Role of Nicotinamide *N*-Methyltransferase in Dorsal Striatum in Cocaine Place Preference

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Nicotinamide *N*-methyltransferase (NNMT) transfers the methyl from *S*-adenosyl-L-methionine (SAM) to nicotinamide (NA) to produce *S*-adenosyl-L-homocysteine (SAH) and 1-methylnicotinamide (MeN). NNMT has been implicated in a variety of diseases; however, the role of NNMT in drug addiction is largely unknown. Here, we found that the expression of *Nnmt* was significantly upregulated in the dorsal striatum (DS) of cocaine-conditioned mice. Cocaine significantly decreased SAM/SAH ratio levels in the DS, which was accompanied with the decreased activities of Rac1 and RhoA. Lentivirus-mediated knockdown of *Nnmt* in the dorsomedial striatum (DMS) attenuated cocaine conditioned place preference (CPP) reward, but increased striatal SAM/SAH ratio levels as well as Rac1 and RhoA activities. In addition, pharmacological inhibition of NNMT through intra-DMS infusion of MeN attenuated cocaine CPP and the activities of Rac1 and RhoA, but increased SAM/SAH ratio. These results suggest that NNMT-dependent transmethylation is involved in the activation of Rac1 and RhoA, which utilize SAM as a methyl donor cofactor. Co-immunoprecipitation assay using a RhoGDIα antibody indirectly captured Rac1 or RhoA that were bound to RhoGDIα. The results showed that cocaine increased the association of RhoGDIα with Rac1 or RhoA, whereas such effect was inhibited by *Nnmt* knockdown. Collectively, our findings show that NNMT regulates cocaine CPP through SAM-mediated modification of Rac1 and RhoA.
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INTRODUCTION

Nicotinamide *N*-methyltransferase (NNMT), a cytoplasmic methyltransferase, transfers the methyl from *S*-adenosyl-L-methionine (SAM) to nicotinamide (NA) to produce *S*-adenosyl-L-homocysteine (SAH) and 1-methylnicotinamide (MeN) (Aksoy *et al*, 1994; Riederer *et al*, 2009). SAM is the sole methyl donor in many important methylation reactions in nervous system, which is involved in the modification of both proteins and DNA. NNMT affects the methylation potential in multiple diseases by consuming methyl units from SAM to create a stable metabolic product MeN (Kraus *et al*, 2014; Sperber *et al*, 2015; Ulanovskaya *et al*, 2013). Studies have revealed the link between NNMT dysregulation and brain disorders. For instance, single nucleotide mutation of *Nnmt* was reported in schizophrenia patients (Wang *et al*, 2014), and NNMT is significantly upregulated in Parkinson's disease (Parsons *et al*, 2002).

Nnmt expression in SH-SY5Y cells increases neurite branching, synaptophysin expression, and dopamine accumulation, which is mediated by MeN (Thomas *et al*, 2013). These results suggest that dysregulation of NNMT and its metabolic product are involved in neuropsychiatric disorders. However, the role of NNMT in drug-induced neuroplasticity is largely unknown.

Cytoskeleton regulation of dendritic signaling and morphology has been thought as an important mediator of plasticity associated with neuropsychiatric disorders (Penzes *et al*, 2011). It has been known that cocaine increases the densities of dendritic spine and promotes dendritic arborization in neurons (Christian *et al*, 2016; Nyberg, 2014; Robinson and Kolb, 1999). Rac1 and RhoA, two major members of Rho GTPase family, regulate neurite branching through controlling the movement of actin, which is critical for neural plasticity (Bradke and Dotti, 2000). Extracellular inhibitory signal molecules activate Rac1 and RhoA to bind to GTP, activate ROCK, and eventually inhibit neurite growth by influencing actin myosin system. On the contrary, after GTP is hydrolyzed to GDP, Rac1 and RhoA become inactivated (Jaffe and Hall, 2005; Tiedje *et al*, 2008). Rho GDP dissociation inhibitor α (RhoGDIα) blocks the dissociation of GDP from Rac1 and RhoA, making them inactive, which enhances the neurite growth, differentiation and neuroplasticity (Hancock and Hall, 1993; Liu *et al*, 2015).

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To be mature and active, Rac1 and RhoA are subjected to post-translational methylation modification at the C-terminal cysteines, utilizing SAM as a methyl donor cofactor (Roberts et al, 2008). This reaction is catalyzed by isoamyl cysteine carboxyl methyl transferase (LCMT1) (Cushman and Casey, 2009).

Studies have shown that inhibition of LCMT significantly decreases the activation of both RhoA and Rac1, which is due to the increase in RhoGDIα binding by both proteins in the absence of their methylation (Cushman and Casey, 2011). Rac1 and RhoA are activated when they are methylated and bound to GTP, and function in inhibiting neurite growth (Bradke and Dotti, 2000; Liu et al, 2015). RhoGDIα blocks GDP's dissociation from methylated or unmethylated Rac1 and RhoA to keep them inactive, therefore promoting neurite growth (Bradke and Dotti, 2000). Cocaine decreases Rac1 and RhoA activities and increases dendritic spines in the nucleus accumbens (NAc) (Dietz et al, 2012; Kim et al, 2009). Dopamine inhibits RhoA activity and enhances neurite growth in cultured neurons (Li et al, 2015a). In spite of these advances, the pathway involved in the activation of RhoA and Rac1 is poorly understood.

Dorsal striatum (DS) includes dorsomedial striatum (DMS) and dorsolateral striatum (DLS). DMS primarily regulates goal-directed behavior, whereas the DLS is more involved in habit (Balleine and O'Doherty, 2010). There is a hypothesis that drug addiction is viewed as a transition from voluntary and goal-directed drug use to compulsive habitual drug-seeking. The goal-directed system depends on the interactions between medial prefrontal cortex (mPFC) and DMS (Everitt and Robbins, 2016). In the DMS, dopamine receptor blockade impairs the initial acquisition of goal-directed cocaine seeking (Murray et al, 2012). Moreover, cocaine administration activates extracellular signal-regulated kinase signal pathway in the DS that is required for the acquisition of cocaine-induced CPP (Valjent et al, 2006).

CPP is a classical (Pavlovian) assay of drug reward in which acquisition of the preference is measured across multiple conditioning cycles (Mucha et al, 1982). It includes differentially pairing two distinct sets of contextual cues (eg, wall color, pattern) with the stimulus of interest (eg, drug). Conditioning involves animals receiving drug stimulus in one context. Another context without the drug stimulus is control. During a subsequent drug-free CPP test, animals receive unrestricted exposure to both contexts. An increase in time spent is taken as evidence that the drug context was rewarding (Bardo and Bevins, 2000). There are two versions of the CPP termed biased and unbiased. In the biased CPP procedure, drug injections are paired with pretest non-preferred context and saline injections with the pretest preferred context. In the unbiased CPP version the subjects do not show initial side preference and the contexts paired with the drug and saline injections are counterbalanced (Cunningham et al, 2006; Wang, 2003). In the present study, we used a biased CPP procedure to explore the role of NNMT in cocaine reward.

MATERIALS AND METHODS

Drugs

Cocaine hydrochloride was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). MeN was purchased from Sigma-Aldrich (St Louis, MO, USA). They were dissolved in 0.9% sterile saline to obtain appropriate concentration for experiment.

Animals

Adult male C57BL/6J mice, weighing 20 to 22 g, were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All of the animal protocols were reviewed and approved by the Experimental Animal Ethics Committee of Sichuan University (Chengdu, China). Complete description of methods can be found in the Supplementary Methods.

CPP Procedure

As described previously (Li et al, 2015b), place conditioning studies were conducted utilizing a shuttle box. Complete description of methods can be found in the Supplementary Methods.

Protein Extraction and Western Blot Analysis

As described previously (Liu et al, 2015; Shang et al, 2016), the protein was extracted by RIPA lysis buffer. Complete description of methods can be found in the Supplementary Methods. The primary antibodies used were as follows: mouse anti-NNMT (29 kDa, 1 : 500, Abcam), mouse anti-Rac1 (21 kDa, 1 : 1000, Abcam), rabbit anti-RhoA (22 kDa, 1 : 1000, Abcam), rabbit anti-LCMT1 (38 kDa, 1 : 1000, Abcam), rabbit anti-RhoGDIα (23 kDa, 1 : 1000, Abcam), rabbit anti-B-tubulin (50 kDa, 1 : 2000, ABclonal).

RNA Extraction and PCR Analysis

As described previously (Shang et al, 2013), the RNA was extracted by Trizol reagent (Invitrogen). Complete description of RNA extraction and PCR analysis can be found in the Supplementary Methods.

Targeted Hydrophilic Interaction Chromatography (HILIC) with Mass Spectrometry

Metabolites NA, MeN, SAM, and SAH were measured by using tandem mass spectrometry. Complete description of methods can be found in the Supplementary Methods.

Assay for Rac1 and RhoA Activation

The assay for Rac1 and RhoA activation was performed by using Rac1 and RhoA Activation Assay Kit (NewEast Bioscience), respectively. Complete method is described in the Supplementary Methods.

Lentivirus Injection

Four shRNA sequences for silencing *Nnmt* (ID: 18113) were designed (Supplementary Table S2) by GeneCopoeia (China). Screening tests revealed that No.4 sequence could significantly downregulate the expression of *Nnmt* mRNA. The target sequence of No.4 was 5′-GAGCAGTTTGAGG TGATTTCT-3′. The lentivirus packaging and mouse treatment are described in the Supplementary Methods.

Co-Immunoprecipitation (Co-IP) Analysis

We used the commercial co-IP assay kit (Pierce Crosslink Magnetic co-IP Kit, Thermo Scientific) and a RhoGDIα antibody to detect the association of RhoGDIα with Rac1 or RhoA. Complete description of methods can be found in Supplementary Methods.

Statistical Analysis

Statistical analysis was performed with IBM SPSS 20.0.0. Statistical analysis was performed by one-way ANOVA for data involving more than two groups. Two groups were analyzed using a Student's *t*-test with a two-tailed *p*-value. Lentivirus or MeN effects on CPP scores were assessed using two-way ANOVAs. All summary statistics of the results are presented as Mean $\pm$ SEM. $p < 0.05$ was considered statistically significant.

RESULTS

Cocaine Upregulates *Nnmt* Expression in the DS

To examine the expression of *Nnmt* in the brain in response to cocaine, mice were conditioned to cocaine in a 6-day CPP protocol (Figure 1a). Mice that received 20 mg/kg cocaine (i.p.) during conditioning spent significantly more time in the non-preferred chamber during the CPP test, and thereby displayed significantly stronger CPP scores than the mice treated with saline during conditioning (Figure 1b, $t_{(28)} = 10.81$, *$p < 0.0001$). Western blotting showed that NNMT level in the DS was significantly increased in the cocaine-conditioned mice when compared with saline-conditioned mice (Figure 1c, $t_{(14)} = 3.47$, *$p = 0.013$). However, there were no significant changes in the hippocampus, medial prefrontal cortex (mPFC) and NAc, suggesting a brain region-specific NNMT upregulation in response to cocaine CPP regimen. The brain regions collected for NNMT test were indicated in Supplementary Figure S1A–D. RT-PCR assay showed that *Nnmt* mRNA level was significantly increased after cocaine CPP procedure in the DS (Supplementary Figure S2A, t(12) = 3.07, *$p = 0.012$). To confirm such upregulation was specific to cocaine CPP regimen, we compared the NNMT level in the DS of mice 30 min and 24 h after single cocaine injection (20 mg/kg, i.p.) and after 7-day repeated cocaine treatment (20 mg/kg per day, i.p.), respectively. NNMT protein level has no significant change 30 min or 24 h after single cocaine challenge. However, 7-day repeated cocaine treatment upregulated NNMT protein level (Figure 1d, $t_{(14)} = 3.93$, *$p = 0.0077$). These results showed that chronic cocaine induces NNMT upregulation in the DS.

Cocaine Decreases the Levels of SAM/SAH Ratio in the DS

To analyze the levels of NNMT-related metabolites in the DS, targeted HILIC-QTOF mass spectrometry was used to separate and identify the metabolites, including SAM and NA, the substrates of NNMT, as well as SAH and MeN, the products of NNMT. Extracted ion chromatogram (EIC) of these 4 metabolites were shown in the Supplementary Figure S3. Retention times and major adducts for each compound are as follows: MeN (*m/z* 137.0715) 2.44 min, NA (*m/z* 123.0558) 0.88 min M+H, SAH (*m/z* 385.1294) 3.05 min M+H, SAM (*m/z* 399.1451) 3.38 min. The result indicated that SAM/SAH ratio levels in the DS was significantly reduced in cocaine-conditioned mice (Figure 1e, $t_{(18)} = 3.60$, *$p = 0.0032$), whereas MeN/NA levels were increased (Figure 1f, $t_{(18)} = 3.48$, *$p = 0.0040$). These results were consistent with the aforementioned finding that *Nnmt* expression was upregulated by cocaine, suggesting that cocaine can upregulate the level and activity of NNMT, leading to the alteration of SAM/SAH ratio levels.

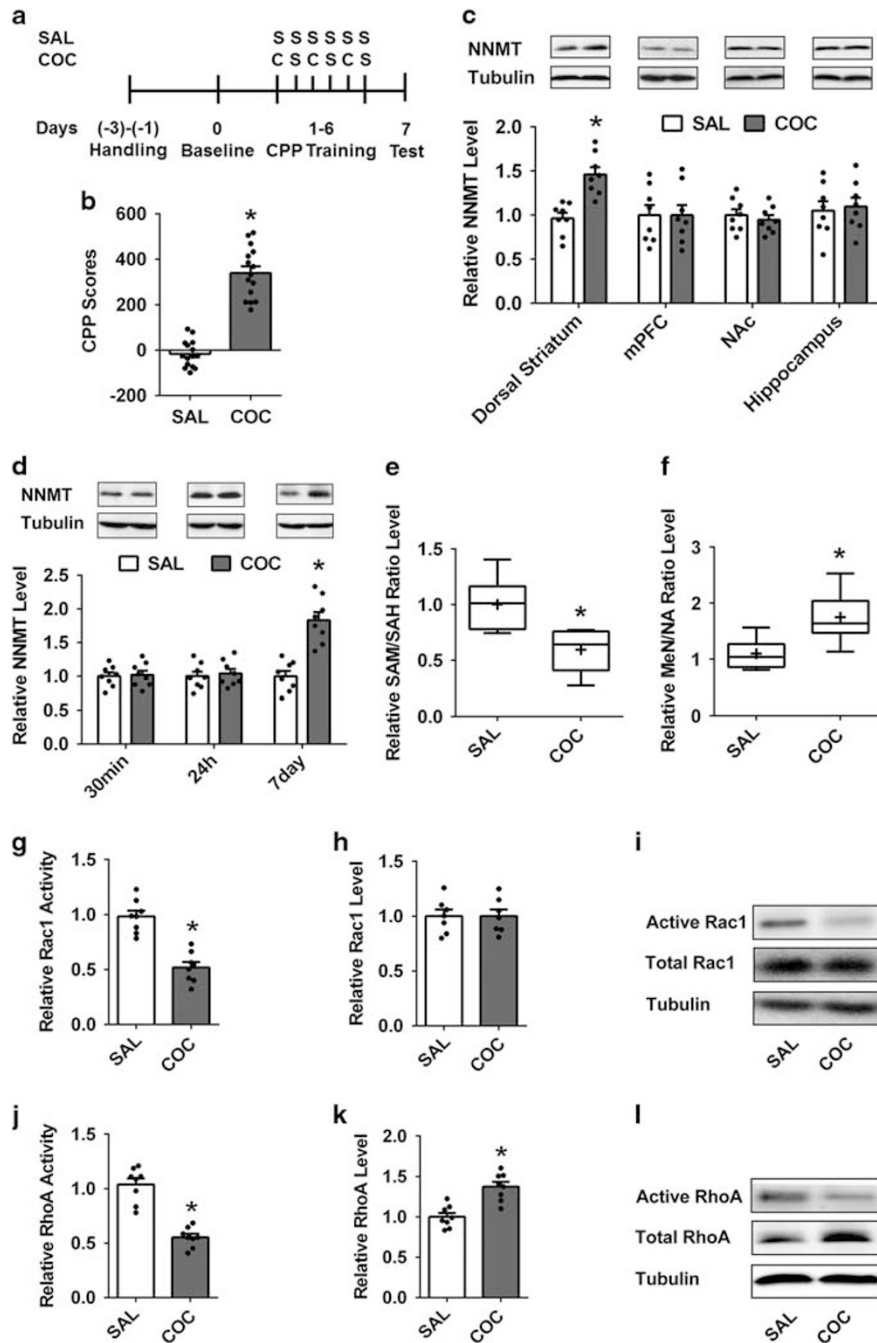### Cocaine Decreases the Activities of Rac1 and RhoA in the DS

To be active, Rac1 and RhoA are subjected to post-translational methylation modification, utilizing SAM as a cofactor(Roberts *et al*, 2008). We first investigated whether the activities of Rac1 and RhoA could be modified by cocaine. Configuration-specific monoclonal antibodies that specifically recognize Rac1-GTP or RhoA-GTP, but not Rac1-GDP or RhoA-GDP, were used for immunoprecipitation assay. We found that the activities of Rac1 ($t_{(14)} = 11.55$, *$p = 0.0003$) and RhoA ($t_{(14)} = 4.41$, *$p = 0.012$) were significantly reduced in the DS of cocaine-conditioned mice (Figure 1g, i, j and l). There was no significant change in the protein level of Rac1 (Figure 1h and i); however, RhoA was upregulated (Figure 1k and l; $t_{(14)} = 4.88$, *$p = 0.0028$). We continued to use RT-PCR to detect the mRNA level of *Rac1* and *RhoA*. We found that there was no significant change in *Rac1* mRNA level in the DS of cocaine-conditioned mice (Supplementary Figure S2B), whereas cocaine significantly elevated the transcription of *RhoA* gene (Supplementary Figure S2C, t(12) = 4.33, *$p = 0.0019$). These results indicated that cocaine could attenuate Rac1 activity but without affecting the transcription and translation of *Rac1* gene. In spite that cocaine elevated *RhoA* expression, but its activity was decreased.

### *Nnmt* Knockdown in the DMS Decreases Cocaine CPP

To test the effect of NNMT on cocaine effect *in vivo*, we knocked down *Nnmt* in the dorsomedial striatum (DMS) of C57BL6/J mice by using lentivirus (LV). Mice were received bilateral intra-DMS injection of LV expressing either scrambled shRNA (LV-NC) or *Nnmt* interference sequence (LV-Sh-*Nnmt*). The coordinate of lentivirus injection site was shown in Figure 2a. After recovery, the mice were subjected to cocaine CPP procedure (Figure 2b). As shown in the Figure 2a, the lentivirus eGFP vector was successfully infected and expressed in the DMS, indicating correct coordinates for intracranial injection. We then detected

Nnmt expression to confirm the silencing effect of LV-Sh-*Nnmt*. As expected, *Nnmt* expression in the DMS receiving LV-Sh-*Nnmt* was significantly decreased as compared with

that receiving LV-NC (mRNA: Figure 2c, $F_{(3, 24)} = 19.17$, COC+LV-NC *vs* SAL+LV-NC *$p < 0.0001$, COC+LV-Sh-*Nnmt* *vs* COC+LV-NC #$p = 0.0004$, SAL+LV-Sh-*Nnmt* *vs*

Figure 1 Cocaine upregulates NNMT and reduces the activities of Rac1 and RhoA in the DS. (a) Timeline of cocaine CPP experiment. After test, mice brain tissues were harvested for molecular experiment. (b) CPP score of cocaine group (20 mg/kg i.p.) was significantly higher than that of saline group. $n = 15$ per group. (c) Western blotting analysis showed that NNMT level in cocaine group was higher than that of saline group in the DS, but not in the mPFC, NAc, and hippocampus. $n = 8$ per group. (d) Acute cocaine did not alter the expression of *Nnmt* in the DS. Repeated cocaine exposure induced upregulation of NNMT in the DS as compared to saline treatment. $n = 8$ per group. (e) Box-and-whisker plots illustrated the changes of SAM/SAH ratio levels in saline and cocaine groups, respectively. SAM/SAH ratio levels were decreased by cocaine. $n = 10$ per group. (f) MeN/NA ratio levels were increased by cocaine. $n = 10$ per group. (g) Rac1 activity in cocaine group was lower than that of saline group. Active Rac1 normalized to total Rac1. $n = 8$ per group. (h) Western blotting analysis indicated that total Rac1 level showed no significant difference between cocaine and saline group. Total Rac1 normalized to β-tubulin. $n = 8$ per group. (i) Immunoblotting of Rac1 and β-tubulin. (j) The activity of RhoA in cocaine group was lower than that of saline group. $n = 8$ per group. (k) Western blotting analysis indicated that total RhoA level in cocaine group was higher than that of saline group. $n = 8$ per group. (l) Immunoblotting of RhoA and β-tubulin. *, compared with SAL group; C, cocaine; COC, intraperitoneal injection of cocaine; CPP, conditioned place preference; MeN, 1-methylnicotinamide; mPFC, medial prefrontal cortex; NA, nicotinamide; NAc, nucleus accumbens; S, saline; SAH, *S*-adenosyl-ʟ-homocysteine; SAL, intraperitoneal injection of saline; SAM, *S*-adenosyl-ʟ-methionine.
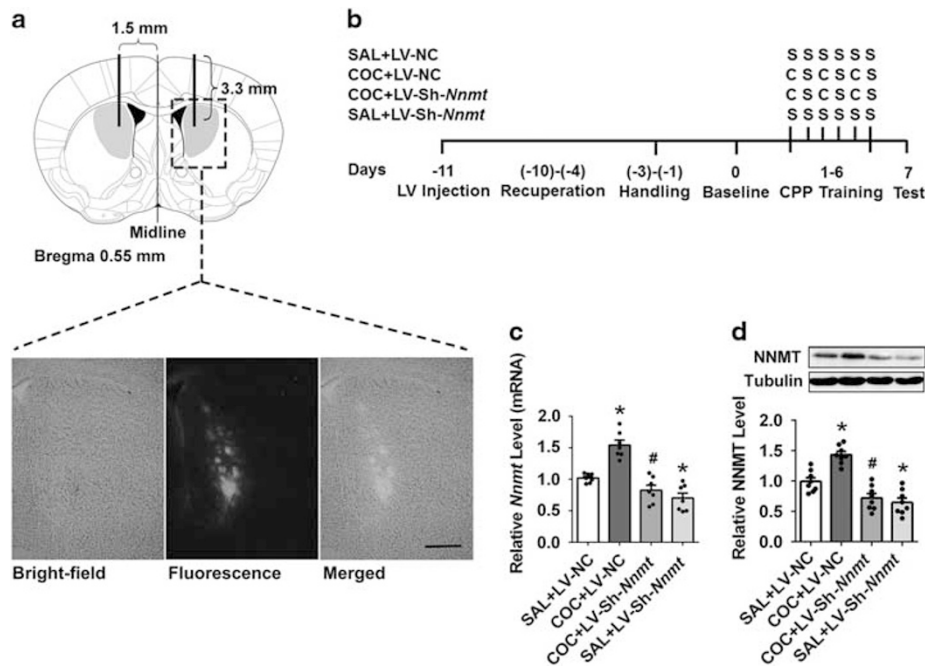
Figure 2 *Nnmt*-shRNA lentivirus decreases *Nnmt* expression in the DMS. (a) Brain atlas showed the lentivirus injection sites in the DMS (blue area). Lentivirus vectors expressing eGFP are successfully transfected and expressed in the DMS. Bar = 400 μm. (b) Before cocaine CPP experiment, mice were subjected to lentivirus injection to knockdown *Nnmt* expression in the DMS. After test, mice brain tissues were harvested for molecular experiment. (c) RT-PCR analysis indicated that *Nnmt*-shRNA lentivirus significantly decreased *Nnmt* transcription in the DMS. SAL+LV-Sh-*Nnmt* compared with SAL+LV-NC; and COC+LV-Sh-*Nnmt* compared with COC+LV-NC. $n = 7$ per group. (d) Western blotting analysis showed that the NNMT level in the DMS receiving LV-Sh-*Nnmt* was decreased significantly as compared with that receiving LV-NC. $n = 8$ per group. *, compared with SAL+LV-NC group; #, compared with COC+LV-NC group; DMS, dorsomedial striatum; C, cocaine; S, saline; COC, intraperitoneal injection of cocaine; SAL, intraperitoneal injection of saline; LV-NC, intra-DMS injection of negative control lentivirus expressing scrambled shRNA; LV-Sh-*Nnmt*, intra-DMS injection of lentivirus expressing *Nnmt* shRNA; CPP, conditioned place preference. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

SAL+LV-NC *$p = 0.0024$; and protein: Figure 2d, $F_{(3, 28)} = 23.17$, COC+LV-NC vs SAL+LV-NC *$p = 0.0014$, COC+LV-Sh-*Nnmt* vs COC+LV-NC #$p = 0.0011$, SAL+LV-Sh-*Nnmt* vs SAL+LV-NC *$p = 0.047$). In CPP experiment, a two-way ANOVA revealed the significant main effects of cocaine ($F_{(1, 56)} = 181.10$, $p < 0.0001$) and lentivirus ($F_{(1, 56)} = 9.19$, $p = 0.0037$), as well as cocaine × lentivirus interactions ($F_{(1, 56)} = 15.22$, $p = 0.0003$). Through *post hoc* test (Tukey's multiple comparisons) we observed that the cocaine-conditioned mice treated with LV-Sh-*Nnmt* showed significantly decreased CPP scores than the control mice treated with LV-NC (Figure 3a, $q_{(4, 56)} = 6.93$, #$p < 0.0001$). Collectively, these results indicated that *Nnmt* knockdown in the DMS could significantly attenuate cocaine effect.

NNMT Regulates Cocaine CPP Possibly though SAM-Rac1/RhoA Pathway

To examine whether *Nnmt* knockdown would modify the ratio levels of SAM/SAH in the DMS, targeted HILIC-QTOF mass spectrometry was used to measure the levels of the metabolites. The results showed that cocaine significantly reduced the levels of SAM/SAH ratio in the DMS, whereas such decreases were inhibited by *Nnmt* knockdown (Figure 3b, $F_{(2, 27)} = 6.46$, *$p = 0.0043$, #$p = 0.015$). Conversely, cocaine significantly elevated MeN/NA ratio levels, whereas such increases were inhibited by *Nnmt* knockdown (Figure 3c, $F_{(2, 27)} = 14.53$, *$p = 0.0007$, #$p = 0.0044$).

We next tested whether NNMT-induced changes in SAM/SAH ratio could affect the activity of protein through methylation events. We measured the activities of Rac1 and RhoA with or without *Nnmt* knockdown in the DMS. Importantly, we found that cocaine reduced the activity of Rac1 and RhoA, whereas such decreases were inhibited by *Nnmt* knockdown (Rac1: Figure 3d and h; $F_{(2, 21)} = 37.22$, *$p = 0.0016$, #$p = 0.0025$; RhoA: Figure 3f and i; $F_{(2, 21)} = 7.44$, *$p = 0.020$, #$p = 0.016$). These results revealed the involvement of NNMT in regulating the activities of Rac1 and RhoA. We continued to confirm the protein level of total Rac1 and RhoA. There was no significant change of Rac1 after *Nnmt* knockdown (Figure 3e and h); however, RhoA was downregulated (Figure 3g and i; $F_{(2, 21)} = 4.59$, *$p = 0.011$, #$p = 0.048$). The RT-PCR results further showed that there were no significant changes in the mRNA level of *Rac1* in the DMS (Supplementary Figure S4A), whereas *Nnmt* downregulation inhibited the transcription of *RhoA* gene (Supplementary Figure S4B, $F_{(3, 24)} = 7.59$, COC+LV-NC vs SAL+LV-NC *$p = 0.0011$, COC+LV-Sh-*Nnmt* vs COC+LV-NC #$p = 0.046$, SAL+LV-Sh-*Nnmt* vs SAL+LV-NC *$p = 0.043$). Collectively, these results indicated that *Nnmt* knockdown can reverse cocaine-induced downregulation of Rac1 and RhoA activities.

As post-translational methylation modification of Rac1 and RhoA is catalyzed by LCMT1 (Cushman and Casey, 2009), we next investigated whether LCMT1 could be involved in the regulation of NNMT on Rac1 and RhoA.
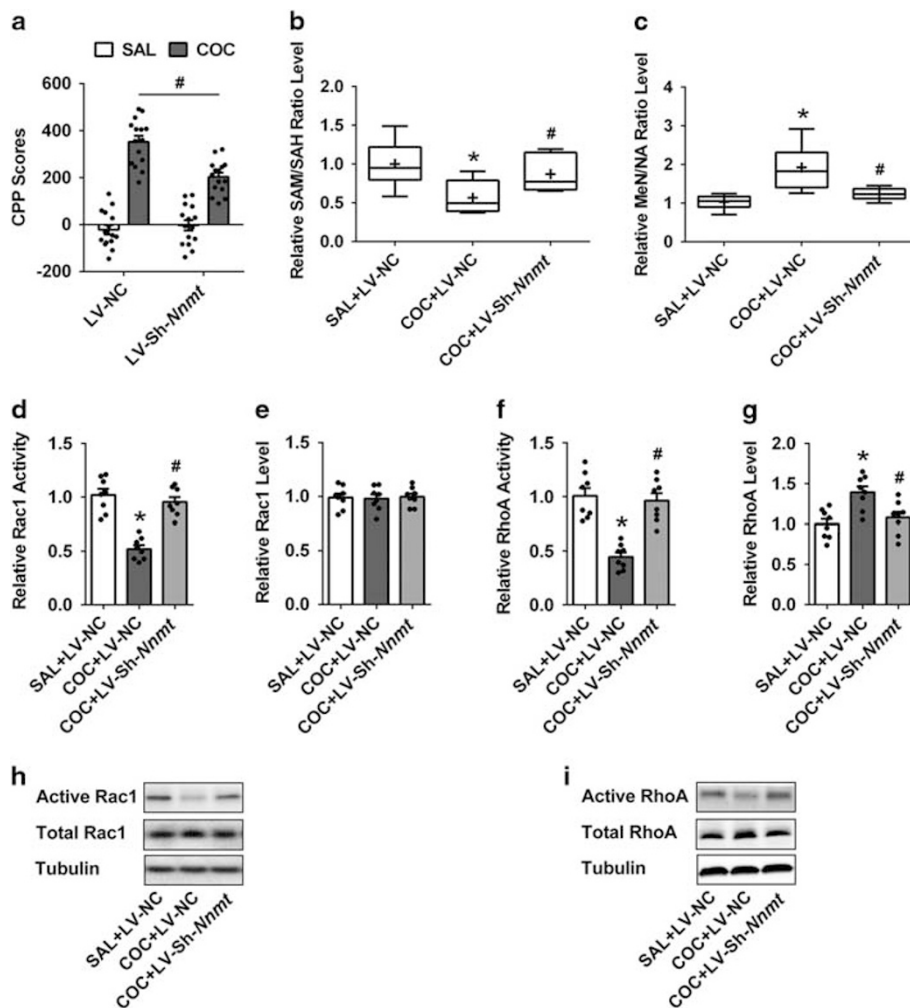
Figure 3 *Nnmt* knockdown in the DMS decreases cocaine CPP and increases the activities of Rac1 and RhoA. (a) *Nnmt* knockdown in the DMS weakened cocaine-induced CPP. $n = 15$ per group. (b) For more clearly showing the effect of lentivirus in cocaine-treated mice, SAL+LV-Sh-*Nnmt* group in subsequent tests was not shown. Box-and-whisker plots illustrated the SAM/SAH ratio levels were decreased by cocaine, but increased by *Nnmt* knockdown. $n = 10$ per group. (c) MeN/NA ratio levels were increased by cocaine, but reduced by *Nnmt* knockdown. $n = 10$ per group. (d) Rac1 activity was reduced by cocaine, but increased by *Nnmt* knockdown. Active Rac1 normalized to total Rac1. $n = 8$ per group. (e) The level of total Rac1 protein showed no difference after COC+LV-NC-treated or COC+LV-Sh-*Nnmt*-treated in the DMS. Total Rac1 normalized to β-tubulin. $n = 8$ per group. (f) RhoA activity was also reduced by cocaine, but increased by *Nnmt* knockdown. $n = 8$ per group. (g) The level of total RhoA protein increased after COC+LV-NC treatment, but decreased after COC+LV-Sh-*Nnmt* treatment. $n = 8$ per group. (h) Immunoblotting of Rac1 and β-tubulin. (i) Immunoblotting of RhoA and β-tubulin. *, compared with SAL+LV-NC group; #, compared with COC+LV-NC group; DMS, dorsomedial striatum; COC, intraperitoneal injection of cocaine; SAL, intraperitoneal injection of saline; LV-NC, intra-DMS injection of negative control lentivirus expressing scrambled shRNA; LV-Sh-*Nnmt*, intra-DMS injection of lentivirus expressing *Nnmt* shRNA; CPP, conditioned place preference. SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; MeN, 1-methylnicotinamide; NA, nicotinamide.

By using RT-PCR and western blotting, we measured the expressions of *Lcmt1*. However, there was no significant change in *Lcmt1* expression in cocaine-conditioned mice with or without LV-Sh-*Nnmt* treatment (Supplementary Figure S4C and D). Therefore, LCMT1 may not mediate the effect of NNMT on the methylation modification of Rac1 and RhoA. As maturation and activation of Rac1 and RhoA rely on post-translational methylation, we consider that *Nnmt* knockdown activates Rac1 and RhoA possibly through elevating SAM/SAH ratio levels in the DMS of mice treated with cocaine. Elevated SAM/SAH levels account for, at least in part, the inhibitory effect of *Nnmt* knockdown on cocaine effect.

Nnmt Knockdown Inhibits the Association of RhoGDIα with Rac1 or RhoA in the DMS

As RhoGDIα plays an important role in GDP binding to Rac1 or RhoA, we detected the RhoGDIα level in extracted total protein (Input protein). We observed that there was no significant change in the RhoGDIα translation level of cocaine-conditioned mice with or without *Nnmt* silence (Figure 4a and e). To explore whether RhoGDIα could mediate the effect of NNMT on Rac1 and RhoA activities, co-IP analysis using a RhoGDIα antibody was performed to detect the association of RhoGDIα with Rac1 or RhoA. Importantly, we found that cocaine increased the interaction of RhoGDIα with Rac1 or RhoA in the DMS, whereas such
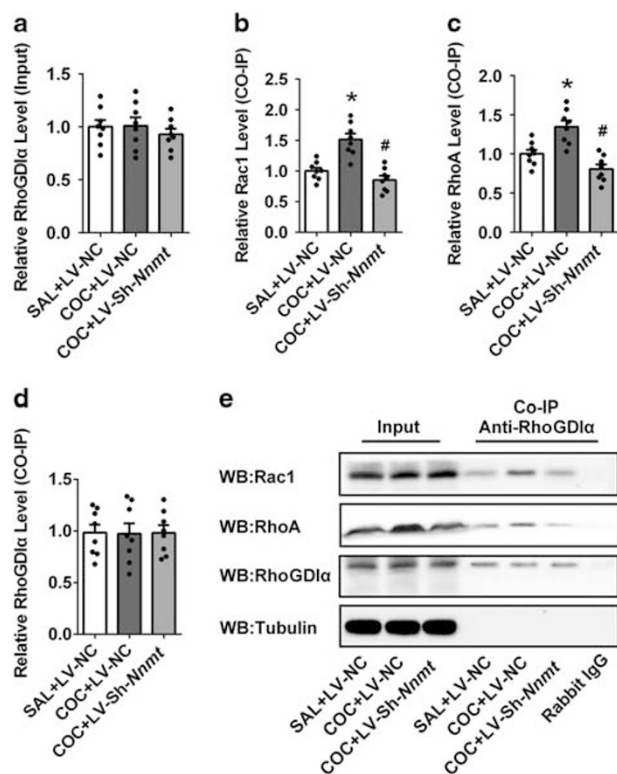
Figure 4 Nnmt knockdown in the DMS inhibits the interaction of RhoGDIα with Rac1 or RhoA. (a) RhoGDIα protein level showed no difference between saline and cocaine group in the DMS. Nnmt knockdown did not influence RhoGDIα translation. Total input RhoGDIα normalized to β-tubulin. $n = 8$ per group. (b) The level of Rac1 enriched by a RhoGDIα antibody in the DMS was increased by cocaine, but reduced by Nnmt knockdown. Co-IP Rac1 normalized to input Rac1. $n = 8$ per group. (c) The level of RhoA enriched by a RhoGDIα antibody in the DMS was also increased by cocaine, but reduced by Nnmt knockdown. $n = 8$ per group. (d) The level of RhoGDIα enriched by a RhoGDIα antibody in the DMS showed no change by cocaine or by Nnmt knockdown. Co-IP RhoGDIα normalized to input RhoGDIα. $n = 8$ per group. (e) Co-IP analysis with a RhoGDIα antibody. *, compared with SAL+LV-NC group; #, compared with COC+LV-NC group; Rabbit IgG, a negative control; Input, total protein before co-IP; COC, intraperitoneal injection of cocaine; SAL, intraperitoneal injection of saline; LV-NC, intra-DMS injection of negative control lentivirus expressing scrambled shRNA; LV-Sh-Nnmt, intra-DMS injection of lentivirus expressing Nnmt shRNA.

increases were blocked by Nnmt knockdown through an infusion of LV-Sh-Nnmt (Rac1: Figure 4b and e; $F_{(2, 21)} = 9.05$, *$p = 0.023$, #$p = 0.012$; and RhoA: Figure 4c and e; $F_{(2, 21)} = 7.51$, *$p = 0.028$, #$p = 0.015$). We also detected the level of RhoGDIα that was immuno-precipitated by RhoGDIα antibody. However, there was no significant change in the RhoGDIα level of cocaine-conditioned mice with or without Nnmt silence (Figure 4d and e). These results showed that the dynamic interaction of RhoGDIα with Rac1 or RhoA, rather than RhoGDIα translation, may mediate the effect of NNMT in regulating cocaine effect.

Pharmacological Inhibition of NNMT in the DMS Inhibits Cocaine CPP and Rac1 and RhoA Activities

MeN, a reaction product of NNMT, is a potent inhibitor of NNMT through a feedback mechanism (Kraus et al, 2014).

To examine whether the effect of NNMT on cocaine CPP would be mediated by its metabolic product, the DMS of mice was intracerebrally infused with MeN solution (2, 10, and 50 mM, respectively) at a volume of 0.5 μl per brain side (Figure 5a). The intracerebral injection was 30 min prior to cocaine injection, and then behavioral training was conducted and cocaine CPP was measured (Figure 5b). Two-way ANOVA revealed the significant main effects of cocaine ($F_{(1, 104)} = 352.00$, $p < 0.0001$) and a significant cocaine × MeN interactions ($F_{(3, 104)} = 3.60$, $P = 0.0465$); however, no significant main effect of MeN was observed ($F_{(3, 104)} = 2.493$, $p = 0.0641$). Through post-hoc test (Tukey's multiple comparisons), we found that the cocaine-conditioned mice treated with 50 mM MeN showed significantly decreased CPP scores than the control mice treated with saline (NC) (Figure 5c, $q_{(8, 104)} = 5.152$, #$p < 0.01$). However, MeN at the dose of 2 mM or 10 mM showed no obvious effect. In order to confirm the inhibitory action of MeN on NNMT activity, we used targeted HILIC-QTOF mass spectrometry to measure the metabolites. The results showed that intra-DMS injections of 50 mM MeN significantly elevated SAM/SAH ratio (Figure 5d, $t_{(18)} = 4.51$, #$p = 0.0006$). Elevated MeN/NA ratio indicated a successful infusion of MeN into the DMS (Figure 5e, $t_{(18)} = 8.07$, #$p < 0.0001$). We continued to measure the activities of Rac1 and RhoA in the DMS, and found that MeN increased the activities of these two molecules (Rac1: Figure 5f and h; $t_{(14)} = 3.31$, #$p = 0.016$; RhoA: Figure 5g and j; $t_{(14)} = 8.13$, #$p = 0.0002$). The total protein level of Rac1 was not significantly changed (Figure 5f and i), whereas RhoA was downregulated by 50 mM MeN (Figure 5g and k; $t_{(14)} = 3.13$, #$P = 0.021$).

DISCUSSION

Altered methyl supply with an increase in brain SAM is associated with methyltransferase level and becomes a crucial factor mediating brain function (Jayaram et al, 2016; Saunderson et al, 2016). NNMT is an important enzyme of one carbon metabolism, using SAM as its cofactor. The roles of NNMT in liver metabolism and cancer have been investigated (Hong et al, 2015; Xu et al, 2016); however, the role of NNMT in drug addiction is largely unknown. Here, we found NNMT decreases SAM level in the DMS of cocaine-conditioned mice, which is accompanied by the reduced post-translational methylation of Rac1 and RhoA. Unmethylated Rac1/RhoA combine with GDP to form inactive Rac1 GDP/RhoA GDP, which interact with RhoGDIα to decrease their function in inhibiting neurite growth. These findings showed that NNMT regulates cocaine CPP possibly through SAM-mediated regulation of Rac1 and RhoA activities (Figure 5l).

Methyl-Modification of DNA, Histone and Nonhistone Protein Play Roles in Cocaine CPP

SAM supplies methyl for many biochemical reactions in vivo (Zhang and Zheng, 2016). In fact, enhancing the pool of available methyl donors with SAM or its precursor increases DNA methylation and alters gene expression. In drug addiction, previous studies most focused on the relationship
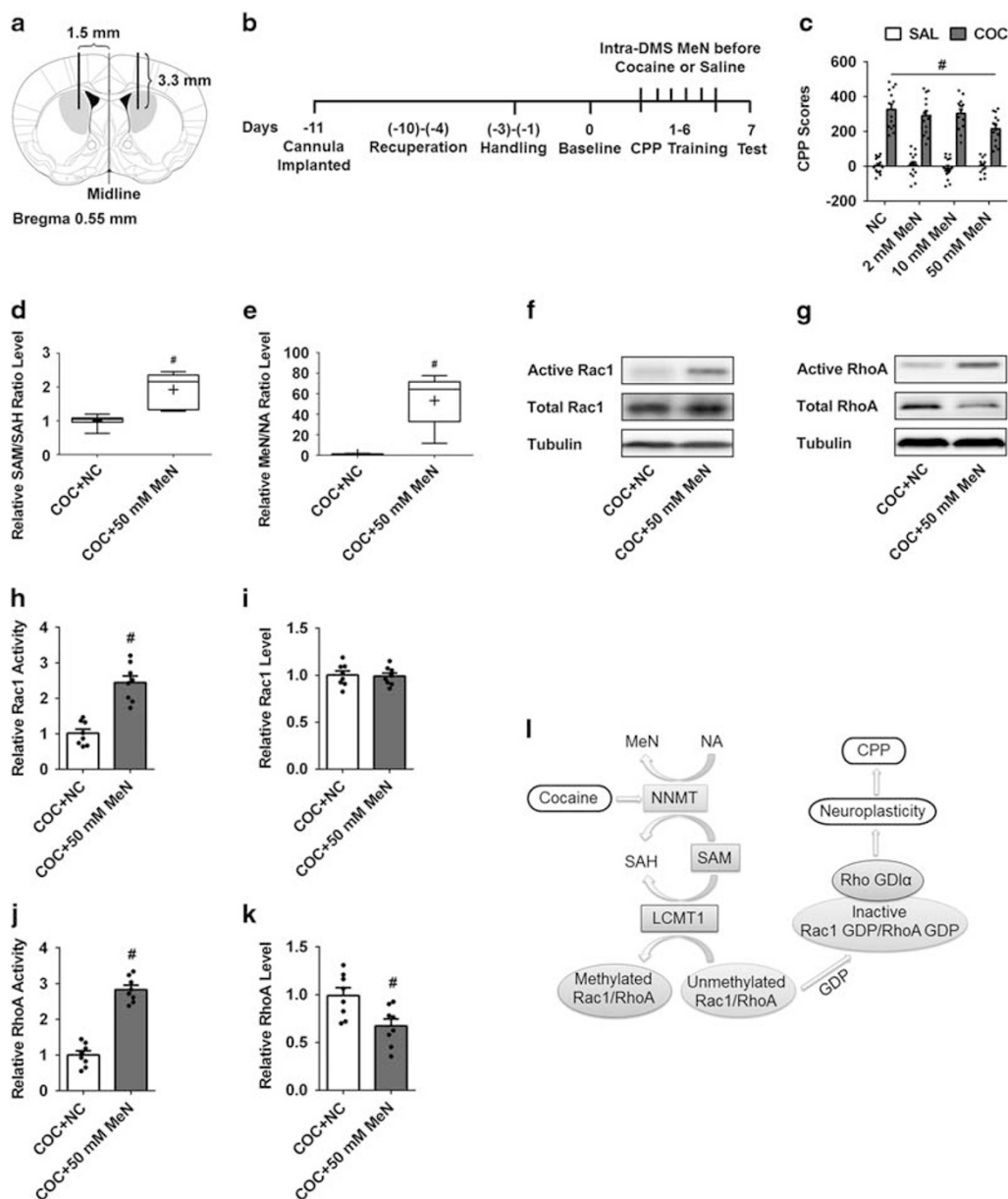
Figure 5 MeN regulate cocaine CPP though Rac1 and RhoA. (a) Brain atlas showed MeN injection sites in the DMS (blue areas). (b) Before cocaine CPP training, mice were subjected to intra-DMS infusion of MeN to inhibit NNMT. (c) Intra-DMS injection of 50 mM MeN decreased cocaine-induced CPP. $n = 14$ per group. (d) SAM/SAH ratio were increased by Intra-DMS injection of 50 mM MeN. $n = 10$ per group. (e) MeN/NA ratio were increased by intra-DMS injection of 50 mM MeN. $n = 10$, per group. (f) Immunoblotting of Rac1 and β-tubulin. (g) Immunoblotting of RhoA and β-tubulin. (h) Rac1 activity was increased by intra-DMS injection of 50 mM MeN. Active Rac1 normalized to total Rac1. $n = 8$ per group. (i) Rac1 protein level showed no difference between COC+NC and COC+50 mM MeN groups in the DMS. Total Rac1 normalized to β-tubulin. $n = 8$ per group. (j) RhoA activity was increased by 50 mM MeN. $n = 8$ per group. (k) RhoA protein level was decreased by intra-DMS injection of 50 mM MeN. $n = 8$ per group. (l) Schematic representation of putative mechanism of NNMT in cocaine-induced CPP. Yellow: upregulation; blue: downregulation; gray: unchanged. #p, compared with COC+NC group; CPP, conditioned place preference; SAL, intraperitoneal injection of saline; COC, intraperitoneal injection of cocaine; NC, intra-DMS injection of saline; MeN, intra-DMS injection of MeN; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; MeN, 1-methylnicotinamide; NA, nicotinamide. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

between SAM and the methylation of DNA or histone (Anier et al, 2010; Black et al, 2006; Jordi et al, 2013; Maze and Nestler, 2011). In addition, there exists some methylation of nonhistone protein assisted by SAM (Aksoy et al, 1995). Rac1 and RhoA, two important members of Rho GTPase family, require post-translational methylation using SAM as the methyl donor (Li et al, 2015a). Rac1 and RhoA are effectors of D1 receptor signaling during dendritic morphogenesis in the PFC (Li et al, 2015a). Blocking Rac1 activity

increases the density of immature dendritic spines of neurons in the NAc (Dietz et al, 2012). However, the mechanism underlying the activation of Rac1 and RhoA is still largely unknown. In the present study, we found that cocaine reduced SAM/SAH ratio levels as well as Rac1 and RhoA activities in the DS, which may be due to the incomplete post-translational methylation as a result of the reduced supply of SAM.

Deficient Methyl-Modification of Rac1 and RhoA may be the Result of Decreased SAM in Cocaine-Conditioned Mice

After binding to GTP, Rho GTPase family becomes activated Rho GTP; on the contrary, after binding to GDP, Rho GTPase family becomes inactivated Rho GDP (Jaffe and Hall, 2005; Tiedje *et al*, 2008). RhoGDIα blocks the dissociation of GDP from Rac1 and RhoA, making them inactive, which enhances the neurite growth, differentiation and neuroplasticity (Hancock and Hall, 1993; Liu *et al*, 2015). It is known that decreased methylation of Rac1 and RhoA increases their binding to RhoGDIα (Cushman and Casey, 2011). We found that cocaine induced a decrease in SAM but an increase in the interaction of RhoGDIα with Rac1 and RhoA in the DMS. However, such effect could be reversed by *Nnmt* knockdown. Therefore, these results suggest that the incomplete post-translational methylations of Rac1 and RhoA may be the result of decreased SAM in the DMS of cocaine-conditioned mice. The exact mechanism by which SAM regulates the methylation of Rac1 and RhoA needs to be addressed in the future.

LCMT1 may not Participate in the Regulation of Rac1 and RhoA Activities after Cocaine Exposure

RhoA and Rac1 are best known as critical regulators of cytoskeleton dynamics and neuronal development. Post-translational methylation of Rac1 and RhoA are catalyzed by LCMT1 (Nishimura and Linder, 2013). It has been known that inhibition of LCMT1 decreases the methylation levels of Rac1 and RhoA, thus weakening the activities of Rac1 and RhoA (Cushman and Casey, 2009). However, in this study, cocaine treatment or *Nnmt* knockdown had no effect on *Lcmt1* expression. So far, there is no evidence showing a post-translational modification that influences LCMT1 activity. Our results might exclude the involvement of LCMT1 in regulating Rac1 and RhoA activities.

DNA Methylation may Contribute to the Discordance of RhoA Expression

We found that NNMT regulated *RhoA* expression in the DMS of cocaine-conditioned mice, which was accompanied by the altered SAM level. Moreover, *RhoA* was upregulated or downregulated along with SAM depletion or elevation. It is known that SAM supplies methyl for DNA methylation (Jones and Takai, 2001; Shang *et al*, 2016). Generally, DNA hypomethylation changes protein-DNA interactions, leading to the alterations in chromatin structure and transcription rate, which mainly promotes gene expression. On the other hand, DNA hypermethylation inhibits gene expression (Jones and Takai, 2001; Zhang and Zheng, 2016). Methylated CpG island regions of DNA exist in the mouse promoter region of *RhoA* gene (NCBI Gene database, NC_000075.6). Considering above, we infer that SAM supply may also influence DNA methylation of *RhoA* gene and regulate its expression responding to cocaine.

Post-translational Methylation is not Necessary for CDC42

Besides Rac1 and RhoA, CDC42 is another important member of Rho GTPase family (Li *et al*, 2002). In the present study, we did not focus on CDC42 because its post-translational modification is different from Rac1 and RhoA. After translation, the C-terminal of Rac1 and RhoA exposes CAAX sequences. The cysteine is isoprentenylated, and AAX peptide is hydrolyzed. The exposed cysteine is then methylated by LCMT1. In this process, SAM provides methyl. However, the C terminal amino sequence of CDC42 is CCIF. Although cysteine is isoprentenylated, the peptide is not hydrolyzed and does not expose cysteine (Nishimura and Linder, 2013). These reports indicate that post-translational methylation and SAM are not necessary for CDC42. Similarly, a previous study showed the activities of Rac1 and RhoA are decreased in the NAc of cocaine-treated mice, but CDC42 activity is not changed (Dietz *et al*, 2012).

Comparison of Biased and Unbiased Design of CPP

There are two different designs for CPP test, biased and unbiased designs. In biased design, mice show a clear preference for one chamber. Cocaine is paired with the less preferred chamber to reverse mice preference. In unbiased design, mice are assigned randomly. Generally, biased design is considered when the average time that untrained animals spend in each test chamber deviates from expectations based on chance. Conversely, unbiased design is considered when the time is consistent with expectations based on chance (eg, average of 50% time in each chamber) (Cunningham *et al*, 2006). The limitation of biased design is that an alternative interpretation of the data is that the drug decreases aversion to the non-preferred side rather than serving as a reinforcing stimulus in the CPP procedure (Bardo and Bevins, 2000; Mucha and Iversen, 1984; Mucha *et al*, 1982). Although the biased design may be susceptible to yielding false-positive results, it is also used due to absolute preference for the initially non-preferred side (Tzschentke, 1998). Moreover, cocaine and other psychoactive drugs produce CPP only when paired with the initially non-preferred side, but not with the initially preferred side (Nomikos and Spyraki, 1988; Tzschentke, 1998).

AUTHOR CONTRIBUTIONS

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