaine treatment. Acute and repeated cocaine treatment also resulted in significant downregulation of $A_{2A}R$ mRNA level 1.5 h after treatment (Figure 3d; one-way ANOVA, Bonferroni post-test, $p < 0.0001$ AC or RC *vs* SAL, $n = 7$). However, there were no significant changes in the mRNA level of *BDNF* gene after cocaine treatment in the NAc (Supplementary Figure S2). As we were interested in studying cocaine-induced long-lasting effects, for future DNA methylation studies, *PP1c* and *fosB* (as reference) genes were selected.

Cocaine Treatment Changed DNA Methylation at *PP1c* and *fosB* Promoter Regions

To shed further light on the role of DNA methylation in cocaine-induced behavioral sensitization, we next looked for an aberrant DNA methylation during acute and repeated cocaine treatments. First, we evaluated cocaine-induced changes of DNA methylation patterns at the *PP1c* promoter region using MeDIP assay. We found that both acute and repeated cocaine treatment resulted in *PP1c* promoter-associated CpG island hypermethylation 24 h after treatment (Figure 4b; one-way ANOVA, Bonferroni post-test, $p < 0.001$ AC or RC *vs* SAL, $n = 6$).

To verify cocaine-induced hypermethylation at the *PP1c* promoter region, methylation-specific quantitative real-time PCR (MSP qPCR) was employed. Data generated using MSP qPCR demonstrated that acute and repeated cocaine treatment elicited DNA hypermethylation associated with the *PP1c* promoter region 24 h after treatment (Figure 4a and c; two-way ANOVA, interaction $F_{2,66} = 8.34$, $p = 0.0006$, treatment effect $F_{2,66} = 2.77$, $p = 0.0699$, methylation effect $F_{1,66} = 31.24$, $p < 0.0001$; Bonferroni post-test, $p < 0.05$ methylated AC *vs* methylated SAL, and $p < 0.001$ methylated RC *vs* methylated SAL). As a DNA methylation control, levels of unmethylated DNA within the *PP1c* promoter after acute and repeated cocaine treatment was measured, using primer sequences designed to amplify unmethylated CpG island sites. Thus, MeDIP and MSP qPCR methods demonstrated that acute and repeated cocaine treatment resulted in hypermethylation at the *PP1c* promoter region 24 h after treatment.

In correlation with upregulated *fosB* mRNA levels, we found hypomethylation at the *fosB* promoter 1.5 h after acute and repeated cocaine treatment, using MeDIP assay (Figure 5a and b; one-way ANOVA; Bonferroni post-test, $p < 0.001$ AC or RC *vs* SAL, $n = 6$). To further confirm that acute and repeated cocaine administration altered DNA methylation at the promoter regions of *PP1c* and *fosB* genes, ChIP assay was performed. Consistent with the MeDIP and MSP qPCR results, ChIP analysis demonstrated that acute and repeated cocaine treatment increased by 1.8- to 2-fold the *PP1c* promoter-associated MeCP2 binding 24 h after treatment in the NAc (Figure 6a; one-way ANOVA, Bonferroni post-test, $p < 0.01$ AC or RC *vs* SAL, $n = 6$).
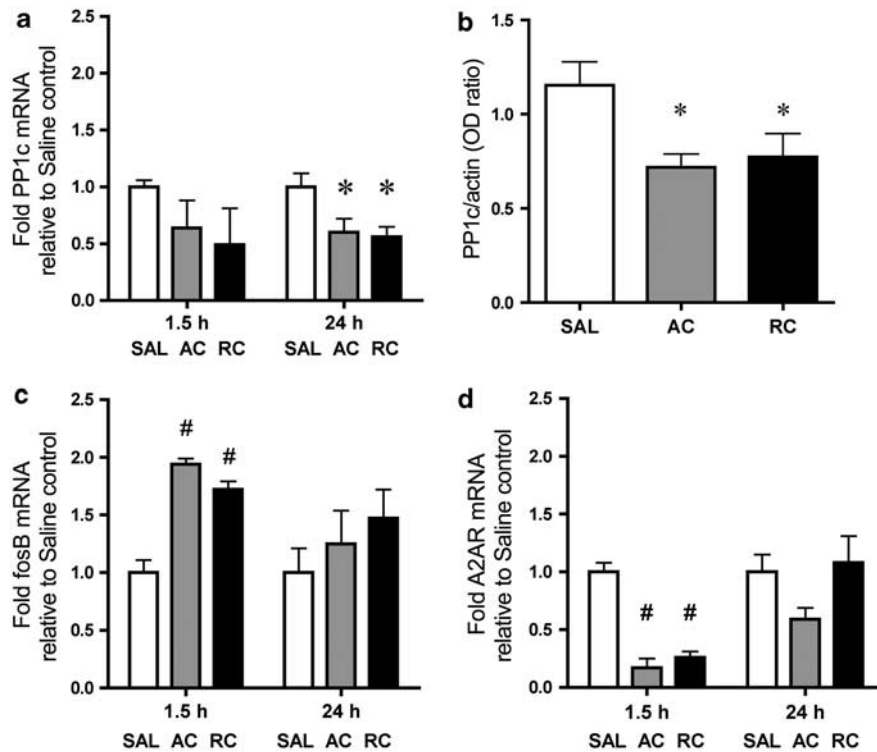
Figure 3 Altered patterns of *PP1c*, *fosB*, and *A2AR* gene expressions after cocaine treatment in the NAc. (a) The effect of acute (AC) and repeated cocaine (RC) administration on *PP1c* mRNA levels at 1.5 and 24 h after treatment, using quantitative real-time PCR. (b) AC and RC treatment resulted in decrease of PP1c protein level 24 h after treatment in the NAc, using western blot analysis. (c) The effect of AC and RC administration on *fosB* mRNA levels, using quantitative real-time PCR. (d) The effect of AC and RC administration on *A2AR* mRNA levels, using quantitative real-time PCR. One-way ANOVA, followed by Bonferroni post-test, *$p < 0.05$, #$p < 0.001$ compared with saline (SAL) group, $n = 11$. Error bars indicate SEM. OD, optical density.

However, in the *fosB* promoter, acute and repeated cocaine treatment was associated with a significant decrease in MeCP2 binding compared with the saline control at 1.5 h after treatment (Figure 6b; one-way ANOVA, Bonferroni post-test, $p < 0.001$ AC or RC vs SAL, $n = 6$). Taken together, these data support the idea that alteration of DNA methylation in promoter regions controls gene expression because there is a correlation of increased *PP1c* promoter-associated CpG island hypermethylation with increased MeCP2 binding and with decreased *PP1c* gene transcription and opposite effect at the *fosB* promoter.

Zebularine Inhibited DNA Hypermethylation at *PP1c* Promoter and Delayed Cocaine-Induced Behavioral Sensitization

As we found that cocaine induced *DNMT3A* and *DNMT3B* expression and *PP1c* promoter hypermethylation, the next goals in this study were to assess the effect of DNMT inhibitor on cocaine-induced molecular changes and development of behavioral sensitization. We selected the DNMT inhibitor zebularine for testing based on reported data (Miller and Sweatt, 2007; Lubin *et al*, 2008). To correlate the molecular changes with cocaine-induced locomotor sensitization, a dose of zebularine (300 ng per 0.5 µl, i.c.v.) that did not affect basal locomotor activity and decreased DNA methylation at the *PP1c* promoter region at 1.5 h after acute treatment (Figure 7a; t-test, $p = 0.034$, $n = 4$) was selected from our pilot study.

For the co-treatment experiment, all mice received i.c.v. infusion of saline or zebularine followed, after 20 min, by i.p. saline or cocaine (15 mg/kg). Locomotor activity was recorded for 60 min immediately after the last i.p. injection (see Materials and Methods). Repeated saline and cocaine (S + C) treatment for 7 days displayed a significantly enhanced locomotion on day 7 compared with that of day 1 (Figure 7b; two-way ANOVA with repeated measures, interaction $F_{3, 39} = 5.69$, $p = 0.0025$, treatment effect $F_{3, 39} = 15.98$, $p < 0.0001$, days effect $F_{1, 39} = 10.26$, $p = 0.0027$; Bonferroni post-test, $p < 0.001$ S + C day 1 vs day 7). However, mice co-treated with zebularine and cocaine (Z + C) did not show any sensitization on day 7 compared with day 1 ($p > 0.05$). There was also no behavioral sensitization after repeated zebularine and saline (Z + S) treatments ($p > 0.05$). Therefore, our results suggest that the inhibition of DNMTs with zebularine did not affect acute cocaine-induced locomotor activity, but instead delayed cocaine-induced behavior sensitization in mice.

Finally, we studied for molecular evidence to confirm that zebularine infusions (i.c.v.) before cocaine treatment altered DNA methylation level at the *PP1c* promoter region in the NAc.

Using MeDIP assay we found that repeated cocaine treatment (S + C) induced, at the *PP1c* promoter region, DNA hypermethylation 24 h after treatment compared with the saline control group (S + S), and repeated zebularine and cocaine (Z + C) co-treatment avoided this effect (Figure 8a; one-way ANOVA, Bonferroni post-test,
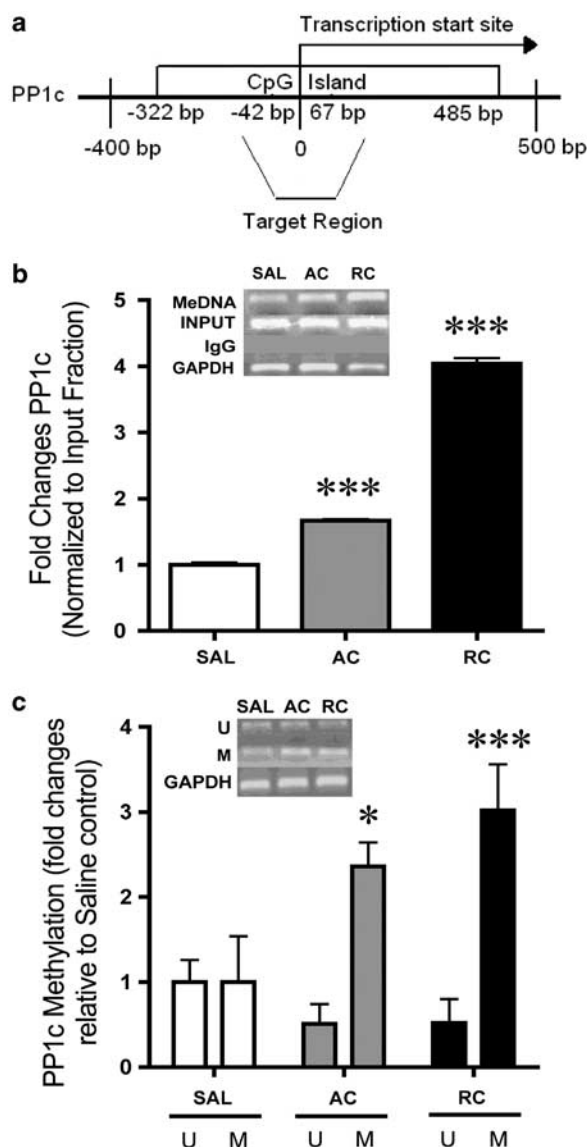
Figure 4 Cocaine treatment was associated with altered DNA methylation at *PP1c* promoters in the NAc at 24 h after treatment. (a) The position of the *PP1c* promoter-associated CpG island is indicated relative to transcription start site. The target region indicates the locations of MSP qPCR primer pairs. (b) Acute (AC) and repeated cocaine (RC) treatment elicited DNA hypermethylation associated with the *PP1c* promoter region, using MeDIP assay. One-way ANOVA, followed by Bonferroni post-test, ***$p < 0.001$ compared with saline (SAL) group, $n = 6$. (c) AC and RC treatment elicited hypermethylation associated with the *PP1c* promoter region, using MSP qPCR. Two-way ANOVA followed by Bonferroni post-test, *$p < 0.05$, ***$p < 0.001$ compared with methylated SAL group, $n = 11$. Error bars indicate SEM. M, methylated; MeDNA, methylated DNA; U, unmethylated.

$p < 0.001$ S + S *vs* S + C, $p < 0.001$ S + C *vs* Z + C, $n = 6$). Similarly, MSP qPCR analysis demonstrated that there was a significant decrease of DNA methylation associated with the *PP1c* promoter region in the Z + C group compared with the S + C group (Figure 8b; two-way ANOVA, interaction $F_{3, 45} = 80.8$, $p < 0.0001$, treatment effect $F_{3, 45} = 39.0$, $p < 0.0001$, methylation effect $F_{1, 45} = 42.8$, $p < 0.0001$; Bonferroni post-test, $p < 0.001$ methylated S + C *vs* methylated Z + C). Interestingly, in both studies we found significant changes
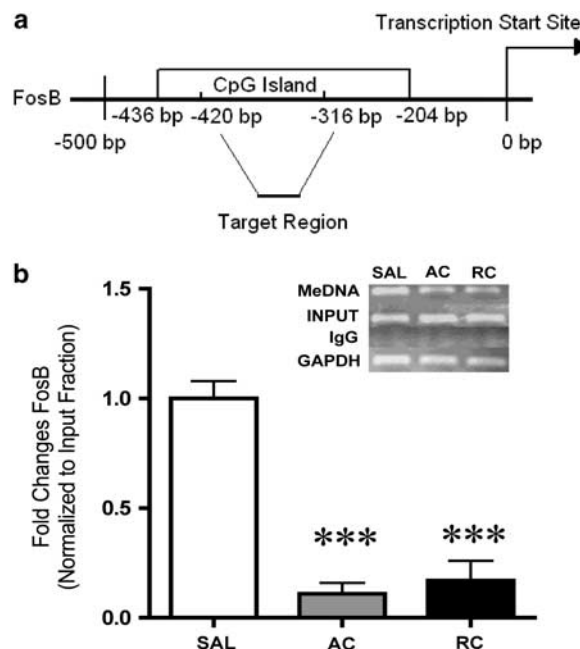
Figure 5 Cocaine treatment was associated with altered DNA methylation at *fosB* promoters in the NAc at 1.5 h after treatment. (a) The position of the *fosB* promoter-associated CpG island is indicated relative to transcription start site. The target region indicates the locations of the qPCR primer pairs. (b) Acute (AC) and repeated cocaine (RC) treatment elicited DNA hypomethylation associated with the *fosB* promoter region, using MeDIP assay. One-way ANOVA, followed by Bonferroni post-test, ***$p < 0.001$ compared with saline (SAL) group, $n = 6$. Error bars indicate SEM. MeDNA, methylated DNA.

of DNA methylation between the zebularine (Z + S) and the saline (S + S) control groups ($p < 0.05$).

In correlation with the *PP1c* promoter-associated CpG island methylation results, we observed that zebularine attenuated cocaine-induced downregulation of *PP1c* mRNA level (Figure 8c; one-way ANOVA; Bonferroni post-test, $p < 0.001$ S + S *vs* S + C, $p < 0.05$ S + C *vs* Z + C, $n = 6$). These results indicate that the inhibition of DNMTs by zebularine reverses cocaine-induced *PP1c* gene hypermethylation and mRNA downregulation in the NAc.

DISCUSSION

In this study we have produced four main findings. First, it was found that acute cocaine treatment results in an upregulated mRNA of *DNMT3A* and *DNMT3B* in the NAc. Second, it was observed that acute and repeated cocaine treatment is associated with DNA hypermethylation and transcriptional downregulation of the *PP1c* gene, and hypomethylation and transcriptional activation of the *fosB* gene in the NAc. Third, it was found that i.c.v. infusions of the DNMT inhibitor zebularine, before cocaine treatment, decreased levels of DNA hypermethylation at the *PP1c* promoter-associated CpG island and attenuated *PP1c* mRNA downregulation in the NAc. Finally, zebularine and cocaine co-treatment were found to delay development of cocaine-induced behavioral sensitization. These observations suggest that DNA hyper- and hypomethylation may be
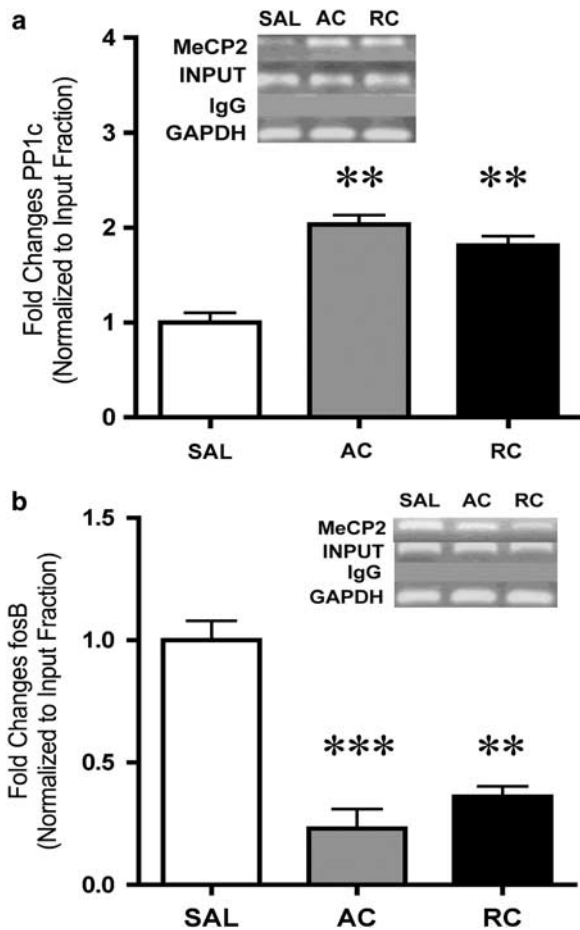
Figure 6 Altered patterns of MeCP2 binding at PP1c and fosB promoter regions in the NAc, using ChIP assay. (a) Acute (AC) and repeated cocaine (RC) treatment increased MeCP2 binding at the PP1c promoter region relative to the saline (SAL) group at 24h after treatment. (b) AC and RC treatment decreased MeCP2 binding at the fosB promoter region at 1.5h after treatment. One-way ANOVA, Bonferroni post-test, **$p < 0.01$, ***$p < 0.001$ relative to SAL, $n = 6$. Error bars indicate SEM.
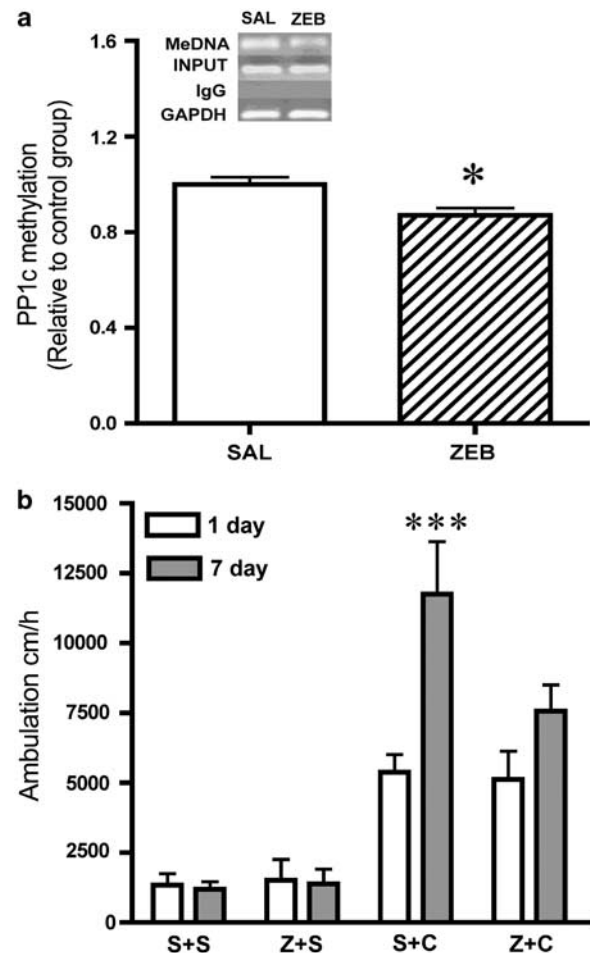

Figure 7 Inhibition of DNMT by zebularine delayed cocaine-induced behavioral sensitization. (a) Acute zebularine (ZEB) (300 ng per 0.5 μl, i.c.v.) treatment elicited DNA hypomethylation associated with the PP1c promoter region at 1.5h after treatment in the NAc, using MeDIP assay. T-test, *$p = 0.034$, $n = 4$. (b) Mice were treated with cocaine (15.0 mg/kg, i.p.) alone or co-treated with zebularine (300 ng per 0.5 μl, i.c.v.) daily for 7 days, and their ambulation was recorded for 1h immediately after treatment. Mice were treated with saline (0.5 μl, i.c.v.) + saline (0.1 ml per 10 g body weight, i.p.) (S + S); zebularine i.c.v. + saline i.p. (Z + S); saline i.c.v. + cocaine i.p. (S + C); or zebularine i.c.v. + cocaine i.p. (Z + C). Two-way ANOVA with repeated measures, followed by Bonferroni post-test, ***$p < 0.001$ S + C first vs seventh day, $n = 7$–12. Error bars indicate SEM. MeDNA, methylated DNA.

important processes during the development of cocaine-induced behavioral sensitization.

Psychostimulants such as cocaine and amphetamine induce neuroadaptations through regulation of gene expression. New concepts have emerged concerning the role of chromatin modification as a requirement for the regulation of gene expression in neurons. In this study we focused on the function of DNA methylation in cocaine-altered gene expression in the NAc. Previous findings have implied that the function of DNMTs in the CNS might be involved in DNA repair and neurodegeneration (Brooks et al, 1996; Endres et al, 2000; Fan et al, 2001; Endres et al, 2001) and that misregulation of DNA methylation and DNMTs might be involved in cognitive disorders such as schizophrenia, Rett syndrome, and Fragile X mental retardation (Veldic et al, 2004; Amir et al, 1999; Sutcliffe et al, 1992).

Recently, Miller and Sweatt (2007) showed that DNA methylation levels were rapidly and dynamically regulated in the hippocampus following the associative training paradigm of contextual fear conditioning. They also found that mRNAs for DNMT3A and DNMT3B were upregulated

in area CA1 of the hippocampus following fear conditioning. The results of the present study were comparable in that they demonstrated that acute cocaine treatment was associated with an upregulation of DNMT3A and DNMT3B genes in the NAc and hippocampus (Figure 2b and c; Supplementary Figure S1B and C, respectively). However, no significant changes of DNMT1 mRNA levels in the NAc and hippocampus were found, suggesting that cocaine induces rather de novo than maintenance of DNA methylation. It was also found that DNMT3A gene expression achieved the highest level after acute cocaine treatment and it diminished after repeated cocaine exposure. In relation to DNMT3A and DNMT3B expression levels in the NAc, cocaine-induced changes in the hippocampus were more dynamic as there were no significant changes 24h after treatment.
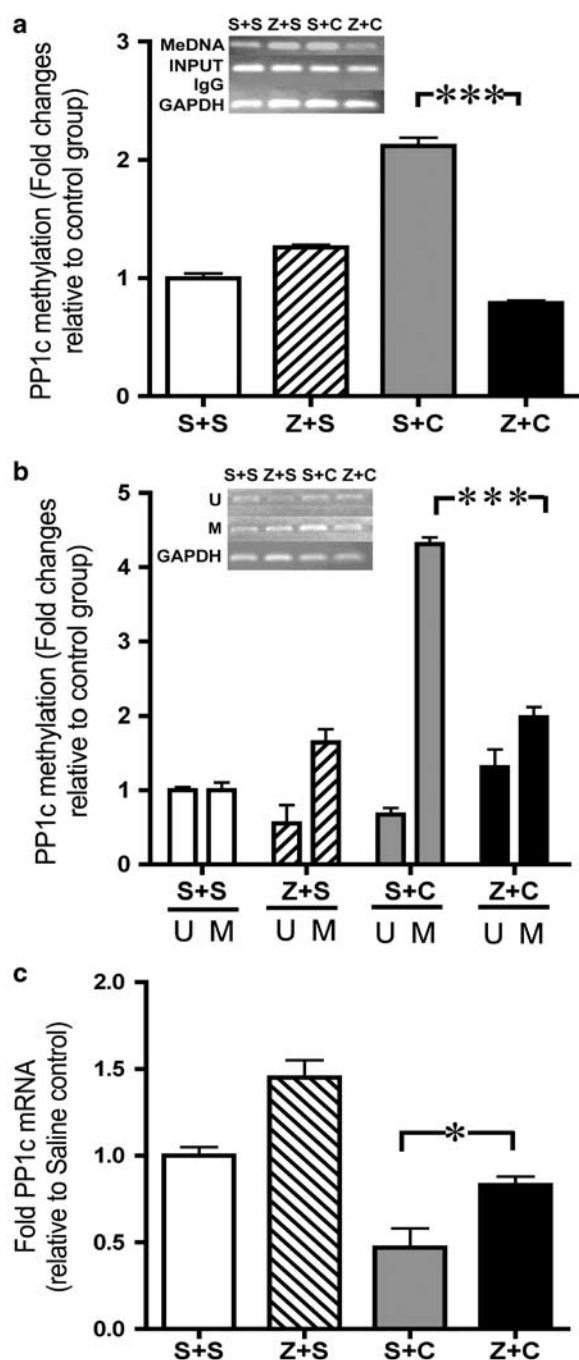
Figure 8 Inhibition of DNMT by zebularine altered DNA methylation at the *PP1c* promoter region 24 h after treatment in the NAc. Mice were treated with saline (0.5 μl, i.c.v.) + saline (0.1 ml per 10 g body weight, i.p.) (S + S); zebularine i.c.v. + saline i.p. (Z + S); saline i.c.v. + cocaine i.p. (S + C); or zebularine i.c.v. + cocaine i.p. (Z + C). (a) Repeated Z + C co-treatment decreased DNA methylation level associated with the *PP1c* promoter relative to the S + C group. (b) Repeated Z + C treatment decreased DNA methylation level associated with the *PP1c* promoter relative to the S + C group. Two-way ANOVA followed by Bonferroni post-test, ***$p < 0.001$ methylated S + C vs methylated Z + C, $n = 5$–7. (c) The blockade of *PP1c* methylation by zebularine was associated with an enhanced *PP1c* mRNA level. One-way ANOVA followed by Bonferroni post-test *$p < 0.05$ S + C vs Z + C, $n = 5$–7. Error bars indicate SEM. M, methylated; U, unmethylated; MeDNA, methylated DNA.

The signal transduction processes that might control cocaine-induced DNMT expression in the adult CNS are unknown at present. Although DNA methylation is a part of the regulation of gene expression, it infers that there may be some overlap of intracellular signaling pathways with other chromatin modification mechanisms, such as histone acetylation and phosphorylation. In the adult, CNS chromatin modification is governed by various intracellular signaling pathways, including the Ras-mitogen-activated protein kinase (MAPK/ERK) cascade (Renthal and Nestler, 2008; Brami-Cherrier et al, 2009). Several studies demonstrated that targeting the Ras signaling pathway with drugs such as methotrexate and inhibitors of MAPK/ERK decreases DNA methylation in malignant hematological diseases and colon cancer cells, indicating a causal relationship between Ras signaling and DNA methylation (MacLeod et al, 1995; Philips, 2004; Morgan et al, 2007; Lu et al, 2007; Diaz-Flores and Shannon, 2007).

In recent years, the MAPK/ERK signaling cascade has been implicated in responses to most drugs of abuse (Berke and Hyman, 2000; Nestler, 2001; Fasano and Brambilla, 2002; Girault et al, 2007). Several studies have demonstrated that in response to cocaine, the MAPK/ERK and the downstream mitogen- and stress-activated protein kinase 1 (MSK1) controls an early phase of histone H3 phosphorylation at the *c-fos* promoter in striatal neurons (Brami-Cherrier et al, 2005). MSK1 action may be potentiated by the concomitant inhibition of PP1 by nuclear translocation of dopamine and cAMP-regulated phosphoprotein Mr 32 000 (DARPP-32) (Stipanovich et al, 2008). Cocaine also phosphorylates the transcription factor cAMP response element binding protein (CREB) via the MAPK/ERK–MSK1 cascade (Swank and Sweatt, 2001; Levine et al, 2005). This phosphorylation and activation of CREB recruits CREB binding protein (CBP), which is a histone acetyltransferase that regulates local chromatin structure as part of CREB-dependent activation of nuclear gene transcription. Therefore, we speculate that cocaine-induced activation of the Ras signaling pathway might trigger both DNA methylation and histone modifications.

Using MeDIP and MSP qPCR methods, we demonstrated that DNMT activation results in increased methylation of the *PP1c* promoter region in the NAc after acute and repeated cocaine treatment (Figure 4b and c). It was also found that *PP1c* promoter region hypermethylation corresponded with decreased expression levels of both mRNA and protein (Figure 3a). These changes were also related to an overall increase in methyl CpG binding domain proteins, such as MeCP2 binding in the *PP1c* promoter region (Figure 6a). It has been shown experimentally that methylation of DNA brings about general deacetylation of histones H3 and H4, prevents methylation at histone H3 K4, and induces methylation of histone H3 K9 (Shahbazian and Grunstein, 2007; Li et al, 2007; Kouzarides, 2007; Patra and Szyf, 2008). Therefore, we speculate that MeCP2 can recruit co-repressor complexes to methylated *PP1c* promoter regions, including histone-modifying enzymes such as histone deacetylases (HDAC) and histone methyltransferases.

Repression of *PP1c* may result in several effects on the intracellular signal transduction level; for example, inhibition of *PP1c* has been shown to increase phosphorylation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

(AMPA) receptor subunit GluR1 and Ca2 + /calmodulin-dependent protein kinase II (Genoux et al, 2002). Reduced PP1 may have important effects for transcriptional regulation by CREB through the ability of PP1 to complex with HDAC. Canettieri et al (2003) demonstrated that a HDAC1–PP1 complex represses CREB activation under basal conditions and dephosphorylates CREB to return the system to baseline after a stimulus. This suggests that repression of PP1 is crucial in providing phosphorylated CREB with the capacity to recruit CBP to the promoter, at which time histones become acetylated and help to drive the transcription of particular genes. Taken together, repression of the *PP1c* gene via methylation of promoter regions should allow aberrant phosphorylation of crucial receptors, protein kinases, and transcription factors during cocaine-induced neuroadaptations.

Previous studies have shown that expression of Fos-family proteins (c-Fos, FosB, Fra-1, and Fra2) is rapidly induced in the NAc after acute exposure to drugs of abuse (Hope et al, 1994). This induction is transient, lasting only 4–12 h after drug exposure. Because acute cocaine treatment caused in *fosB* promoter-associated CpG island hypomethylation (Figure 5b), and decreased MeCP2 binding (Figure 6b) and *fosB* mRNA upregulation (Figure 3c), this suggests that cocaine may also induce DNA demethylation and increase expression in certain genes. At present, there is no clearly identified DNA demethylase in mammals and the biochemical mechanism for active DNA demethylation is unknown (Ooi and Bestor, 2008). Several reports discovered that the demethylation process is initiated by DNMT3A and DNMT3B (Kangaspeska et al, 2008; Métivier et al, 2008). These studies proposed that DNMT3A and DNMT3B possess deaminase activity and that both enzymes are involved in a dynamic demethylation–methylation pathway that operates during gene transcription (Kangaspeska et al, 2008; Métivier et al, 2008). Recently, Ma et al (2009) proposed that active DNA demethylation can be potentially carried out through three mechanisms: direct removal of the methyl group from the 5-methylcytosine base, base excision of 5-methylcytosine through DNA glycosylases, and nucleotide excision of 5-methylcytosine through endonucleases. Base and nucleotide excisions are followed by similar subsequent fill-in repair with unmethylated cytosine.

In this study we demonstrate that DNMT inhibition by zebularine blocked hypermethylation on the *PP1c* promoter region and delayed the development of cocaine-induced behavioral sensitization. The decrease of *PP1c* hypermethylation was triggered using i.c.v. infusion of zebularine and it was sufficient to re-establish *PP1c* gene expression in the NAc. Zebularine is a potent inhibitor, affecting all DNMT subtypes (Weisenberger et al, 2004; Marquez et al, 2005). However, we were unable to determine which subtype of DNMT is important for the development of behavioral sensitization. It is possible that both DNMT3A and DNMT3B work together to hypermethylate the promoter-associated CpG islands and to then transcriptionally repress these genes, aiding in the development of behavioral sensitization.

In Figure 7a we show that a single dose of zebularine caused hypomethylation at the promoter of *PP1c* at 1.5 h after treatment. However, the effect of repeated zebularine (Z + S) administration resulted in *PP1c* promoter region hypermethylation 24 h after last treatment (Figure 8a and b). Based on zebularine pharmacokinetics (Holleran et al, 2005), we speculate that 24 h after the last administration of zebularine (Z + S), the DNMT inhibition effect is ended. Therefore, it is possible that a slight increase of methylation in *PP1c* promoter-associated CpG island in Z + S groups (Figure 8 and b) is caused by a compensatory increase of DNMT activity after inhibition by zebularine. Despite slight hypermethylation at *PP1c*, we found 1.5-fold increased mRNA level in the Z + S group compared with the saline control (S + S, Figure 8c), suggesting that smaller changes of DNA methylation may not alter gene transcription. Other DNA methylation-associated epigenetic mechanisms (histone acetylation, phosphorylation, and methylation) may also alter gene transcription activity. Our results demonstrated that zebularine blocked hypermethylation at the *PP1c* promoter region and downregulation of *PP1c* mRNA level. We speculate that in addition to *PP1c*, zebularine may also inhibit hypermethylation in promoter-associated CpG islands of other genes, re-establish these gene transcriptions, and therefore inhibit cocaine-induced neuroplasticity in the brain.

At the behavioral level, we found that repeated co-administration of zebularine with cocaine markedly delayed the development of behavioral sensitization, whereas zebularine by itself did not affect locomotor activity. Interestingly, zebularine did not modify acute cocaine effect, suggesting that the promoter region hypermethylation might be an important underlying mechanism for altered gene expression during development of behavioral sensitization. Therefore, we hypothesize that zebularine via normalization of gene transcription at cellular level stabilized neuronal network function and therefore delayed the development of psychostimulant-induced behavioral sensitization. As DNA methylation has an important role in learning and memory formation (Miller and Sweatt, 2007), zebularine may also delay cocaine-induced behavioral sensitization by inhibiting DNA methylation in the hippocampus and altering associated learning and memory formation.

In conclusion, our study demonstrated that cocaine treatment might cause a dynamic increase in *DNMT3A* and *DNMT3B* expression and consequently hypermethylation of promoter-associated CpG islands in the NAc. It is not known which subtype of DNMT is important for DNA methylation during the development of behavioral sensitization. However, our results suggest that cocaine induces rather *de novo* than maintenance DNA methylation. The results of DNMT inhibitor zebularine and cocaine co-treatment experiments demonstrated that zebularine effectively inhibited cocaine-induced *PP1c* hypermethylation and delayed the development of behavioral sensitization. Therefore, DNMT3A and DNMT3B may be important molecular targets for modifying psychostimulant-induced neurochemical and behavioral effects.

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DISCLOSURE

The authors declare no conflict of interest.

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