Behavioral/Cognitive

Methyl Supplementation Attenuates Cocaine-Seeking Behaviors and Cocaine-Induced c-*Fos* Activation in a DNA Methylation-Dependent Manner

Katherine N. Wright,[1] Fiona Hollis,[1,2] Florian Duclot,[1] Amanda M. Dossat,[1] Caroline E. Strong,[1] T. Chase Francis,[3] Roger Mercer,[1] Jian Feng,[4] David M. Dietz,[5] Mary Kay Lobo,[3] Eric J. Nestler,[4] and Mohamed Kabbaj[1]

[1]Program in Neuroscience, Department of Biomedical Sciences, Florida State University, Tallahassee, Florida 32306, [2]Federal Polytechnic School of Lausanne, CH-1015 Lausanne, Switzerland, [3]Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, Maryland 21201, [4]Fishberg Department of Neuroscience and Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, New York 10029, and [5]Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, New York 14214

Epigenetic mechanisms, such as histone modifications, regulate responsiveness to drugs of abuse, such as cocaine, but relatively little is known about the regulation of addictive-like behaviors by DNA methylation. To investigate the influence of DNA methylation on the locomotor-activating effects of cocaine and on drug-seeking behavior, rats receiving methyl supplementation via chronic L-methionine (MET) underwent either a sensitization regimen of intermittent cocaine injections or intravenous self-administration of cocaine, followed by cue-induced and drug-primed reinstatement. MET blocked sensitization to the locomotor-activating effects of cocaine and attenuated drug-primed reinstatement, with no effect on cue-induced reinstatement or sucrose self-administration and reinstatement. Furthermore, upregulation of DNA methyltransferase 3a and 3b and global DNA hypomethylation were observed in the nucleus accumbens core (NAc), but not in the medial prefrontal cortex (mPFC), of cocaine-pretreated rats. Glutamatergic projections from the mPFC to the NAc are critically involved in the regulation of cocaine-primed reinstatement, and activation of both brain regions is seen in human addicts when reexposed to the drug. When compared with vehicle-pretreated rats, the immediate early gene c-*Fos* (a marker of neuronal activation) was upregulated in the NAc and mPFC of cocaine-pretreated rats after cocaine-primed reinstatement, and chronic MET treatment blocked its induction in both regions. Cocaine-induced c-*Fos* expression in the NAc was associated with reduced methylation at CpG dinucleotides in the c-*Fos* gene promoter, effects reversed by MET treatment. Overall, these data suggest that drug-seeking behaviors are, in part, attributable to a DNA methylation-dependent process, likely occurring at specific gene loci (e.g., c-*Fos*) in the reward pathway.

Key words: **addiction; c-Fos; cocaine; epigenetics; methionine**

Introduction

Cocaine (COC) addiction is characterized by compulsive and pathological drug use despite negative consequences (Koob and Le Moal, 1997; Robinson and Berridge, 2003; Everitt and Robbins, 2005). Relapse is a major obstacle that many individuals with addiction face on their path to recovery (Simpson et al., 1999). As such, there is a critical need to gain better understanding of the neurobiological mechanisms of addiction and relapse to improve treatments and reduce the likelihood of relapse. Epigenetic mechanisms regulate transcriptional changes that underlie long-lasting neuroadaptations occurring with chronic COC exposure (Kumar et al., 2005; Brami-Cherrier et al., 2009; Renthal et al., 2009; Maze et al., 2010; Robison and Nestler, 2011; Malvaez et al., 2013). DNA methylation, the addition of methyl groups onto the 5-position of cytosines by DNA methyltransferases (*Dnmt1*, *Dnmt3a*, *Dnmt3b*), is usually a repressive marker when occurring in close proximity to transcription start sites (Jones, 2012). Previously considered a static process in the adult CNS, DNA methylation dynamically regulates neural functions associated with synaptic plasticity and learning and memory (Szyf et al., 2005; Levenson et al., 2006; Miller and Sweatt, 2007; Nelson et al., 2008; Feng et al., 2010; Day et al., 2013). In fact, enhancing the pool of available methyl donors with *S*-adenosylmethionine (SAM) or its precursor, L-methionine (MET), increases DNA methylation and alters gene expression (Dong et al., 2008; Anier et al., 2010). Additionally, MET blocks conditioned place preference to COC (LaPlant et al., 2010; Tian et al., 2012), indicating that DNA methylation may play a role in

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Correspondence should be addressed to Mohamed Kabbaj, Florida State University, College of Medicine, Department of Biomedical Sciences, 1115 West Call Street, Tallahassee, FL 32306. E-mail: mohamed.kabbaj@med.fsu.edu.

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COC reward processing. However, the aforementioned studies did not address the shared mechanism underlying both sensitization, a defining characteristic of addiction in which repeated drug exposure induces neuroadaptations to behavioral and synaptic plasticity, and relapse (Robinson and Berridge, 1993; Vezina, 2004; Steketee and Kalivas, 2011). Thus, one of the aims of this study was to investigate the role of DNA methylation in locomotor sensitization to COC and reinstatement to COC and associated cues.

We hypothesized that chronic MET treatment would reduce addictive-like behaviors via DNA methylation-dependent processes. First, we investigated the effect of MET on locomotor sensitization, in which repeated exposure to COC results in subsequent increases in locomotor activity. Next, we assessed the effects of MET on intravenous COC self-administration and subsequent drug-seeking behaviors during cue-induced and COC-primed reinstatement. We also assessed the effects of MET on sucrose pellet reinstatement to determine whether its effects extend to natural reward. We then measured expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b,* and global DNA methylation in two areas of the neural circuitry responsible for drug-seeking behavior, the nucleus accumbens core (NAc) and the medial prefrontal cortex (mPFC; McFarland and Kalivas, 2001; Kalivas and Volkow, 2005), both of which are shown to be hypoactive at rest and overactive in response to COC in rats and humans (Goldstein and Volkow, 2002; Sun and Rebec, 2006). Finally, given the rapid induction by COC of c-*Fos*, an immediate early gene product and marker of neuronal activation (Graybiel et al., 1990; Neisewander et al., 2000), we examined whether MET affects the reward circuitry as indicated by blocking COC-induced c-*Fos* expression.

Materials and Methods

Animals

All experiments were performed according to the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (National Research Council, 1996) and were approved by the Florida State University Institutional Animal Care and Use Committee of Florida State University. Male Sprague Dawley rats weighing 225–250 g (Charles River) were initially pair housed in 43 × 21.5 × 25.5 cm Plexiglas cages on a 12 h light/dark cycle. Locomotor activity was assessed during the first 4 h of their light cycle. Operant training was conducted at least 2 h after the onset of the dark cycle. Food and water were provided *ad libitum*. After catheter implantation, animals were single housed for the duration of the experiment.

For jugular catheter surgery, heparin-coated catheters (Instech Laboratories) were attached with epoxy to back-mounted cannulae with a 10 mm upward projection (Plastics One). Surgery was conducted under sterile conditions. Rats were anesthetized with an intramuscular injection of ketamine (70 mg/kg) and xylazine (10 mg/kg), a 1 cm incision was made, and the right external jugular vein was dissected bluntly. After creating a subcutaneous passage for the catheter to pass from the midscapular region to the front, the jugular vein was incised halfway with artery scissors, and the catheter tubing was installed and tied into place using silk suture. Finally, incision sites were sutured, and rats were treated with a topical analgesic (0.25 mg/kg bupivacaine) and an anti-inflammatory (5 mg/kg carprofen, i.p.). Catheters were flushed twice daily with 0.1 ml of heparinized saline (SAL; 50 U/ml heparin) and ampicillin (30 mg/ml). After 2–3 d of recovery, the self-administration procedure began. Catheter patency was tested with 20 mg/kg ketamine 4 d after surgery and after 10 d of acquisition. These heparin-coated catheters have been shown previously to maintain patency for at least 30 d with twice weekly heparin flushes (Foley et al., 2002). One rat was removed from the study because of a failed catheter.

Drugs

Cocaine hydrochloride (generously donated by National Institute on Drug Abuse) was dissolved in 0.9% sterile SAL at a dose of 0.75 mg/kg per infusion for the COC self-administration experiment and 10 mg/kg intraperitoneally for the sensitization experiment and for drug-induced reinstatement. MET (10.4 mmol/ml, dissolved in SAL and injected at 1 ml/kg, s.c.) or SAL was injected once daily, 1–2 h before behavioral testing, starting on the first day of behavioral sensitization or self-administration and lasting the duration of the experiment. This dose of MET, used previously by our group, is within a range that has been shown to increase SAM levels in the brain (Tremolizzo et al., 2002; LaPlant et al., 2010).

Behavioral testing

Novelty response. Because individual differences in novelty-seeking behaviors influences response to drugs of abuse (Kabbaj, 2006), classification as high responders (HRs) or low responders (LRs) was determined to balance HR and LR rats equally between experimental groups, based on the group median split of their exploratory behavior described previously (Dietz et al., 2008). Briefly, at the beginning of the light cycle, all rats underwent a 1 h locomotor test in a novel donut-shaped arena 71.2 cm in diameter with four equidistant photobeam sensors (Med Associates), and locomotor activity was quantified by the number of beam breaks. This classification was only used for assigning experimental groups, and no other statistical analyses are reported.

Drug-induced locomotor sensitization. A separate cohort of rats was used for this experiment. On day 1, rats were given 1 h to habituate to a donut-shaped arena, injected with SAL (1 ml/kg), and given 1 additional hour to habituate to the stress of injection. The rats were then removed briefly from the arena and given an injection of COC (10 mg/kg, i.p.), followed by 2 h in which they were allowed to move freely within the arena. All 4 h of locomotor activity were measured, and the number of photobeam breaks in 10 min time bins were recorded. Then, the rats were given an injection of COC in their home cage on days 4 and 7, before repeating the same locomotor procedure on day 10 as was done on day 1.

Operant training. Animals were trained to respond for 45 mg sucrose pellets under an fixed ratio 1 (FR1) schedule of reinforcement for 1 h each day in standard operant chambers (30.5 × 24.1 × 21.0 cm; Med Associates) in sound-attenuating cabinets. The chambers were equipped with two nose-poke holes: an active response resulted in a sucrose pellet reward, with the house light turning off for a 7.2 s timeout duration during which time no responses were reinforced, and a cue light turning on for 10 s. An inactive response had no programmed consequences. Rats trained daily until they met the criteria of a minimum of three trials with at least 15 active responses with <20% variability between them and active responses at least 75% higher than inactive responses. Number of sucrose pellets, active responses, and inactive responses were recorded.

Sucrose pellet training, measurement of body weights, and food and water intake. A separate cohort of rats was used for this experiment. Animals were housed two per cage and trained to self-administer sucrose pellets as described above. Once rats reached criteria for advancement, they continued self-administering sucrose pellets for 10 d under the same conditions, and they were treated daily with MET or SAL. Additionally, their food and water intake and their body weights were measured daily.

COC self-administration procedure, extinction, and reinstatement trials. Rats self-administered 0.75 mg/kg per infusion intravenous COC or SAL in the same operant chamber in which they initially trained using sucrose pellets, for a 2 h trial with an FR1 schedule of reinforcement with 100 maximum infusions possible. During the trial, an active response resulted in the following: a cue light turned on for 10 s, a COC infusion was delivered intravenously over the course of 2.8 s, and house lights turned off for a 7.2-s timeout period, during which time active and inactive responses were counted but no infusions were available until the house light turned back on. Selection of the inactive nose-poke hole had no programmed function. Animals trained once daily until criteria for advancement was achieved (a minimum of 10 trials, with 25 or more infusions on the last 3 d). Infusions, active responses, and inactive responses were recorded. At the end of each trial, catheters were flushed with heparinized SAL, and rats were returned to their home cages. After reaching the minimum criteria for acquisition, animals underwent daily 2 h extinction trials in which the house light was off, COC was unavailable, and there were no programmed responses for either active or inactive selec-

Table 1. Primer sequences used for RT-qPCR and bisulfite sequencing

Primer	Forward	Reverse	Reference
NADH	CTATTAATCCCCGCCTGACC	GGAGCTCGATTTGTTTCTGC	Duclot and Kabbaj, 2013
Dnmt1	CAGATGTTCCATGCACACT	TGTGGATGTAGGAAAGTTGCA	Zhou et al., 2013
Dnmt3a	ACGCCAAAGAAGTGTCTGCT	CTTTGCCCTGCTTTATGGAG	LaPlant et al., 2010
Dnmt3b	TCACCCGAGAGACCAAGGAT	CGTGATTCTGGGGGAGGTTC	Own design
Hprt1	GCAGACTTTGCTTTCCTTGG	GTCTGGCCTGTATCCAACACT	Sarkar et al., 2014
c-*Fos*	ACCTCAAGGACTTGAAAGCATC	ACATCTCCGGAAGAGGTGAG	Echeverry-Alzate et al., 2012
c-*Fos_Bis*	TAATTGTGAATATTTATAGGTGAAAGTTAT	ACTCTATCCAATCTTCTCAATTACTAA	Dyrvig et al., 2012

Bis, Bisulfite.

tions. Active and inactive responses were recorded. When the rats reached criteria for advancement (10 trials, a 75% reduction from their final acquisition trial, and <20% variability in active responses compared across the last three trials), they underwent a single cue-induced reinstatement trial, in which contextual cues that previously indicated COC availability were present but COC was not available. Active and inactive responses were recorded. Two days of extinction trials identical to previous extinction trials followed, because this was sufficient to return all rats to levels of responding comparable with extinction before cue reinstatement. Next they underwent a COC-primed reinstatement trial, in which rats were injected with COC (10 mg/kg, i.p.) and placed immediately in the operant chamber for one 2 h trial with no cues or programmed responses. Animals were killed by rapid decapitation immediately after the end of the trial. Brains were dissected, flash frozen in cold 2-methylbutane, and stored at −80°C for future use.

Sucrose self-administration procedure, extinction, and reinstatement trials. A separate cohort of rats underwent an experiment identical to the one described above to see whether the effects of MET generalize to natural reinforcers. After determining HR/LR status and initial sucrose pellet training as described above, rats underwent 10 d of acquisition identical to the COC self-administration experiment, except active responses were reinforced with 45 mg sucrose pellets instead of intravenous COC (FR1 schedule of reinforcement, 2 h trial, 100 pellets maximum). Extinction trials and reinstatement trials were also the same as the previous cohort. Finally, rats underwent a pellet-primed reinstatement trial, in which five sucrose pellets were delivered noncontingently at the beginning of the trial, which was otherwise identical to the extinction trials. Active and inactive responses were recorded.

Tissue processing

Brains from the COC reinstatement experiment were sectioned at 200 μm, and the mPFC and NAc were punched bilaterally according to Paxinos and Watson (2006). DNA and RNA were extracted using Tri-Reagent (Molecular Research Center) according to the instructions of the manufacturer.

Real-time RT-qPCR

RT-qPCR was performed as described previously (Hollis et al., 2011), with some modifications. Briefly, RNA was reverse transcribed using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). cDNA was analyzed in triplicates in a 384-well plate using a CFX thermal cycler (Bio-Rad), with SYBR Green as the fluorescent detector for the qPCR reaction. Nicotinamide adenine dinucleotide dehydrogenase (*NADH*) was used as a reference gene except when noted in Results. Results are depicted as percentage change from controls (SAL/SAL). For primer sequences, see Table 1.

Global DNA methylation

Genomic DNA samples were purified using QIAquick PCR Purification kit (Qiagen) and hydrolyzed using DNA Degradase Plus (Zymo Research) according to the instructions of the manufacturer. DNA electrophoresis was performed on a 1% agarose gel to confirm thorough digestion of samples (data not shown). C18 reverse-phase liquid chromatography separation followed by electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) was used to detect levels of 5-methyl-2′-deoxycytidine (5mdC) in the NAc and mPFC as described previously (Song et al., 2005). LC was performed using a Waters Symmetry C18 5 μm 0.180 × 20 mm trap column and a Waters HSS T3 1.8 μm 0.075 × 150 mm analytical column. The LC buffers were 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B). Two microliter injections were loaded on the trap column for 1 min at 15 μl/min 1% buffer B. Elution from the analytical column was at 400 nl/min using a gradient of 1% buffer B at run start, 50% buffer B at 5 min, 85% buffer B from 7–10 min, and 1% buffer B at 11 min. Samples were run in duplicate on a Xevo TQ-S Triple Quadupole mass spectrometer (Waters) using multiple reaction monitoring transitions of 228.2/112.2 [deoxycytidine (dC)], 242.1/126/3 (5mdC). 243.3/127.2 [thymidine (T)], 252.3/135.9 [2′-deoxyadenosine (dA)], and 268.1/152.3 [2′-deoxyguanosine (dG)], all at 3 ms dwell time, 20 V cone voltage, 0.20 bar spray gas, and 8 eV collision energy. Chromatograms were acquired and compared with a standard curve of calibration solutions containing 40 pmol/μl each of 5mdC + dC, dG, dA, and T (160 pmol/μl total) with 5mdC concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10% ($r = 0.9984$). Data are presented as percentage of 5mdC to total dC.

Bisulfite sequencing

Genomic DNA was bisulfite treated using EpiTect Bisulfite kit (Qiagen), and a portion of the c-*Fos* promoter from −163 to 16 bp was amplified using JumpStart REDTaq DNA Polymerase (Sigma), resulting in a PCR product of 236 bp containing 12 CpG dinucleotides (Dyrvig et al., 2012). Thirty nanograms of bisulfite-converted DNA were amplified as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 2.5 min, 72°C for 1 min; and 72°C for 5 min. The samples were run on a 1% agarose gel to confirm primer specificity. PCR products were then extracted and purified using GenElute Gel Extraction kit (Sigma) and sent to the sequencing facility of Florida State University, where samples were sequenced by capillary electrophoresis against the reverse primer. DNA standards of known methylation were processed alongside experimental samples, and a standard curve was derived to confirm accuracy of sequencing (EpigenDx). Chromatograms were analyzed, and the peak ratio was calculated as follows: $C/(C + T) \times 100$, to determine the percentage of DNA methylation at each of the 12 CpG dinucleotides and the average methylation across all CpGs.

Statistical analysis

For locomotor sensitization, two-way repeated-measures ANOVA was used with phase of experiment (habituation, SAL, or COC) or 10 min time bins as the within-groups factor and treatment (SAL or MET) as the between-groups factor. One-way repeated-measures ANOVA was used to compare treatment over time during the COC portion of the experiment. For the self-administration data, repeated-measures two-way ANOVA was used with trial as the within-subjects factor, and treatment (SAL or MET) and self-administration (SAL or COC) as the between-subjects factors. For reinstatement trials, paired two-tailed *t* tests were used to compare the respective extinction response of each group to reinstatement. Two-way ANOVA was used to analyze RT-qPCR, global methylation, and c-*Fos* methylation results with treatment and self-administration as the independent variables. Regression analysis was used to compare active responses during COC-primed reinstatement and c-*Fos* mRNA levels. GraphPad Prism 4.3 (GraphPad Software) and StatView 5.0.1 (SAS Institute) were used for data analyses. Data are depicted as mean ± SEM, and the level of significance was set to 0.05. *Post hoc* comparisons were made with Bonferroni's corrections when appropriate.

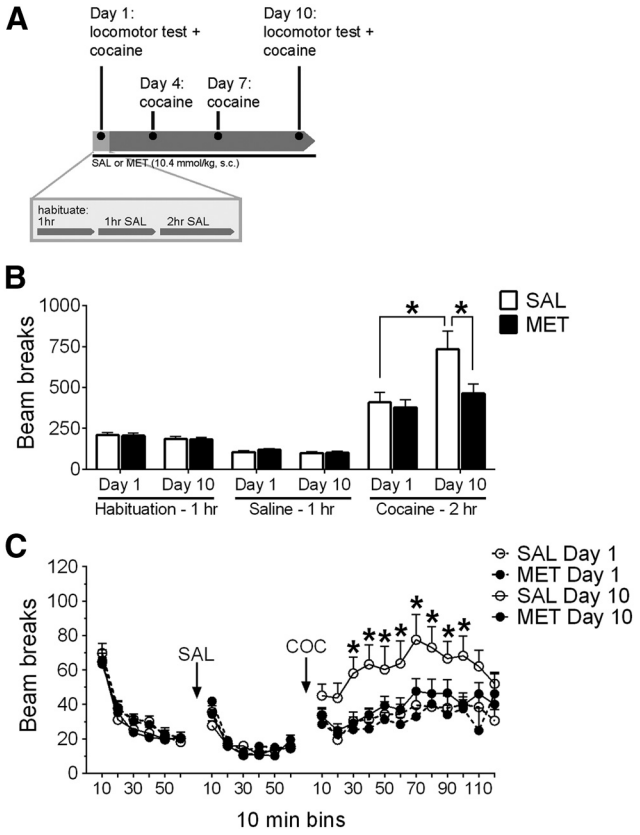

Figure 1. MET treatment blocks COC-induced locomotor sensitization. ***A***, The experimental timeline. ***B***, Locomotor activity in each phase of the experiment, measured by beam breaks. No differences were observed between SAL- and MET-treated rats on day 1 versus day 10 for the 1 h habituation or 1 h with SAL injection. On day 1 during the 2 h of COC, no differences were observed between SAL and MET. On day 10, SAL-treated rats exhibited a significantly higher locomotor response compared with their day 1, whereas MET-treated rats exhibited no differences between day 1 and day 10. ***C***, Locomotor activity in 10 min bins. SAL-treated rats had a significantly higher response on day 10 compared with day 1. Data are presented as mean ± SEM. *$p < 0.05$, Bonferroni's *post hoc* test, $n = 18–20$ per group.

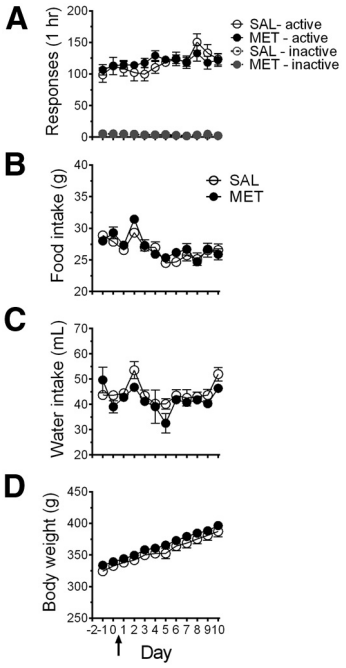

Figure 2. MET treatment had no effect on sucrose pellet self-administration and consummatory behaviors. ***A***, Active and inactive responses to sucrose pellets. Food intake (***B***), water intake (***C***), and body weights (***D***) were measured daily during 10 d of MET or SAL treatment and sucrose self-administration, and no differences were found. Data are presented as mean ± SEM. $n = 10$ per group. Arrow indicates onset of MET treatment.

Results

COC-induced locomotor sensitization

To determine the effects of methyl supplementation on the psychomotor-activating effects of COC, we administered 10.4 mmol/kg MET or SAL daily and 10 mg/kg COC every third day for 10 d (for the timeline, see Fig. 1*A*). We then examined total locomotor scores on the first and last days of treatment (Fig. 1*B*). One-way repeated-measures ANOVA indicated a significant increase in locomotor response to COC from day 1 to day 10 for SAL-treated rats ($F_{(1,19)} = 7.756, p = 0.012$) but not MET-treated rats ($F_{(1,17)} = 3.099, p = 0.096$), indicating that SAL-treated rats sensitized to COC but MET-treated rats did not. On day 1, two-way repeated-measures ANOVA indicated no significant effect of treatment on locomotor activity across the three phases of the experiment: habituation, SAL, and COC ($F_{(1,36)} = 0.044$, $p = 0.8360$, $n = 18–20$ per group). Day 10 two-way repeated-measures ANOVA indicated a significant interaction of treatment and phase of trial ($F_{(2,36)} = 4.678, p = 0.012, n = 18–20$ per group). Bonferroni's *post hoc* analysis showed that SAL-treated rats exhibited a significant increase in locomotor response compared with MET on day 10 during the 2 h COC measurement ($t_{(36)} = 4.242, p < 0.001$), indicating a heightened response to COC from SAL-treated, but not MET-treated, animals. Figure 1*C* shows 10 min time bins for each phase of the experiment. Two-way repeated-measures ANOVA indicated a significant interaction of time × treatment ($F_{(3,69)} = 3.198, p < 0.0001$). Bonferroni's *post hoc* analysis shows day 10 SAL as statistically significant from day 1 SAL, day 1 MET, and day 10 MET ($p < 0.05$).

Sucrose pellet acquisition, body weights, and food and water intake with MET treatment

To determine whether the effects of MET extend to sucrose pellet acquisition and whether there are potential side effects of MET, a separate cohort of rats were treated with MET for 10 d, and body weights along with food and water intake were measured daily during sucrose self-administration. There was a significant main effect of time ($F_{(1,198)} = 4.245, p < 0.001$) but no effect of treatment on active responses ($F_{(1,18)} = 0.081, p = 0.7796$, one-way repeated-measures ANOVA; Fig. 2*A*). Daily food intake is shown in Figure 2*B*. There was a significant main effect of day ($F_{(11,198)} = 19.07, p < 0.0001$) but no effect of MET treatment ($F_{(1,18)} = 0.414, p = 0.528$). Daily water consumption showed a main effect for day ($F_{(11,198)} = 5.847, p < 0.0001$) but no effect of treatment ($F_{(1,18)} = 1.729, p = 0.205$; Fig. 2*C*). Finally, there was a significant main effect of day ($F_{(11,198)} = 197.1, p < 0.0001$) but no effect of MET treatment ($F_{(1,18)} = 1.418, p = 0.249, n = 10$ per group) on body weights 1–2 d before and during 10 d of MET treatment (Fig. 2*D*).

The effects of MET on COC self-administration, extinction, and reinstatement

Given the ability of MET to attenuate the psychomotor-activating and place conditioning effects of COC (LaPlant et al., 2010), we hypothesized that MET treatment might also block the reinforcing properties of the drug. Thus, after completion of sucrose pellet training, we treated rats with either MET or SAL and

allowed them to self-administer COC (0.75 mg/kg per infusion) or SAL, followed by 10 d of extinction (timeline shown in Fig. 3*A*). There were no differences observed in sucrose pellet training before MET treatment (data not shown). During acquisition, COC self-administering rats had significantly higher rates of infusions than SAL self-administrating rats (two-way repeated-measures ANOVA, $F_{(3,64)} = 5.039$, $p = 0.0034$; Fig. 3*B*). A significant main effect of self-administration on active responses during acquisition was observed ($F_{(1,64)} = 9.718$, $p = 0.0027$), but no effect of MET was observed ($F_{(1,64)} = 0.148$, $p = 0.702$, $n = 11–23$ per group; Fig. 3*C*). Similarly, during extinction, we observed a significant main effect of active responses ($F_{(1,45)} = 11.761$, $p = 0.0013$) but no MET effect ($F_{(1,45)} = 0.050$, $p = 0.8170$). Notably, the number of inactive responses remain unchanged throughout the procedure ($F_{(1,61)} = 0.8132$, $p = 0.7366$). Therefore, MET treatment did not affect acquisition or extinction of COC self-administration.

After 10 d of extinction, cues were returned to the operant chambers for a single trial of cue-induced reinstatement (Fig. 3*D*). Although the number of active responses was significantly higher during the cue-induced reinstatement compared with the last day of extinction ($F_{(1,45)} = 27.72$, $p < 0.0001$, main effect of trial), no effect of treatment was observed ($F_{(1,45)} = 1.014$, $p = 0.395$, $n = 11–13$ per group), suggesting that MET did not affect cue-induced reinstatement. Additionally, all groups exhibited increased active responses during cue-induced reinstatement compared with their final extinction trial (SAL/MET, $t_{(20)} = 2.958$, $p = 0.0078$; COC/SAL, $t_{(24)} = 2.897$, $p = 0.0079$; and COC/MET, $t_{(26)} = 2.620$, $p = 0.0145$), with a trend toward significance for SAL/SAL ($t_{(20)} = 2.034$, $p = 0.0554$, two-tailed paired *t* tests), demonstrating that cue-induced reinstatement was observed regardless of MET treatment. After 2 d of extinction training to return their responses to baseline levels, rats were injected with COC (10 mg/kg, i.p.) immediately before the trial to assess drug-seeking behavior under COC-primed reinstatement (Fig. 3*E*). Two-way repeated-measures ANOVA revealed a significant main effect for trial ($F_{(1,42)} = 18.58$, $p < 0.0001$), treatment ($F_{(1,42)} = 2.96$, $p = 0.041$), and an interaction ($F_{(3,42)} = 2.908$, $p = 0.045$, $n = 11–13$ per group). Bonferroni's *post hoc* comparisons were performed to analyze pairwise comparisons by trial. There was a significant difference between SAL/SAL versus COC/SAL ($t = 3.483$, $p < 0.05$), SAL/MET versus COC/SAL ($t = 3.735$, $p < 0.001$), and COC/SAL versus COC/MET ($t = 2.759$, $p < 0.05$). Notably, although rats that self-administered SAL did not exhibit more active responses during COC-primed reinstatement compared with their final extinction trial ($t_{(20)} = 1.425$, $p = 0.1697$ for SAL/SAL; $t_{(20)} = 1.689$, $p = 0.1068$ for SAL/MET), there was a significant increase for rats that self-administered COC ($t_{(20)} = 2.722$, $p = 0.0131$ for COC/SAL; $t_{(24)} = 2.510$, $p = 0.0192$ for COC/MET). Together, rats that had self-administered COC reinstated to a priming injection compared with their responses during extinction; however, the degree of response was significantly lower in MET-treated animals, indicating that MET treatment attenuated COC-primed reinstatement.

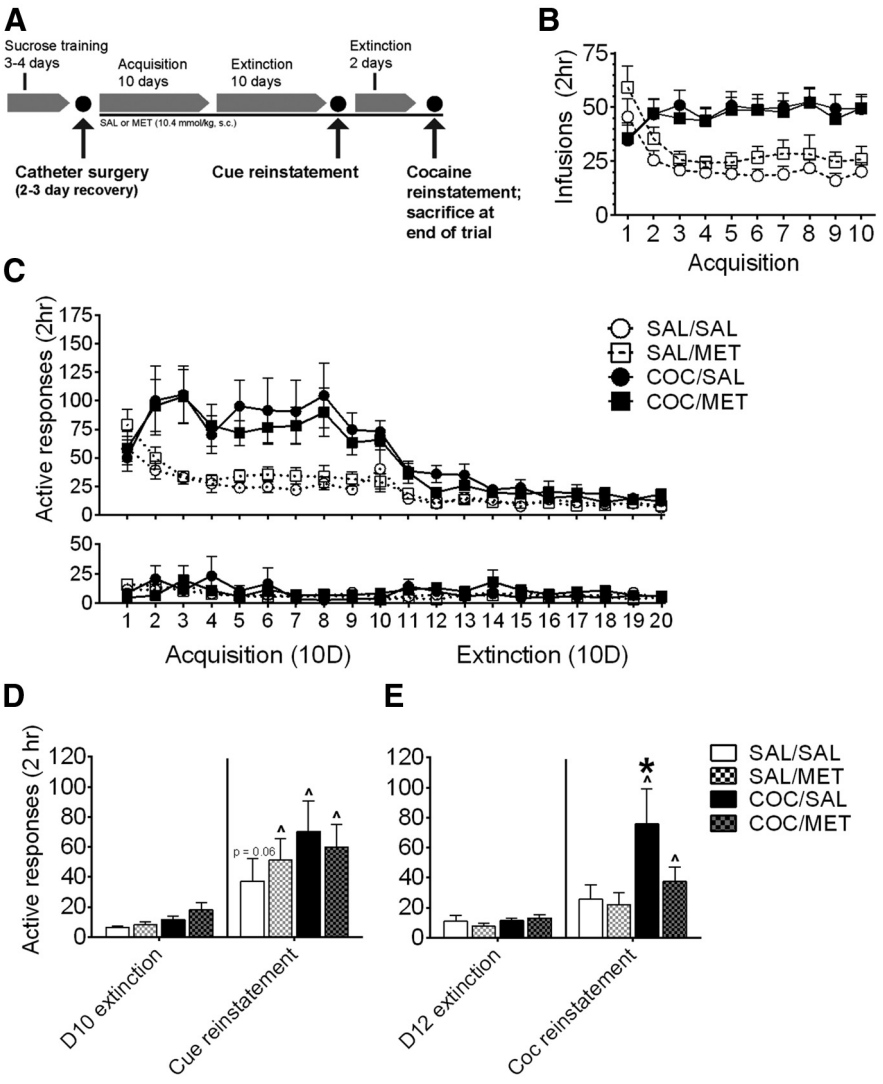

Figure 3. MET treatment effects on COC self-administration, extinction, and reinstatement. ***A***, Timeline of experimental design. ***B***, Number of infusions during 10 d of acquisition of COC or SAL self-administration. No effect of MET treatment was observed. ***C***, Number of active and inactive responses during 10 d of acquisition and 10 d of extinction. No effect of MET treatment was observed. ***D***, Active responses during the final extinction trial and the subsequent cue reinstatement trial. MET treatment had no effect on cue-induced reinstatement; all groups experienced an increase in active responses compared with day 10 extinction. ***E***, Active responses during the final extinction trial and the subsequent COC reinstatement trial. Whereas rats that self-administered COC reinstated to a COC prime, SAL-treated rats exhibited a significantly higher active response that was not observed in MET-treated rats. Data are presented as mean ± SEM. ^$p < 0.05$ compared with extinction, *$p < 0.05$ compared with SAL/SAL, Bonferroni's *post hoc* test. $n = 11–23$ per group for ***B*** and ***C***; $n = 11–13$ per group for ***D*** and ***E***.

The effects of MET on sucrose self-administration, extinction, and reinstatement

To assess the effects of MET on sucrose pellet reinstatement, rats underwent a similar procedure as in the COC self-administration experiment (for the timeline, see Fig. 4*A*). There were no initial differences observed in sucrose pellet training before MET or SAL

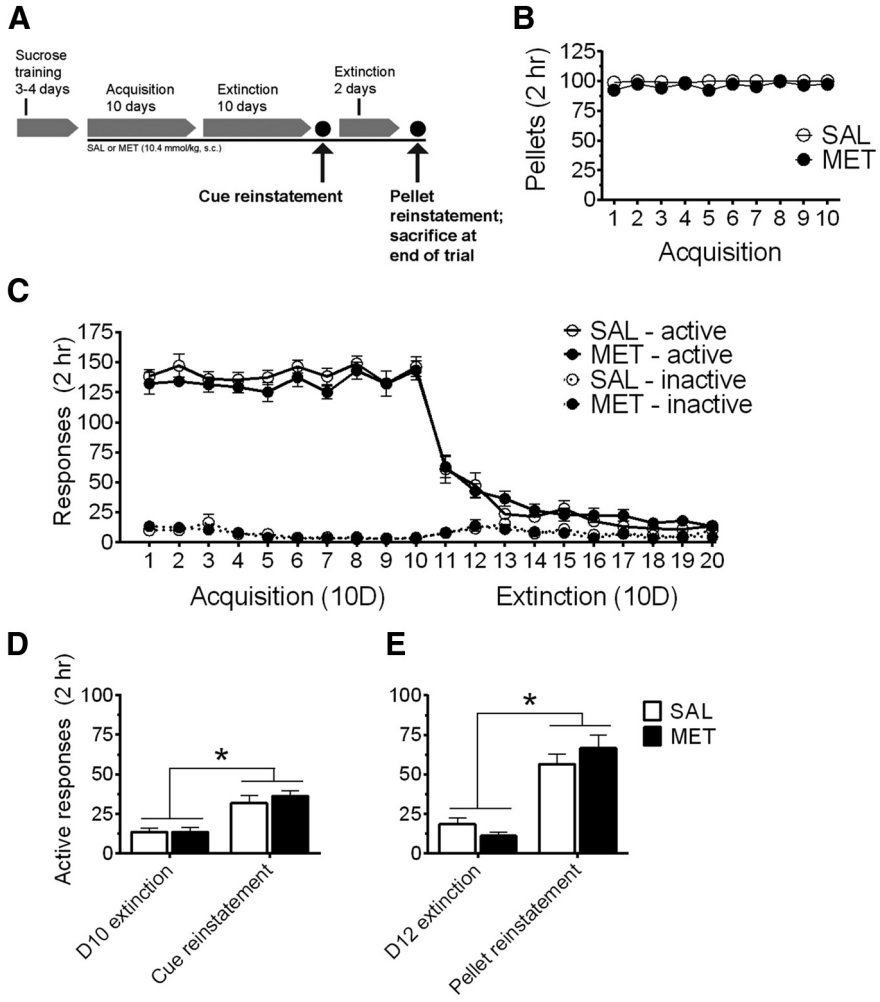

Figure 4. MET treatment has no effect on reinstatement to sucrose pellets after sucrose self-administration. ***A***, Timeline of experimental design. ***B***, Number of pellets taken during acquisition. ***C***, Active and inactive responses during 10 d acquisition and 10 d extinction. No differences were observed between SAL- and MET-treated rats. ***D***, Active responses during cue-induced reinstatement show that both groups reinstated to cues equally compared with their respective active responses on day 10 extinction. ***E***, Active responses during pellet-primed reinstatement show that both groups exhibited pellet-seeking behavior compared with their responses on day 12 extinction. Data were analyzed by two-way repeated-measures ANOVA and presented as mean ± SEM. $n = 10$ per group. *$p < 0.05$ compared with extinction trial.

treatment (data not shown). During acquisition for the number of pellets self-administered, there was a main effect of trial (two-way repeated-measures ANOVA, $F_{(1,9)} = 1.961$, $p = 0.0471$), but no effect of MET treatment was observed ($F_{(1,18)} = 3.201$, $p = 0.0904$; Fig. 4*B*). Similarly, for active responses during acquisition, there was a main effect of trial ($F_{(1,9)} = 2.252$, $p = 0.0213$), but no effect of treatment ($F_{(1,18)} = 1.218$, $p = 0.2843$; Fig. 4*C*). Inactive responses during acquisition revealed a main effect of trial ($F_{(1,9)} = 7.199$, $p < 0.0001$) but no effect of treatment ($F_{(1,18)} = 0.02782$, $p = 0.869$). For active responses during extinction, there was a main effect of trial ($F_{(1,9)} = 19.25$, $p < 0.0001$) but no effect of treatment ($F_{(1,18)} = 3.872$, $p = 0.4350$). Inactive responses during extinction revealed a main effect of trial ($F_{(1,9)} = 2.080$, $p = 0.0341$) but no effect of treatment ($F_{(1,18)} = 0.8445$, $p = 0.3703$). Together, MET treatment had no effect on sucrose pellet acquisition and extinction.

When comparing active responses on the last extinction trial with cue-induced reinstatement (Fig. 4*D*), there was a significant main effect of trial ($F_{(1,18)} = 37.82$, $p < 0.0001$) but no main effect of MET treatment ($F_{(1,18)} = 0.3230$, $p = 0.5789$, two-way repeated-measures ANOVA). There was a significant increase in active responses during cue-induced reinstatement compared with the last day of extinction for both SAL-treated rats ($t_{(9)} = 3.166$, $p = 0.0114$) and MET-treated rats ($t_{(9)} = 6.708$, $p < 0.0001$, $n = 10$ per group), indicating that both groups reinstated to cues. Unpaired *t* tests comparing SAL and MET cue-induced reinstatement shows no differences ($t_{(18)} = 0.7414$, $p = 0.4680$). For pellet-primed reinstatement (Fig. 4*E*), there was a significant main effect of trial ($F_{(1,18)} = 64.54$, $p < 0.0001$) but no main effect of treatment ($F_{(1,18)} = 0.05057$, $p = 0.8246$, two-way repeated-measures ANOVA). There was a significant increase in active responses during pellet-primed reinstatement compared with day 12 extinction for both SAL-treated rats ($t_{(9)} = 5.247$, $p = 0.0005$) and MET-treated rats ($t_{(9)} = 6.092$, $p = 0.0002$, $n = 10$ per group), indicating that both groups reinstated to a pellet priming. Unpaired *t* tests comparing SAL and MET pellet-primed reinstatement responses revealed no differences ($t_{(18)} = 0.9810$, $p = 0.3396$). Together, MET treatment had no effect on operant behaviors to obtain a natural reward, and thus its effects do not generalize to sucrose reward.

The effects of COC self-administration and MET on *Dnmt* expression

After the COC-primed reinstatement trial, rats were killed, and mRNA was extracted from the NAc and mPFC to measure mRNA levels of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* by RT-qPCR (Fig. 5*A*,*B*). In the NAc, whereas *Dnmt1* expression remained unaffected ($F_{(1,20)} = 0.0456$, $p = 0.8330$ for self-administration effect; $F_{(1,20)} = 0.4104$, $p = 0.5290$ for MET effect), *Dnmt3a* and *Dnmt3b* were upregulated in COC self-administering rats, regardless of SAL or MET treatment ($F_{(1,20)} = 9.265$, $p = 0.006$ and $F_{(1,17)} = 10.89$, $p = 0.004$ for self-administration effect, respectively; $F_{(1,20)} = 0.1043$, $p = 0.750$ and $F_{(1,17)} = 0.1674$, $p = 0.688$ for main effect of MET, respectively). In the mPFC, *Dnmt1* ($F_{(1,20)} = 1.310$, $p = 0.2658$ for self-administration main effect; $F_{(1,20)} = 0.9352$, $p = 0.3451$ for MET effect), *Dnmt3a* ($F_{(1,20)} = 0.1488$, $p = 0.7038$ for self-administration main effect; $F_{(1,20)} = 1.094$, $p = 0.3081$ for MET effect), and *Dnmt3b* ($F_{(1,20)} = 0.00243$, $p = 0.9612$ for self-administration main effect; $F_{(1,20)} = 0.1012$, $p = 0.7537$ for MET main effect, $n = 5–6$ per group) expression were unaffected. To account for possible regulation of NADH by COC exposure (del Castillo et al., 2009), we compared *NADH* against a different reference gene, hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*). No differences were observed ($F_{(1,18)} = 1.451$, $p = 0.2440$, self-administration main effect; $F_{(1,18)} = 0.0022$, $p = 0.9633$, MET effect, $n = 5–6$ per group), supporting that NADH is not regulated by the treatment. To further confirm our results, we normalized *Dnmt3a* against *Hprt1* and confirmed the upregulation of *Dnmt3a* mRNA in the NAc by COC self-administration ($F_{(1,18)} = 7.803$, $p = 0.0120$, main effect of self-

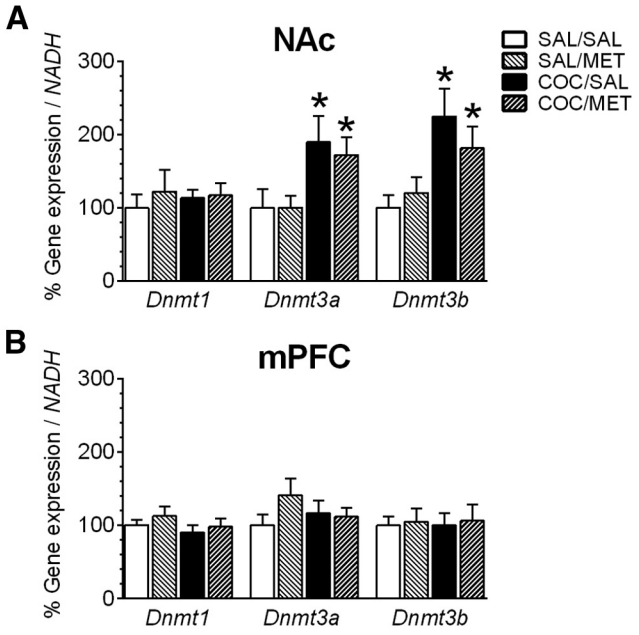

Figure 5. *Dnmt1*, *Dnmt3a*, and *Dnmt3b* mRNA levels assessed by RT-qPCR. ***A***, In the NAc, no differences were observed in *Dnmt1* mRNA levels. *Dnmt3a* and *Dnmt3b* were upregulated in rats that self-administered COC, independent of MET treatment. ***B***, In the mPFC, *Dnmt1*, *Dnmt3a*, and *Dnmt3b* levels were unchanged. Data were analyzed by two-way ANOVA and are presented as mean ± SEM, normalized to 100% of SAL/SAL. $n = 5–6$ per group. $^*p < 0.05$ compared with SAL.

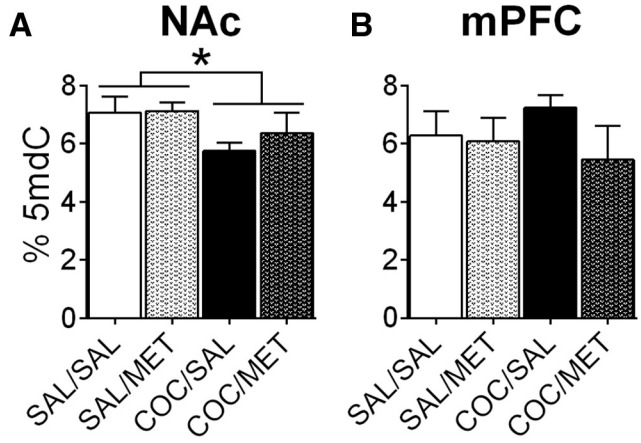

Figure 6. Global DNA methylation assessed by LC-ESI MS/MS. ***A***, Global DNA hypomethylation was observed in the NAc as a result of COC self-administration. ***B***, No differences were observed in the mPFC. Data were analyzed by two-way ANOVA and are presented as mean ± SEM, 5mdC. $n = 5–6$ per group. $^*p < 0.05$ compared with SAL.

administration; $F_{(1,18)} = 0.3405$, $p = 0.5668$, MET effect; $n = 5–6$ per group; data not shown). Together, this suggests that *Dnmt3a* and *Dnmt3b*, but not *Dnmt1*, mRNA levels were upregulated specifically in the NAc after COC self-administration, but remained unaffected by MET treatment.

Global DNA methylation

Because MET is a methyl donor for DNMTs (Ross, 2003), we investigated whether MET treatment resulted in changes in global DNA methylation. Using LC-ESI MS/MS, we determined the abundance of 5′-methylcytosine in genomic DNA from the NAc and mPFC of rats after COC-primed reinstatement. In the NAc, we observed a significant reduction in global DNA methylation in rats that self-administered COC ($F_{(1,20)} = 4.412$, $p = 0.048$, main effect of self-administration, $n = 5–6$ per group; Fig. 6*A*), but there was no effect of MET treatment ($F_{(1,20)} = 0.473$, $p = 0.499$). In the mPFC, global DNA methylation remained unchanged ($F_{(1,17)} = 0.03574$, $p = 0.8523$ for self-administration effect; $F_{(1,17)} = 1.353$, $p = 0.2607$ for MET effect, $n = 5–6$ per group; Fig. 6*B*).

Regulation of c-*Fos* mRNA levels and c-*Fos* promoter methylation

Because the immediate early gene c-*Fos* is induced by both acute COC exposure and during COC-primed reinstatement (Young et al., 1991; Kumar et al., 2005; Larson et al., 2010), we determined whether the blockade of COC-primed reinstatement by MET was associated with impaired c-*Fos* expression. We examined c-*Fos* mRNA levels after COC-primed reinstatement in the NAc and the mPFC. We observed a significant interaction between treatment and self-administration in both the NAc ($F_{(1,20)} = 4.587$, $p = 0.0447$, $n = 6$ per group) and the mPFC ($F_{(1,20)} = 4.362$, $p = 0.0497$, $n = 6$ per group; Fig. 7*A*). Bonferroni's *post hoc* analysis identified a significant increase in c-*Fos* expression between SAL/SAL and COC/SAL in the NAc and mPFC ($t = 2.305$, $p < 0.05$; $t = 2.514$, $p < 0.05$, respectively). Notably, the magnitude of active responses during the COC-primed reinstatement is correlated positively with c-*Fos* mRNA levels in both structures, although falling short to reach significance in the NAc [$r^2 = 0.1495$, df = 22, $p = 0.062$ in the NAc (Fig. 7*B*); $r^2 = 0.5158$, df = 22, $p < 0.0001$ in the mPFC (Fig. 7*C*)].

Figure 7*D* shows the sequence of the c-*Fos* promoter that was analyzed by bisulfite sequencing. Figure 7*E* shows the methylation state of the 12 CpGs at the c-*Fos* promoter in the NAc, with the inset showing the average of all 12 CpG dinucleotides. Consistent with mRNA levels, the c-*Fos* gene promoter exhibits an overall reduction of DNA methylation in the NAc after COC self-administration ($F_{(1,12)} = 6.835$, $p = 0.0226$), which is prevented by MET treatment ($F_{(1,12)} = 4.918$, $p = 0.0466$). This profile is observed throughout most of the individual CpG dinucleotides investigated. Of note, there was a significant effect of self-administration at the second CpG ($F_{(1,12)} = 10.10$, $p = 0.008$) and a significant effect of MET treatment at the third CpG ($F_{(1,12)} = 6.711$, $p = 0.0236$). Additionally, the ninth CpG had a significant interaction ($F_{(1,12)} = 6.203$, $p = 0.0284$); *post hoc* analysis indicates that COC/SAL is significantly lower than COC/MET ($t = 3.35$, $p < 0.05$). However, in the mPFC, no differences in DNA methylation were observed across the 12 CpG dinucleotides ($F_{(1,10)} = 1.065$, $p = 0.3264$, $n = 3–4$ per group; Fig. 7*F*, inset). Nevertheless, hypermethylation of the second CpG was observed as a result of MET treatment ($F_{(1,10)} = 9.267$, $p = 0.014$), regardless of COC self-administration. Together, these findings suggest that MET treatment might have reversed COC-induced DNA hypomethylation at specific CpGs to reduce c-*Fos* expression after COC reinstatement.

Discussion

In this study, we demonstrate that a methyl donor, MET, reduces behavioral sensitization to the locomotor-activating and drug-seeking effects of chronic COC. We show that MET blocks COC-induced c-*Fos* activation after reinstatement in two key brain regions responsible for drug-seeking behavior and relapse: the NAc and mPFC. Furthermore, COC pretreatment induced global hypomethylation in the NAc and decreased DNA methylation at the c-*Fos* promoter, which was blocked by MET treatment.

Drug-induced locomotor sensitization is defined as a progressive increase in locomotor activity when a subject is exposed re-

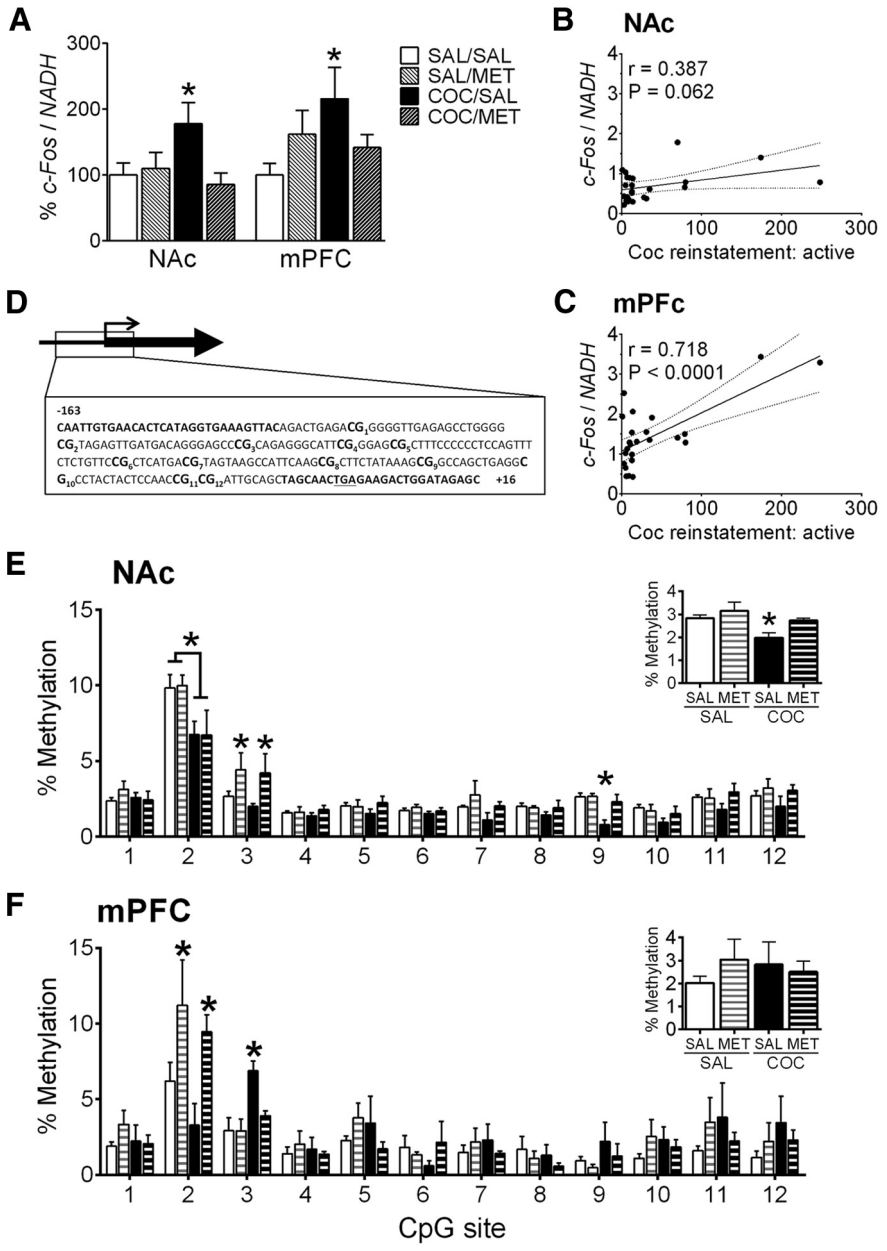

Figure 7. c-*Fos* expression, correlation with reinstatement, and promoter DNA methylation in the NAc and mPFC. ***A***, COC-primed reinstatement significantly upregulated c-*Fos* in the NAc and mPFC of rats that self-administered COC and were treated with SAL, but this expression was blocked in MET-treated rats. ***B***, A trend toward a correlation was observed between the magnitude of active nose-poke responses during COC-primed reinstatement and c-*Fos* activation in the NAc. ***C***, A significant positive correlation between the magnitude of active responses during COC-primed reinstatement and c-*Fos* in the mPFC was observed. ***D***, The sequence of the c-*Fos* gene promoter analyzed, with the 12 CpG dinucleotides in bold. ***E***, Percentage of DNA methylation observed at each CpG dinucleotide in the NAc. Inset, Average methylation across the 12 CpGs. In the NAc, COC self-administration hypomethylates the c-*Fos* promoter region, whereas MET treatment returns methylation levels to that of controls. At individual CpGs, the second one is hypomethylated in COC self-administered rats, the third is hypermethylated in MET-treated rats, and the ninth CpG is significantly hypomethylated in COC self-administered rats but MET treatment returns it to control levels. ***F***, In the mPFC, no differences in average methylation was observed (inset), but there was a significant increase in methylation of the second CpG as a result of MET treatment. Data were analyzed by two-way ANOVA with Bonferroni's *post hoc* comparisons when needed and are presented as mean ± SEM, normalized to 100% of SAL/SAL in ***A***. *$p < 0.05$ compared with SAL/SAL, $n = 5–6$ per group in ***A–C***, $n = 3–5$ per group in ***E*** and ***F***.

peatedly to a drug of abuse and is indicative of underlying reorganization of the mesocorticolimbic dopamine reward system (Steketee and Kalivas, 2011). MET treatment during the 10 d of COC exposure blocks locomotor sensitization, suggesting that MET could be preventing drug-induced neural reorganization. Although the effects of MET on sensitization to other rewarding compounds have not been investigated, conditioned place preference studies suggest that its inhibitory effects are not generalized to food reward or morphine (Tian et al., 2012). Thus, the effects of MET and, in turn, the contribution of DNA methylation may be specific to psychostimulants (Numachi et al., 2007; Jayanthi et al., 2014) and have less of an influence on the rewarding effects of opioid compounds and natural reward. This is not surprising, because a better behavioral and pharmacological overlap is observed between opioids and natural reward than COC and natural reward (Kelley et al., 2002; Romieu et al., 2008). We did not observe any differences in self-administration or reinstatement of sucrose pellets, in accordance with previous findings, suggesting that MET does not have a generalized effect on other natural motivated behaviors. Our sensitization results differ from those of Anier et al. (2013), who found that SAM increased locomotor sensitization to COC. This could be attributable to differences in species, behavioral procedures, and pharmacokintetics of SAM compared with MET. MET is actively transported across the blood–brain barrier, whereas SAM is synthesized in the brain from MET (Oldendorf and Szabo, 1976), and, in fact, systemic MET administration increases brain levels of endogenous SAM more effectively than systemic SAM (Young and Shalchi, 2005).

The main goal of our study was to examine how methyl supplementation affected drug-seeking behaviors. Animal reinstatement models using cues, drugs, and stress are indispensable tools for gaining insight on human addiction and relapse because of their high construct and face validity (de Wit and Stewart, 1981; De Vries et al., 1998; Shaham et al., 2003; Epstein et al., 2006). In our hands, although rats experienced with COC self-administration robustly reinstated to a priming injection of COC, MET treatment attenuated COC-seeking behavior without affecting acquisition, indicating that the blockade of COC-primed reinstatement cannot be attributed to differences in initial COC intake. Interestingly, the inhibitory effects of MET did not extend to cue-induced reinstatement, because all animals exhibited some degree of responding regardless of treatment. This could be attributable to the fact that reinstatement to cues involves recruitment of additional brain areas, including the basolateral amygdala (Di Ciano and Everitt, 2004; Ambroggi et al., 2008), that might not be affected by MET treatment. Furthermore, because all groups un-

derwent sucrose pellet training before MET treatment, the motivational incentive to self-administer had already been established before MET treatment began, as evidenced by the fact that SAL self-administrating rats also reinstated to cues (Fig. 3*D*). Therefore, it is possible that MET may only have an effect prophylactically and when alterations in DNA methylation occur, as is the case with repeated COC exposure.

It is compelling that MET treatment blocked both locomotor sensitization and COC-primed reinstatement because of the shared neural substrates mediating both behaviors. Repeated exposure to COC induces molecular and structural neuroadaptations in the reward circuitry that result in COC being assigned a higher incentive salience over other motivating stimuli, contributing to the habit-forming nature of the drug (Robinson and Berridge, 1993; Nestler, 2001). A sensitized locomotor response is one behavioral manifestation of these neuroadaptations, and others have shown that self-administration predicts subsequent sensitization (Hooks et al., 1994; Phillips and Di Ciano, 1996; Vezina, 2004) and vice versa (Piazza et al., 1990; Ferrario and Robinson, 2007). Furthermore, because sensitization is also observed in human patients (Leyton, 2007), comparing these two behavioral measures is critical to understanding the transition to addiction and the potential for relapse. It has also been shown that MET treatment attenuates conditioned place preference to COC, another commonly used index of drug reward (LaPlant et al., 2010; Tian et al., 2012). The fact that MET treatment blocks sensitization, conditioned place preference, and COC-primed reinstatement (but not cue-induced reinstatement, morphine conditioned place preference, or natural reward) suggests that DNA methylation may only mediate very specific COC-induced neuroadaptations. Identifying the exact molecular substrates for these changes will be an important task for future research.

In our hands, chronic COC pretreatment increased both *Dnmt3a* and *Dnmt3b* expression while inducing a globally hypomethylated state in the NAc (Figs. 5*A*, 6*A*, respectively). These findings raise the question of why increased *Dnmt3a* and *Dnmt3b* would result in a decrease in the product of its reaction. It is possible that COC-induced *Dnmt3a* and *Dnmt3b* increases might be a counter response to the global DNA hypomethylation, and the increased availability of methyl groups via MET treatment might enable DNMTs to methylate more DNA. Furthermore, a global decrease in DNA methylation is only indicative of a net hypomethylation, but there are likely numerous genes that are also hypermethylated in response to COC and/or MET, or not affected at all. In fact, SAM and MET treatment can alter expression of a very discrete population of genes without affecting global DNA methylation (Weaver et al., 2006; Anier et al., 2013), suggesting that, for MET treatment to have any effect on transcription, it requires careful coordination of the enzymatic machinery and CpG accessibility. This is especially relevant in postmitotic cells in which DNA methylation and DNMTs are not nearly as prevalent as in dividing cells (Bird, 2002). COC induces several alterations in the epigenetic landscape, including upregulation of *Dnmt3a* and *Dnmt3b*, which allows MET to exert the behavioral and molecular effects observed in this study, and perhaps explains why no effect of MET was observed in SAL or sucrose self-administering rats. Additionally, active DNA demethylation events come into play as well, as ten-eleven translocation 1, which is responsible for the conversion of 5-methylcytosine to the transcriptionally permissive 5-hydroxymethylcytosine, is regulated by learning and memory processes, associated cortical and hippocampal c-*Fos* expression, and exposure to COC (Rudenko et al., 2013; Feng et al., 2015).

In the current study, one of the genes affected by this COC-induced hypomethylation was c-*Fos*, the expression of which was increased in the NAc and mPFC and correlated with reinstatement of COC-seeking behavior. This enhanced c-*Fos* expression is likely, as seen in human addicts, indicative of enhanced activation of the reward circuits as a result of chronic COC exposure (Bannon et al., 2014). Similarly, there is a correlation between c-*Fos* induction and COC-primed reinstatement of conditioned place preference in mice (Brown et al., 2010), supporting the notion that neuronal activation is critical for the reinstatement of drug-seeking behaviors. Here, MET treatment normalized the COC-induced hypomethylation observed at the c-*Fos* gene promoter in the NAc. This restoration in DNA methylation likely contributed to the blockade of COC-primed c-*Fos* induction. Furthermore, it is possible that downstream transcriptional targets of the AP-1 complex (of which c-Fos is a component, along with c-Jun) are differentially activated, which is an intriguing point to address in future studies.

In the CNS, dynamic DNA methylation changes have been observed in models of learning and memory (Levenson, 2007; Miller et al., 2008; Feng et al., 2010), synaptic plasticity (Guo et al., 2011), and responsiveness to drugs of abuse (Anier et al., 2010; LaPlant et al., 2010; Tian et al., 2012; Pol Bodetto et al., 2013; Bodetto et al., 2014). Additionally, individuals with addiction exhibit changes in DNA methylation patterns in peripheral blood and brain (Nielsen et al., 2009, 2012; Zhang et al., 2013). This is the first study to demonstrate the inhibitory effects of chronic MET on behavioral sensitization and drug-seeking behavior, as well as gene expression in rats. We show that COC self-administration upregulates *Dnmt3a* and *Dnmt3b* in the NAc, regardless of MET treatment, but chronic MET treatment selectively increases methylation of the c-*Fos* promoter that COC exposure rendered transcriptionally permissive. At the histone level, COC alters markers of both euchromatin and heterochromatin, histone methyltransferase expression, regulation of micro-RNAs, and other posttranslational modifications (Kumar et al., 2005; Im et al., 2010; Maze et al., 2010, 2011). More research is needed to understand how histone modifications, DNA methylation, and DNA demethylation are orchestrated to regulate the behavioral and neurobiological adaptations that occur with chronic COC exposure. Relapse is the biggest hurdle to overcome on the path to recovery from addiction, and it is critical to gain a better understanding of the mechanisms behind relapse with the hopes of developing more targeted therapeutics. With these findings, we suggest additional investigation of MET as a potential treatment for COC addiction.

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