S-adenosylmethionine modifies cocaine-induced DNA methylation and increases locomotor sensitization in mice

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Abstract
Several studies suggest that individual variability is a critical component underlying drug addiction as not all members of a population who use addictive substance become addicted. There is evidence that the overall epigenetic status of a cell (epigenome) can be modulated by a variety of environmental factors, such as nutrients and chemicals. Based on these data, our aim was to investigate whether environmental factors like S-adenosylmethionine (SAM) via affecting epigenome could alter cocaine-induced gene expression and locomotor sensitization in mice. Our results demonstrate that repeated SAM (10 mM/kg) pretreatment significantly potentiated cocaine-induced locomotor sensitization. Using mouse nucleus accumbens (NAc) tissue, whole-genome gene expression profiling revealed that repeated SAM treatment affected a limited number of genes, but significantly modified cocaine-induced gene expression by blunting non-specifically the cocaine response. At the gene level, we discovered that SAM modulated cocaine-induced DNA methylation by inhibiting both promoter-associated CpG-island hyper- and hypomethylation in the NAc but not in the reference tissue cerebellum. Finally, our in vitro and in vivo data show that the modulating effect of SAM is in part due to decreased methyltransferase activity via down-regulation of Dnmt3a mRNA. Taken together, our results suggest that environmental factors that affect the NAc-cell epigenome may alter the development of psychostimulant-induced addiction and this may explain, at least partly, why some individuals are more vulnerable to drug addiction.

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Key words: Cocaine, DNA methylation, gene expression, locomotor sensitization, S-adenosylmethionine.

Introduction
Several studies have shown that individual variability is a critical component that underlies the development of drug addiction as not all members of a population will exhibit signs of addiction despite equivalent drug availability or drug history (Deroche-Gamonet et al., 2004; Kreek et al., 2005). Roughly half of an individual’s risk for drug addiction is genetic, but the specific genes that confer the risk for drug addiction remain largely unknown (Nestler, 2001; Goldman et al., 2005; Hyman et al., 2006). Other potential factors for vulnerability to addictive disease are the long-lasting epigenetic modifications, including DNA methylation, which are critical regulators of gene expression in the adult central nervous system (Day and Sweatt, 2011; Robison and Nestler, 2011). DNA methylation is catalysed by DNA methyltransferase (Dnmt) and occurs at the 5’ position of cytosine residues within cytosine-phospho-guanine ( CpG) dinucleotides (Bestor, 2000). Several recent studies have provided crucial evidence of the role of DNA methylation in cocaine-induced neuronal plasticity in the mouse nucleus accumbens (NAc) and hippocampus (Anier et al., 2010; Han et al., 2010; LaPlant et al., 2010). However, it is not clear whether the aberrant DNA methylation is involved in the subject’s sensitivity to psychostimulants.

There is a growing acceptance that the overall epigenetic status of a cell (epigenome) can be modulated by a variety of environmental factors, including nutrients, chemicals and the early-life environment (Weaver et al., 2004; Waterland et al., 2006; Roth et al., 2009). The epigenome therefore provides an important
interface between genes and the environment and may be viewed as a potential mechanism underlying the rapid form of environmentally driven adaptation (Franklin and Mansuy, 2010). Based on these data, we hypothesize that the complex effect of environment on the epigenome may affect the development of psychostimulant-induced addiction and this may be (at least partially) the underlying mechanism why some individuals are more prone to take drugs and/or to develop addiction.

The environmental factors S-adenosylmethionine (SAM, also known as AdoMet), L-methionine (Met) and folic acid may increase DNA methylation and alter gene expression (Ross, 2003). Of these agents, SAM is the primary biochemical methyl donor and it reacts with nucleophilic acceptors in association with various methyltransferases including Dnmt (Lu, 2000; Ulrey et al., 2005). A number of data support the notion that exogenous SAM administration can increase the levels of intracellular SAM and trigger hypermethylation of DNA (Watson et al., 1999; Lu, 2000; Fusso et al., 2001). It has been shown that SAM treatment also inhibits intracellular demethylase activity, which results in hypermethylation of DNA (Detich et al., 2003). Therefore, in this study we used exogenous SAM treatment to affect the epigenome. We demonstrate that SAM pretreatment modifies cocaine-elicited gene expression (at genome and gene level), alters the development and expression of cocaine-induced locomotor sensitization and these changes are in part due to reduced Dnmt3a and -3b expression in the NAc.

**Method**

**Animals**

Adult male C57BL/6 mice (aged 5–6 months, weight 28–32 g) were obtained from Scanbur BK (Karlslunde, Sweden) and housed under standard laboratory conditions (12 h light–dark cycle, lights on 07.00 hours). Animals were allowed to acclimatize to laboratory conditions and were handled at least 4 d before use in behavioural experiments. All experiments were performed in accordance with 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.

**Drug treatment and behavioural procedures**

Mice were treated for 7 d i.p. with sterile saline (0.1 ml/10 g body weight) or SAM (4520 mg/kg, 10 nmol/kg, Sigma-Aldrich Co, USA) 20 min prior to cocaine hydrochloride administration (10 mg/kg i.p.; Oriola Oy, Finland). Animals (n=17–22) were randomly assigned to one of the following treatment groups: (1) saline+saline (S+S); (2) SAM+saline (M+S); (3) saline+cocaine (S+C); (4) SAM+cocaine (M+C). To reduce inter-individual variability, mice (four mice in S+C and five mice in M+C groups) whose cocaine-induced ambulation was <2000 cm/90 min on the first day were excluded. Locomotor activity was recorded for 90 min after the second injection on days 1, 3, 5 and 7 and analysed using VideoMot2 software (TSE Systems, Germany). Mice were killed 24 h after the end of the repeated treatment.

On days 14 and 28, all groups were tested for locomotor activity for 90 min after cocaine challenge (7 mg/kg i.p., n=8). On days 8–13 and 15–27 mice did not receive any treatment.

**Cell culture**

Rat pheochromocytoma cells (PC12) purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) were cultured in a humidified 5% CO₂/95% air atmosphere at 37 °C. To differentiate cells, RPMI 1640 medium (Gibco, USA) was supplemented with 10% heat-inactivated horse serum and 5% foetal bovine serum (Gibco) on polyethyleneimine (Sigma-Aldrich, USA) pre-coated plastic dishes (Nunc; Thermo Fisher Scientific, USA) at a density of 10⁵ cells/ml (2 ml cell suspension per dish). The cells from the fourth to seventh passages were treated with 0.5 mM SAM and used for Dnmt activity, gene expression and DNA methylation experiments. Two dishes combined as a sample, four samples per group were used. Experiments were repeated twice.

**Gene expression profiling**

Total RNA was extracted from the mouse NAc as previously described (Anier et al., 2010). Two tissues were combined to a sample, four samples per group. RNA quantity and quality were assessed using the NanoDrop-1000 spectrophotometer and the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Labelled cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit according to the manufacturer’s instructions using 750 ng total RNA as a template (Ambion Inc, USA). The Illumina BeadChip platform (Illumina, USA) and the corresponding whole-genome Mouse Ref-8 v2.0 BeadChip (approximately 25,698 transcripts; over 19,100 genes) were used for the gene expression analysis. The raw data were analysed with Illumina BeadStudio Gene Expression Module.
v3.3.7 (Illumina). Further data analysis was performed with R version 2.13.0 (http://www.r-project.org)/Bioconductor software (www.bioconductor.com) using lumi (Du et al., 2008) and limma packages (Smyth, 2005). The ‘fdr’ method to adjust the \( p \) values for multiple testing was used to control the false discovery rate (Benjamini and Hochberg, 1995). With a statistical discrimination \( p \) value set at <0.05, limma software and B-statistics analyses were used to identify up- and down-regulated genes and filtered for 1.5-fold or greater differences in expression in accordance with standards for microarray analysis (Allison et al., 2006). Gene ontology (GO) analysis was conducted using DAVID Bioinformatics Resources (Huang et al., 2009). Microarray data have been submitted to the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/).

**DNA methylation assay**

Methylated DNA immunoprecipitation (MeDIP) for NAc, cerebellum and PC12 cells was performed using the EpiQuik Methylated DNA and Tissue Methylated DNA Immunoprecipitation Kit’s (Epigentek Group, USA). Methylated DNA (750 ng) was subjected to qPCR using primers from SABioscience (Dnmt3a, Dnmt3b, Cck, Gal, Slc17a7) and methylation between samples was calculated as previously reported (Anier et al., 2010).

**Nuclear extract preparation and Dnmt activity measurement**

For single SAM treatment (SST), 0.5 mM SAM solution (prepared in RPMI medium) was added to growing cells at time-points 0, 2, 6, 12 and 24 h. For the repeated SAM experiment, 0.5 mM SAM was added to the cells once per day for 7 d and nuclear extract was prepared 0, 2, 6, 12 and 24 h after the last treatment. Control measurements were performed with vehicle (RPMI medium) treated cells.

Nuclear extract was isolated from cells using EpiQuik Nuclear Extraction kit (Epigentek Group). Total Dnmt activity was determined using an EpiQuik Dnmt activity assay kit (Epigentek Group). Dnmt activity [optical density (OD)/h.mg] was calculated according to the formula:

\[
\frac{(\text{sample OD} - \text{blank OD})}{\text{protein amount} \times h} \times 1000
\]

**Statistics**

Data were analysed with one-way or repeated two-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test using the GraphPad Prism software (GraphPad Software Inc, USA). Results from PC12 cells were analysed using \( t \) test, one-way or two-way ANOVA. All data are expressed as mean±S.E.M., significance was set at \( p<0.05 \).

**Table 1. Primers used in gene expression analysis**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA qPCR primers for mouse</td>
<td></td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>GCCGAATTGTGTCTTGGTGGATGACA CTCGTTGGAATGCACTGCAGAAGGA</td>
</tr>
<tr>
<td>Dnmt3b</td>
<td>TTTCTGTGACACCTCAGACAGAA TCAGAAGTGTTGACACACCTGCTT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>TGCATATTTTCCTGGTGTGACACC CTGATATTTGCCTGAGTGCTCTGG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>ATGGTGAGAATGGGTCAGAAGA TCTCCATGTCGTCCACTGG</td>
</tr>
<tr>
<td>mRNA qPCR primers for rat</td>
<td></td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>CAGCGTCACACAGAACATATCC GGTCTCTACATTTGGTAACCTTGG</td>
</tr>
<tr>
<td>Dnmt3b</td>
<td>GAATTGTGAGCGCCCAAGTTG TGAAGAACCCCTCCATGG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>TCAGATCAGACACACAGGATACATT GTCCATCAATCGGTGATGAC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>ATGGGCTGAATCACCCATT CCATCAAATCGGTGATGAC</td>
</tr>
</tbody>
</table>

qPCR, Quantitative PCR.
Results

SAM pretreatment potentiated the development and expression of cocaine-induced locomotor sensitization in mice

First, we evaluated the effect of SAM (4520 mg/kg.d) on locomotor sensitization to cocaine (10 mg/kg.d) in adult mice. Repeated cocaine treatment (S+C) and repeated SAM and cocaine treatment (M+C) for 7 d displayed a significantly enhanced locomotion on day 7 compared with day 1 (Fig. 1a), indicating the development of locomotor sensitization. There were also significant differences ($p<0.001$) between S+C and M+C groups on day 7. However, we did not find a difference in locomotor activity between SAM (M+S) and saline control (S+S) groups.

In the cocaine (7 mg/kg i.p) challenge study (on days 14 and 28), S+C and M+C groups demonstrated a robust sensitization exhibiting more locomotor activities than the S+S group (Fig. 1b). Importantly, the M+C group had a higher expression of sensitization compared to the S+C group. Cocaine challenge also increased locomotor activity in the M+S group compared to the S+S group, but these changes were not substantial. Taken together, our results demonstrate that exogenous SAM pretreatment did not affect acute cocaine-induced locomotor response, but instead potentiated the development and expression of cocaine-induced locomotor sensitization in mice.

SAM-modified cocaine-induced gene expression

In this study, we focused on persistent changes in gene expression in the NAc following repeated SAM and/or cocaine treatment. Therefore, the samples for gene expression profiling were collected 24 h after the final treatment. An Illumina microarray was used to study changes in NAc expression of 25,698 unique mRNA transcripts (represents >19100 unique genes). Four different treatment groups (four samples per group) were compared: S+S; M+S; S+C; M+C. Differentially expressed genes were identified by a combination of statistical significance ($p<0.05$) and a fold change (FC) filter (FC>1.5).

In total, 482 separate transcripts were expressed differently between the M+S, S+C and M+C groups, representing 1.88% of the total number of transcripts analysed, whereas 98.12% of the transcripts remained unaltered.

To examine the direction of gene expression changes induced by the treatments, three different treatment groups were compared to the S+S group. In the M+S group, a total of 18 transcripts (36%) were up-regulated (Supplementary Table S1) and 32 transcripts (64%) were down-regulated (Supplementary Table S2), representing 0.19% ($n=50$) of the total number of transcripts analysed. In the S+C group, 93 transcripts (38.6%) were up-regulated (Table 2) and 148 transcripts (61.4%) were down-regulated (Table 3), representing 0.94% ($n=241$) of the total number of transcripts analysed. In the M+C group, 54 transcripts (42.5%) were up-regulated (Supplementary Table S3) and 73 transcripts (57.5%) down-regulated (Supplementary Table S4), representing 0.49% ($n=127$) of the total number of transcripts analysed.
Comparisons between M+S, S+C and M+C groups were also performed. To illustrate the numbers of up- and down-regulated transcripts in all those groups Venn diagrams were presented. Our analysis showed that 28 of 32 (87.5%) SAM-responsive transcripts were down- and nine of 18 (50%) were up-regulated in the S+C group (Supplementary Figs. S1a, S2a, respectively). Comparisons between the M+S vs. M+C groups demonstrated that 12 of 32 (37.5%) SAM-responsive transcripts were down- and eight of 18 (44.4%) were up-regulated in the M+C group (Supplementary Figs. S1b, S2b, respectively). Interestingly, we found that 64 transcripts (43.2%) of the 148 cocaine-responsive genes were down-regulated (Supplementary Fig. S1c) and 50 transcripts (33.8%) of the 93 cocaine-responsive genes were up-regulated in the M+C group (Supplementary Fig. S2c). These data suggest that SAM pretreatment reduced 56.8 and 46.2% (of genes down- and up-regulated by cocaine, respectively) of cocaine-induced transcripts.

GO analysis was performed to group significantly regulated genes into similar biological or molecular functional categories. GO analysis showed an over-representation of down-regulated genes in the M+S, S+C and M+C groups encoding proteins involved in: (i) cell cycle, differentiation and proliferation; (ii) developmental process; (iii) signal transduction (Supplementary Table S5). The up-regulated genes in all those groups were mainly aggregated into the categories of: (i) multi-cellular organismal process; (ii) cell cycle, differentiation and proliferation; (iii) signal transduction; (iv) developmental process and/or ion transport (Supplementary Table S6).

Table 2. The most up-regulated genes (at least two-fold) in the mouse NAc after cocaine (S+C) treatment

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Accession number</th>
<th>Gene title</th>
<th>Fold change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxt</td>
<td>NM_011025.3</td>
<td>Oxytocin</td>
<td>15.89</td>
<td>2.32E-05</td>
</tr>
<tr>
<td>Gal</td>
<td>NM_010253.3</td>
<td>Galanin</td>
<td>9.38</td>
<td>0.000249</td>
</tr>
<tr>
<td>Trh</td>
<td>NM_009426.2</td>
<td>Thyrotropin releasing hormone</td>
<td>5.13</td>
<td>8.13E-06</td>
</tr>
<tr>
<td>Arp</td>
<td>NM_009732.1</td>
<td>Arginine vasopressin</td>
<td>4.38</td>
<td>3.19E-05</td>
</tr>
<tr>
<td>Sytl4</td>
<td>NM_013757.1</td>
<td>Synaptotagmin-like 4</td>
<td>3.48</td>
<td>3.98E-06</td>
</tr>
<tr>
<td>Igsf1</td>
<td>NM_183335.1</td>
<td>Immunoglobulin superfamily, member 1</td>
<td>3.43</td>
<td>0.000127</td>
</tr>
<tr>
<td>Agt</td>
<td>NM_007428.3</td>
<td>Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)</td>
<td>3.23</td>
<td>9.93E-05</td>
</tr>
<tr>
<td>Itih3</td>
<td>NM_008407.1</td>
<td>Inter-a trypsin inhibitor, heavy chain 3</td>
<td>2.99</td>
<td>0.000245</td>
</tr>
<tr>
<td>Dgkk</td>
<td>NM_177914.2</td>
<td>Diacylglycerol kinase k</td>
<td>2.99</td>
<td>2.32E-05</td>
</tr>
<tr>
<td>Prlr</td>
<td>NM_011169.4</td>
<td>Prolactin receptor</td>
<td>2.97</td>
<td>5.41E-05</td>
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<tr>
<td>Calb2</td>
<td>NM_007586.1</td>
<td>Calbindin 2</td>
<td>2.93</td>
<td>6.72E-06</td>
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<tr>
<td>Slc17a6</td>
<td>NM_080853.2</td>
<td>Solute carrier family 17 (sodium-dependent inorganic phosphate co-transporter), member 6</td>
<td>2.81</td>
<td>5.41E-05</td>
</tr>
<tr>
<td>Gpx3</td>
<td>NM_008161.2</td>
<td>Glutathione peroxidase 3</td>
<td>2.46</td>
<td>0.000127</td>
</tr>
<tr>
<td>Resp18</td>
<td>NM_009049.1</td>
<td>Regulated endocrine-specific protein 18</td>
<td>2.41</td>
<td>0.000182</td>
</tr>
<tr>
<td>Calc</td>
<td>NM_007588.2</td>
<td>Calcitonin receptor</td>
<td>2.41</td>
<td>6.37E-05</td>
</tr>
<tr>
<td>Sparc</td>
<td>NM_009242.1</td>
<td>Secreted acidic cysteine rich glycoprotein</td>
<td>2.36</td>
<td>0.000249</td>
</tr>
<tr>
<td>Clq2l2</td>
<td>NM_010273.1</td>
<td>Complement component 1, q subcomponent-like 2</td>
<td>2.33</td>
<td>0.001113</td>
</tr>
<tr>
<td>Gbh2</td>
<td>NM_010262.3</td>
<td>Gastrulation brain homeobox 2</td>
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<td>2.33E-05</td>
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<tr>
<td>Nkr2−1</td>
<td>NM_009385.2</td>
<td>NK2 homeobox 1</td>
<td>2.07</td>
<td>0.000649</td>
</tr>
<tr>
<td>Tnslie</td>
<td>NM_146260.2</td>
<td>Transmembrane inner ear</td>
<td>2.00</td>
<td>0.000125</td>
</tr>
</tbody>
</table>

NAc, Nucleus accumbens; S+C, saline+cocaine group.

qPCR analysis was performed to validate the subset of gene expression changes observed in the microarray analyses. Genes chosen for qPCR validation were selected based on their potential roles in cocaine-induced neuronal plasticity and on in silico analysis that revealed CpG islands located within their promoter regions. From the microarray data (Tables 2 and 3), we selected three genes for validation: (a) Slc17a7 and Cck as down-regulated genes; (b) Gal as up-regulated gene after repeated cocaine treatment.
Using the same RNA samples as in the gene expression profiling, transcription analysis of *Slc17a7* and *Cck* revealed a significant decrease in mRNA levels following repeated M+S, S+C or M+C treatments in the NAc. There were significant differences (*p*<0.001) in both genes between the S+C and the M+C groups. Using mouse cerebellum as a reference region, we found that the marker gene mRNA was altered in the cerebellum as well, but these changes were not as extensive as in the NAc. *Slc17a7* mRNA level was significantly (*p*<0.01) decreased in the cerebellum following M+S treatment compared to the S+S group, but there was no significant changes in *Cck* mRNA levels. Marker gene comparisons in both brain tissues demonstrated that the *Slc17a7* mRNA level was significantly different (*p*<0.001) between the S+C group in the NAc vs. S+C group in the cerebellum and the *Cck* mRNA level between the S+C and M+C groups in the NAc and cerebellum.

### Table 3. The most down-regulated genes (at least two-fold) in the mouse NAc after cocaine (S+C) treatment

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Accession number</th>
<th>Gene title</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc17a7</td>
<td>NM_182993.1</td>
<td>Solute carrier family 17 (sodium-dependent inorganic phosphate co-transporter), member 7</td>
<td>17.15</td>
<td>2.09E-08</td>
</tr>
<tr>
<td>Ctgf</td>
<td>NM_010217.1</td>
<td>Connective tissue growth factor</td>
<td>7.16</td>
<td>5.80E-09</td>
</tr>
<tr>
<td>Cck</td>
<td>NM_031161.2</td>
<td>Cholecystokinin</td>
<td>4.72</td>
<td>1.19E-08</td>
</tr>
<tr>
<td>Slc12a4</td>
<td>NM_172892.1</td>
<td>Solute carrier family 13 (sodium/sulfate symporters), member 4</td>
<td>3.58</td>
<td>6.72E-06</td>
</tr>
<tr>
<td>Tbr1</td>
<td>NM_009322.3</td>
<td>T-box brain gene 1</td>
<td>3.12</td>
<td>9.49E-05</td>
</tr>
<tr>
<td>Ogt</td>
<td>NM_008760.2</td>
<td>Osteoglycin</td>
<td>3.03</td>
<td>3.09E-05</td>
</tr>
<tr>
<td>Slc6a13</td>
<td>NM_144512.2</td>
<td>Solute carrier family 6 (neurotransmitter transporter, GABA), member 13</td>
<td>2.81</td>
<td>2.05E-05</td>
</tr>
</tbody>
</table>

**NAc, Nucleus accumbens; S+C, saline+cocaine group.**
increased ($p<0.001$) Gal expression compared to the S+S group. Furthermore, Gal mRNA was significantly different ($p<0.001$) between the S+C and M+C groups. In the cerebellum, we found that M+S and S+C treatments significantly ($p<0.001$) up-regulated Gal expression compared to the saline control. There were also statistical differences ($p<0.001$) between the S+C and M+C groups. Gal mRNA level comparison in both brain regions demonstrated that there were significant differences ($p<0.001$) between the S+C and M+C groups in the NAc vs. S+C and M+C groups in the cerebellum (Fig. 2c).

Using MeDIP assay, Slc17a7, Cck and Gal promoter-associated CpG island methylation analysis in the NAc was performed. For the Slc17a7 promoter, MeDIP analysis revealed that both M+S and S+C treatments resulted in promoter hypermethylation compared to the S+S group (Fig. 3a). We also found that repeated SAM pretreatment significantly ($p<0.001$) decreased Slc17a7 promoter hypermethylation compared to the S+C group. With regard to Cck promoter methylation, M+S and S+C treatments resulted in promoter hypermethylation (Fig. 3b). However, there was an additive increase in Cck promoter methylation levels in the M+C group when compared with S+C treatment ($p<0.001$). Gal MeDIP analysis demonstrated that M+S and S+C treatments induced promoter-associated CpG island hypomethylation (Fig. 3c). Remarkably, we found that repeated M+C treatment essentially reversed Gal promoter hypomethylation compared to the S+C treatment ($p<0.001$).

MeDIP data in the mouse cerebellum showed that repeated M+S and S+C treatments significantly ($p<0.001$) enhanced (Slc17a7, Cck) promoter-associated CpG island methylation (Supplementary Fig. S3a, b). Moreover, Slc17a7 and Cck promoter methylation data comparison in both brain tissues demonstrated that there were significant differences ($p<0.001$) between the S+C and M+C groups in the NAc vs. S+C and M+C groups in the cerebellum. Gal MeDIP data comparison in both brain regions showed that there were significant changes ($p<0.001$) only between the S+C groups in the NAc vs. S+C groups in the cerebellum (Supplementary Fig. S3c).

**SAM decreased Dnmt3a and -3b expression and methyltransferase activity in PC12 cells**

To evaluate the underlying mechanism of the SAM modifying effect at the gene and genome level, we first studied the effects of a single and repeated dose (7 d) of 0.5 mM SAM on methyltransferase (Dnmt) activity in PC12 cells. Time-course analyses showed

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**Fig. 2.** The effect of repeated S-adenosylmethionine and cocaine administration on marker gene mRNA levels in the mouse nucleus accumbens (NAc; n=8 in each group) and cerebellum (n=6). (a) Slc17a7 mRNA, two-way analysis of variance; treatment $F_{3,44}=27.80$, $p<0.0001$; tissue effect $F_{1,44}=8.44$, $p=0.0057$; interaction $F_{3,44}=7.51$, $p=0.0004$; Bonferroni’s post-test, $*** p<0.001$ S+C in the NAc vs. S+C in the cerebellum; (b) Cck mRNA, treatment $F_{3,44}=27.67$, $p<0.0001$; tissue effect $F_{1,44}=36.35$, $p<0.0001$; interaction $F_{3,44}=7.66$, $p=0.0003$; Bonferroni’s post-test, $*** p<0.001$ S+C and M+C in the NAc vs. S+C and M+C in the cerebellum; (c) Gal mRNA, treatment $F_{3,44}=2045.35$, $p<0.0001$; tissue effect $F_{1,44}=1269.06$, $p<0.0001$; interaction $F_{3,44}=885.82$, $p<0.0001$; Bonferroni’s post-test, $*** p<0.001$ S+C and M+C in the NAc vs. S+C and M+C in the cerebellum. For details of treatment groups, see main text.
Methylated DNA.

For details of treatment groups, see main text. MeDNA,

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levels in the mouse nucleus accumbens. (The effect of repeated S-adenosylmethionine and

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methylation, one-way analysis of variance, \( F_{3,28}=1583, \ p<0.001; \) Bonferroni’s post-test; \( a \) \( p<0.05, \) S+S vs. M+S; \( * \) \( p<0.05, \) S+S vs. M+C; \( \rho \) \( p<0.05, \) M+S vs. S+C; \( \gamma \) \( p<0.05, \) S+C vs. M+C; \( n=8 \) in each group; (b) Cck promoter methylation, \( F_{3,28}=2587, \ p<0.0001; \) Bonferroni’s post-test; \( b \) \( p<0.05, \) S+S vs. M+S; \( * \) \( p<0.05, \) S+S vs. S+C; \( \gamma \) \( p<0.05, \) M+S vs. M+C; \( n=8 \) in each group; (c) Gal promoter methylation, \( F_{3,28}=39.58, \ p<0.0001; \) Bonferroni’s post-test, \( c \) \( p<0.05, \) S+C vs. M+C; \( * \) \( p<0.05, \) S+C vs. S+C; \( \gamma \) \( p<0.05, \) M+S vs. S+C; \( n=8 \) in each group. For details of treatment groups, see main text. MeDNA, Methylated DNA.

that SAM altered methyltransferase activity in a biphasic manner: SST enhanced and repeated SAM treatment (RST) decreased Dnmt activity compared with vehicle controls (Fig. 4a, b).

Next, to link methyltransferase activity with Dmnt3a and -3b mRNA levels, we measured the SAM-altered Dnmt activity on the first, third, fifth and seventh treatment days. On the first day, Dnmt activity was similar to vehicle control, on the third day its activity was reduced approximately by 50%, on the fifth day by 65% and on the seventh day by 82% compared to controls (Fig. 4c). We also discovered that the decrease in Dnmt activity in PC12 cells correlated with down-regulation of the Dmnt3a mRNA level (Fig. 5a), but not with the Dmnt3b mRNA level (Fig. 5b) after RST.

In silico analysis of Dmnt3a revealed CpG islands located within the promoter region; therefore, we assessed Dmnt3a promoter methylation following RST. The MeDIP results showed that RST resulted in a significant increase in methylation of the Dmnt3a promoter (Fig. 5c), thereby decreasing Dmnt3a gene transcription in PC12 cells.

**SAM hypermethylated promoter and down-regulated the Dmnt3a mRNA level in mouse NAc**

To bridge our PC12 cells and mice data, we assessed Dmnt3a and -3b promoter methylation patterns and transcriptional activity in the NAc. Using MeDIP, we discovered that M+S and M+C treatments increased Dmnt3a promoter methylation (Fig. 6a), but there was no significant change in Dmnt3b promoter (Fig. 6b).

Next, we evaluated whether aberrant promoter methylation was associated with altered Dmnt3a expression. We found that Dmnt3a mRNA was significantly decreased following M+S and M+C treatments and increased following S+C treatment compared with the saline-treated group (Fig. 6c). There were significant \((p<0.001)\) differences in both Dmnt3a and -3b mRNA levels after the S+C and M+C treatments in the NAc (Fig. 6c, d). These data indicate that RST might decrease methyltransferase activity in vitro and RST is associated with hypermethylation of Dmnt3a promoter region both in vitro and in vivo.

**Discussion**

The present work aimed to investigate whether the environmental factor SAM, via affecting epigenome, could change cocaine-induced gene expression and locomotor sensitization in mice. The concentration of
SAM (10 mM/kg) used in this study was at pharmacological level. We selected this dose of SAM based on a study by Tremolizzo et al. (2002) and on our pilot data showing that this dose produced a significant increase of protein phosphatase-1 catalytic subunit promoter region methylation in the mouse NAc. Our whole-genome gene expression study demonstrated that repeated treatments with SAM significantly altered 50 transcripts. Of these transcripts, 36% were up-regulated and 64% were down-regulated, suggesting that exogenous SAM treatment primarily silences gene expression, as expected from the typical silencing effect of DNA hypermethylation. Compared with SAM, cocaine treatment has a broader impact upon gene expression. Repeated cocaine treatment changed 241 transcripts and predominantly down-regulated these transcripts (Table 3). Thirty-seven transcripts of the 50 SAM-responsive genes were also altered in the S+C group. The other transcripts were uniquely responsive to either SAM or cocaine treatment, indicating that the effects of SAM and cocaine on the NAc transcriptome are gene-specific.

Interestingly, the number of significantly affected genes in the M+C group was lower than that of the S+C group. These data demonstrate that 46 and 57% (respectively up- and down-regulated) SAM and cocaine treatment-group transcripts were no longer significantly different compared to the respective transcripts from the S+C group, suggesting that SAM pretreatment modified the cocaine-induced gene expression pattern in NAc. GO analysis revealed that SAM pretreatment decreased expression of several cocaine-induced genes in different functional groups, demonstrating that SAM’s blunting effect is non-specific.

Other microarray studies have demonstrated that several genes are affected by cocaine (S+C) treatment (Freeman et al., 2010; Rodríguez-Borrero et al., 2010; Maze and Nestler, 2011). However, there have been only a few reports demonstrating an effect of SAM or Met (a precursor of SAM) at the genome level. In SK-N-BE neuroblastoma cells, SAM modulates seven genes (of a total of 588 genes analysed) of which three were up-regulated and four down-regulated, showing low levels of modulation (Cavallaro et al., 2006). Weaver et al. (2006) demonstrated that repeated Met intraventricular treatment

![Fig. 4. S-adenosylmethionine (SAM) altered Dnmt activity in PC12 cells in a biphasic manner: (a) a single SAM treatment (SST) enhanced two-way analysis of variance (ANOVA), treatment $F_{1,30}=15.64$, $p=0.0004$; time $F_{4,30}=6.77$, $p=0.0005$; interaction $F_{4,30}=4.91$, $p=0.0037$; Bonferroni’s post-test, $^{5}$ $p<0.001$ SST SAM 2 h vs. SST control 2 h; SST SAM 0, 6, 12, 24 h vs. SST control was non-significant, $n=4$ in each group] whereas (b) repeated SAM treatment (RST) decreased Dnmt activity compared to the control (two-way ANOVA, treatment $F_{1,30}=206.74$, $p<0.0001$; time $F_{4,30}=0.18$, $p=0.9461$; interaction $F_{4,30}=0.13$, $p=0.9717$, $n=4$ in each group). (c) The effect of RST SAM treatment on Dnmt activity in cells, two-way ANOVA, treatment $F_{1,12}=141.58$, $p<0.0001$; time $F_{3,12}=5.65$, $p=0.0447$; interaction $F_{3,12}=6.22$, $p=0.0086$; Bonferroni’s post-test, $^{16}$ $p<0.001$ RST SAM vs. RST control on the indicated days; RST SAM on the first day vs. RST control revealed non-significant changes; $n=4$ (RST SAM and RST control). OD, Optical density.
altered 337 transcripts, representing 1.08% of the population of genes on the DNA microarray. Of these altered transcripts, 217 (64%) were down-regulated and 120 (35.6%) were up-regulated in rat hippocampus. It is difficult to compare our results with these data but, nevertheless, the ratio of up- and down-regulated genes is similar between all of the studies. Both SAM and Met only affect the expression of a limited number of genes and did not affect the vast majority of the genome, suggesting that SAM or Met treatments, despite their global nature, do not result in a general silencing of gene expression. The basis for the specificity of SAM or Met gene expression effects remains unknown. Thus, exogenous SAM treatment induces minor effects on whole-genome gene expression; however, SAM pretreatment significantly modified cocaine-induced gene expression by blunting non-specifically the cocaine response.

To investigate the effect of SAM and cocaine at the gene level, we selected (from the gene expression profiling data) Cck, Slc17a7 and Gal genes for analysis based on their possible participation in cocaine-induced neuroadaptions in the NAc (Hökfelt et al., 1980; Josselyn et al., 1997; Fremeau et al., 2001; Narasimhaiah et al., 2009). We found that RST caused both hyper- and hypomethylation in the promoter regions of the selected genes and these changes are associated with down- and up-regulated mRNA expression, respectively (Figs. 2a–c, 3a–c; Supplementary Fig. S3a–c). These results are comparable in part with the studies that demonstrated that exogenous SAM treatment elicits gene silencing via promoter hypermethylation (Watson et al., 1999; Fusco et al., 2001; Pulukuri et al., 2007). Similarly, repeated treatment with Met is also associated with hypermethylation of reelin and GAD67 promoter regions in mouse frontal cortex and striatum (Tremolizzo et al., 2002; Dong et al., 2008). We also found that SAM pretreatment inhibited cocaine-induced hyper- and hypomethylation (Slc17a7 and Gal, respectively) in the NAc. Interestingly, SAM additively enhanced cocaine-induced hypermethylation at the Cck promoter, which was associated with transcriptional down-regulation of the Cck gene in the NAc (Figs. 2b, 3b). Thus, it seems that SAM pretreatment may both increase and decrease cocaine-induced DNA methylation on gene level.

To evaluate whether the effects of SAM are NAc-specific or general effects that are seen in the brain regions that are unrelated to drug addiction, we also assessed the marker gene expression and promoter region methylation levels in the mouse cerebellum. We found that RST significantly changed Slc17a7 and Cck promoter methylation levels in both brain regions (Supplementary Fig. S3a, b). As expected, the cocaine effects on the gene expression were more prominent in the NAc than in the cerebellum.
Despite the fact that effects of SAM in the mouse brain are rather non-specific and not directly related to drug addiction, our data indicate that SAM treatment modifies cocaine (another epigenetic factor) specific effects in the NAc. To further study the mechanism underlying SAM’s modifying effects on gene expression, we analysed the consequences of acute and RSTs on Dnmt’s expression and methyltransferase activity in PC12 cells. We found that exogenous SAM treatment induced a biphasic effect on methyltransferase activity (Fig. 4a, b), since SST enhanced whereas repeated daily treatment significantly reduced methyltransferase activity. Both in vitro and in vivo data show that RST increases Dnmt3a promoter-region hypermethylation, which is associated with down-regulation of the Dnmt3a mRNA (Figs. 5a, c, 6a, c). SAM and cocaine treatment also increased Dnmt3a promoter methylation compared with the S+C group. As we found a significantly down-regulated Dnmt3b mRNA level in the M+C group (Fig. 6d), without methylation of Dnmt3b promoter, we speculate that SAM-induced histone methylation might be involved in Dnmt3b expression.

SAM is synthesized in the cytosol of every cell (Lu, 2000). Methionine adenosyltransferase is the enzyme responsible for the synthesis of SAM, using the substrates Met and adenosine-5’-triphosphate (Mato
et al., 1997), whereas S-adenosylhomocysteine (SAH) is the product of the transmethylation reactions and inhibits Dnmt activity (Chiang, 1998). Based on accumulating data, we propose the following model for the regulation of DNA methylation/demethylation. A single dose of SAM stimulates methyltransferase activity, resulting in increased DNA methylation. It is possible that SAM increases DNA methylation by also decreasing demethylation via inhibition of methyl-CpG-binding domain protein 2 (MBD2; Detich et al., 2003). Repeated administration of exogenous SAM decreases methyltransferase activity via decreased Dnmt3a and -3b expression and via increased levels of intracellular SAH. Therefore, we propose that the blunting effect on cocaine-induced gene expression in the NAc following SAM treatment may result from an altered balance between methylation/demethylation activities. We also speculate that decrease of SAM-induced methyltransferase activity is reversible after discontinuing exogenous SAM treatment. While we found that SAM and cocaine treatment may also cause promoter-associated CpG-island hypermethylation (e.g. Cck), we speculate that other factor(s) may participate in fine-tuning the DNA methylation/demethylation balance.

In the present study, we found that RST alone did not affect locomotor activity (Fig. 1a); a related finding has been described following repeated Met treatment (Tremolizzo et al., 2002). Several recent studies indicate that injections of Dnmt inhibitors into different brain regions may affect inversely the development of cocaine-induced locomotor sensitization and conditioned place preference (CPP) in mice. For example, continuous intra-NAc infusion over 7 d of RG108 increased cocaine-induced CPP and enhanced the induction of locomotor sensitization to chronic cocaine (LaPlant et al., 2010). Dnmt inhibitor 5-aza-2-deoxycytidine (5-aza) injections into the hippocampus CA1 area restrained acquisition of cocaine-induced CPP; however, 5-aza had no effect on acquisition after injection into the prelimbic cortex (Han et al., 2010). We previously demonstrated that repeated zebularine (Zeb) intracerebroventricular injections decreased cocaine-induced locomotor sensitization, suggesting that Zeb (due to diffusion to brain tissue) affected Dnmt activity in the hippocampus more than in the NAc (Anier et al., 2010). In this study, we found that SAM pretreatment significantly potentiated cocaine-induced ambulations during the development and expression of locomotor sensitization in mice. Therefore, our behavioural data, coupled with the decreased Dnmt3a and -3b mRNA levels in the NAc, support the findings that decreased methyltransferase activity in the NAc positively regulates cocaine-induced locomotor sensitization (LaPlant et al., 2010). Increased locomotor activity in M+C group on the 5–7 treatment days is also in line with decreased Dnmt3a mRNA level and methyltransferase activity in PC12 cells (Figs. 4c, 5a). It is not known how exogenous SAM affects Dnmt3a and -3b expression in other brain regions, but we speculate that reduced Dnmt3a and -3b expression in the NAc might have had a higher impact on cocaine-induced locomotor activity compared with other brain regions (e.g. CA1 area).

The cocaine challenge study suggests that increased locomotor activity in the M+C group (compared to the S+C group) is persistent, indicating that the effects of SAM on cocaine are long-lasting even after the withdrawal period of cocaine. In general, our behavioural data demonstrated that altered DNA methylation in the NAc may play a critical role in both the development and expression of locomotor sensitization.

Recently, it has been reported that chronic Met (0.78 g/kg, twice per day s.c.) diminishes the rewarding effects of cocaine in the CPP procedure (LaPlant et al., 2010). There are a number of methodological differences between LaPlant’s study and this study that could account for the disparate findings, including the drug (Met vs. SAM), dose of Met and SAM and different pretreatment regimens. We think that an especially important factor is the pretreatment regimen as SAM or Met might affect different genes, depending on the interval between pretreatment and cocaine administration.

In conclusion, our results demonstrate that repeated SAM pretreatment modifies cocaine-induced gene expression at genome and gene level, increases cocaine-induced locomotor sensitization and these changes are associated with reduced Dnmt3a and -3b expression in the NAc. Therefore, we conclude that environmental factors, which affect the NAc-cell epigenome, may alter the development and expression of psychostimulant-induced addiction. Thus, understanding the epigenetic modifications that affected the response of psychostimulants is fundamental to unlocking the neurobiology of a subject’s sensitivity to psychostimulants.

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Statement of Interest
None.

Supplementary material
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