Published in final edited form as:

Neuropharmacology. 2018 October ; 141: 113–125. doi:10.1016/j.neuropharm.2018.08.030.

The histone demethylase KDM6B in the medial prefrontal cortex epigenetically regulates cocaine reward memory

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Abstract

Epigenetic remodeling contributes to synaptic plasticity via modification of gene expression, which underlies cocaine-induced long-term memory. A prevailing hypothesis in drug addiction is that drugs of abuse rejuvenate developmental machinery to render reward circuitry highly plastic and thus engender drug memories to be highly stable. Identification and reversal of these pathological pathways are therefore critical for cocaine abuse treatment. Previous studies revealed an interesting finding in which the mRNA of histone lysine demethylase, KDM6B, is upregulated in the medial prefrontal cortex (mPFC) during early cocaine withdrawal. However, whether and how it contributes to drug-seeking behavior remain unknown. Here we used a conditioned place preference paradigm to investigate the potential role of KDM6B in drug-associated memory. We found that KDM6B protein levels selectively increased in the mPFC during cocaine withdrawal. Notably, systemic injection of KDM6B inhibitor, GSK-J4, disrupted both reconsolidation of cocaine-conditioned memory and cocaine-primed reinstatement, suggesting dual effects of KDM6B in cocaine reward memory. In addition, we found that NMDAR expression and function were both enhanced during early cocaine withdrawal in mPFC. Injection of GSK-J4 selectively reversed this cocaine-induced increase of NR2A expression and synaptic function, suggesting that mal-adaptation of cocaine-induced synaptic plasticity in mPFC largely underlies KDM6B-mediated cocaine-associated memory. Altogether, these data suggest that KDM6B plays an essential role in cocaine-associated memory, which mainly acts through enhancing cocaine-induced synaptic

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Author Contributions

Y.C. Li, W.G. Gao and Y.X. Zhang designed the research; Y.X. Zhang performed the experiments and analyzed the data; R.C. Akumuo assisted with the Western blotting; Y.X. Zhang, Y.C. Li, and W.G. Gao wrote the manuscript; C.X. Yan supervised Y.X. Zhang and edited the manuscript, and R.A. España consulted on the project and edited the manuscript.

Conflict of Interests

The authors declare no conflicts of interest.

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plasticity in the mPFC. Our findings revealed a novel role of KDM6B in cocaine-associated memory and inhibition of KDM6B is a potential strategy to alleviate drug-seeking behavior.

Keywords

KDM6B; NR2A; memory reconsolidation; cocaine-primed reinstatement; prelimbic cortex

Introduction

Epigenetic regulation underlies drug-induced adaptations. Post-translational modification of histone and dynamic changes of chromatin remodeling proteins alter the expression of specific genes in the brain's reward circuitry, and thus mediate the actions of drugs of abuse (Ajonijebu *et al*, 2018; Godino *et al*, 2015; Jangra *et al*, 2016; Maze *et al*, 2010; Qiang *et al*, 2011; Renthal *et al*, 2007). An attractive scenario of drug addiction is the rejuvenation hypothesis, in which drugs of abuse elicit developmental and/or even embryonic mechanisms to produce abnormally robust and durable forms of memories associated with addiction (Dong and Nestler, 2014; Huang *et al*, 2015).

Recent evidence shows histone lysine demethylase, KDM6B, is significantly increased during cocaine withdrawal in the mPFC (Li *et al*, 2017; Sadakierska-Chudy *et al*, 2017); however, the mechanisms involved in this change are unknown. KDM6B, also known as one of the JmjC domain-containing proteins (JMJD3), catalyzes the removal of methyl marks from histone 3 at lysine 27 (H3K27). The degree of methyltransferases and demethylases will change the balance between mono-(me1), di-(me2) and trimethyl (me3) marks, thus dynamically altering the chromatin structures and regulating gene expression. Methylation of H3K27 is generally associated with gene repression. By removing di- or tri-methylation at histone3 lysine 27 (H3K27me2/3), KDM6B interacts with either promoter regions or enhancer elements to regulate a variety of critical genes involved in development, neurogenesis (Park *et al*, 2014), neuronal differentiation and plasticity, and cancer (Dai *et al*, 2017).

Compared with the nucleus accumbens (NAc) which is extensively studied in the field of drug addiction, the role of mPFC in cocaine addiction is understudied. Excitatory inputs from the mPFC provide major glutamatergic projections to the NAc, which contributes to drug priming-, cue-, context- and stress-induced drug relapse and craving (Capriles *et al*, 2003; Gipson *et al*, 2013a; McFarland and Kalivas, 2001; Parrilla-Carrero *et al*, 2018). Inhibition of PFC also blocks cocaine-induced reinstatement (Schmidt *et al*, 2005).

Repeated cocaine exposure generated silent synapses that are characterized by N-methyl-D-aspartate receptor (NMDAR)-mediated synaptogenesis during the early phase of withdrawal (Graziane *et al*, 2016; Ma *et al*, 2014). NMDARs are heteromeric ion channels containing NR1 and NR2 subunits, and the NR2A/2B subunit composition can substantially alter the properties of synaptic transmission (Quinlan *et al*, 1999), thus altering the cocaine-induced memory. Besides, up-regulated NR2A and NR2B, which are usually accompanied with synaptic plasticity, contribute to cue-induced relapse to nicotine use (Gipson *et al*, 2013b).

However, most of the efforts highlighted the key role of NR2B in synaptic plasticity in both cocaine addiction and development, leaving the NR2A subunit uncharacterized.

We report here that a histone lysine-specific demethylase, KDM6B, is significantly increased in the prelimbic (PL) area of mPFC after 3 days of withdrawal from repeated cocaine exposure. Importantly, GSK-J4, a selective inhibitor of KDM6B, not only inhibited cocaine-conditioned memory reconsolidation but also diminished cocaine-primed reinstatement. Additionally, NR2A but not NR1 or NR2B protein levels in mPFC coincidentally increased with the change of KDM6B during the early withdrawal. Consistently, NMDAR/AMPA ratio and NMDA-mediated current recorded from layer 5 pyramidal neurons were enhanced by cocaine exposure while GSK-J4 not only selectively prevented the increase of NR2A protein expression but also reduced cocaine-induced enhancement of NR2A-mediated synaptic transmission. Given that NR2A is a key player in silent synapse genesis and drug-induced memory, KDM6B likely acts as an endogenous contributor to withdrawal development in the cocaine-exposed mPFC.

Materials and Methods

Animals

Male C57BL/6 mice (8–12 weeks old) were purchased from Jackson Laboratory and provided by the animal center at Drexel University College of Medicine. Four animals were housed each cage in a colony room with controlled ambient temperature ($25 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$) and under a regular (12/12 h) light-dark cycle with access to food and water *ad libitum*. All protocols and animal procedures were performed in accordance with the National Institutes of Health Guide for care and use of laboratory animals under the supervision of the Institutional Animal Care and Use Committee at Drexel University College of Medicine.

Drugs and drug treatment

Cocaine hydrochloride (obtained from the National Institute on Drug Abuse) was prepared in saline with two different concentrations either for conditioning or reinstatement. Mice received either vehicle or GSK-J4 (0.5 mg/kg, i.p.; Abcam, ab144395, Cambridge, MA) daily and 30 min before each test or immediately after the retrieval test (specified in each experiment). GSK-J4 was prepared daily, in saline containing 2% DMSO (Sigma-Aldrich, St Louis, MO) and mixed at room temperature for at least 1 h before use. Saline containing 2% DMSO used to prepare GSK-J4 was used as a vehicle. Euthasol (2.5 ml/kg, Virbac, Fort Worth, TX) was used for anesthesia of animals.

Conditioned Place Preference (CPP) Apparatus and Procedure of Cocaine-Induced CPP

Cocaine reward was examined using the CPP paradigm. The CPP apparatus consisted of a rectangular 3-chamber Plexiglas box. Two conditioning chambers ($L \times W \times H$: 15 cm $\times$ 15 cm $\times$ 15 cm) were identical except that the walls and floor of one chamber were patterned with a black and rectangular net; while the other was patterned with white walls and perforated holes on the floor. Providing distinct tactile and visual cues, the two chambers were separated from the neutral chamber by two doors. Patterns were counterbalanced

across the left and right chambers, and drug pairing was further counterbalanced across both side and pattern. Transparent Plexiglas lids allowed recording the experiments on a video system.

Procedure: An unbiased design was used to determine the CPP for cocaine (Shaw *et al*, 2017). In the pre-test, mice were allowed access to both chambers for 15 min, and the time spent in each chamber was recorded. Animals demonstrating 15% preference for one chamber in pre-test were removed from the study. During the conditioning phase, mice received either intraperitoneal (i.p.) 20 mg/kg cocaine or saline (Contarino *et al*, 2017; Fumagalli *et al*, 2007; Itzhak and Martin, 2002; Malvaez *et al*, 2010) and were confined to one chamber for 30 min. The mice were then put back in their home cage. After 8 hr, mice were injected with either cocaine or saline which they had not previously received and were immediately put in the other chamber for another 30 min. The conditioning phase continued for 4 consecutive days with 2 pairings per day, one for each side, alternating saline and cocaine as the first injection each day. Animals that only received saline injection served as control groups among behavior, molecular and electrophysiological experiments. CPP was assessed on the withdrawal days (WD) 5 (WD 5) and 7 (WD 7) and was determined by the amount of time spent in each chamber over a 15-min period in a drug-free condition. On WD 14, mice received post-test 3 immediately after the saline or cocaine (10 mg/kg) i.p. injection for reinstatement (McFarland *et al*, 2003). The schedule of cocaine-induced behaviors and drug administration are shown in Figure S1.

The time spent in each chamber of the CPP apparatus and total distance traveled were tracked automatically from Moving Pictures Experts Group video using EthoVisionXT software (EthoVision 13; Noldus Information Technology, Wageningen, The Netherlands) (Malvaez *et al*, 2010). The videos were recorded using digital video cameras mounted above the CPP chambers.

Western Blotting and Analysis Procedure

Brain tissues containing the mPFC, nucleus accumbens and hippocampus were collected for Western blotting. Tissues were dissected and homogenized in ice-cold lysis buffer. Homogenates were incubated on ice for 30 min and then centrifuged at 10,000 *g* for 15 min at 4°C. Supernatants were collected and stored at –80° C until use. Sample protein levels were measured using a bicinchoninic acid (BCA) protein assay, and then normalized to microgram per microliter with laemmli sample buffer. The same amount of loading proteins (15 µg) were separated on acrylamide gels comprised of a 3.0% acrylamide stacking gel on top of a 7.5% acrylamide separating gel, and then transferred to pore size 0.45 µm polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA).

Membranes were blocked with 5% nonfat milk and then probed for anti-rabbit KDM6B (RRID:AB_2722742; ABCAM, AB154126, 1 : 1000, Cambridge, MA), anti-mouse GluR1 (RRID: AB_11212678; EMD-Millipore, MAB2263, 1 : 2000, Temecula, CA), anti-mouse GluR2 (RRID: AB_10806492; EMD-Millipore, MABN71, 1 : 2000), anti-mouse NR1 (RRID:AB_2533060; Invitrogen, 32–0500, 1 : 5000, Carlsbad, CA), anti-rabbit NR2A (RRID:AB_1163481; EMD-Millipore, 04–901, 1 : 4000), anti-mouse NR2B

(RRID:AB_417391; EMD-Millipore, 05–920, 1 : 2000), and anti-mouse actin (RRID:AB_476743; Sigma, A5316, 1 : 100 000, St Louis, MO), which was used as a loading control.

For histone proteins, tissues from the mPFC, NAc, and hippocampus were also collected as described above. The EpiQuik Total Histone Extraction Kit (OP-0006, EpiGentek, Farmingdale, NY, USA) was used to isolate the histone fraction. The same BCA protein assay was used to determine protein concentration. After boiling in laemmli buffer for 5 min, 15 µg of protein were loaded on acrylamide gels comprised of a 3.0% acrylamide stacking gel on top of a 15% acrylamide separating gel. After electrophoresis, blots were transferred to pore size 0.20 µm PVDF membranes (Millipore). After blocking, membranes were separately probed with anti-mouse H3K27me3 (RRID: AB_305237; Abcam, ab6002, 1: 2000) and total anti-rabbit H3 (RRID: AB_10001790; Novus, NB500–171, 1: 100000, Littleton, CO) with anti-H3 as a loading control. All the blots were incubated with horseradish peroxidase-coupled anti-rabbit or anti- mouse IgG secondary antibody (Vector Laboratories), and proteins were visualized by chemiluminescence (western blotting detection reagents, Amersham Biosciences, Buckinghamshire, UK).

Densitometry of each protein was evaluated by the Image-J (NIH). Additionally, samples from each animal were run at least 4 times to minimize interblot variance. Raw values were normalized to actin or H3.

Histology and Quantification

On WD 7, after anesthetization, all saline- and cocaine-treated mice (4 mice for each group) were perfused with 0.01M PBS followed by 4% paraformaldehyde. Brains were then removed, fixed overnight, transferred to 30% sucrose, and stored at 4°C. Coronal sections (25 µm) containing mPFC were cut in a cryostat and antigen retrieval was executed by incubation with 10 mM sodium citrate (pH 6.5) in a 60°C water bath for 20 min (Hanlon *et al*, 2016). After washing in 0.01M PBS, sections were pretreated in PBS containing 30% methanol and 10% H₂O₂ for 15 min at room temperature. After washing in PBS, sections were then blocked in 5% normal goat serum, 0.3% Triton X-100 in PBS for 1 hour. After blocking, sections containing mPFC were incubated with primary antibody anti-rabbit KDM6B (RRID: AB_2722742; ABCAM, AB154126, 1: 1000) for 48 hr at 4°C.

After incubation with primary antibody, sections were washed in PBS, incubated with Alexa Fluor® 488 secondary antibodies (goat anti-rabbit IgG, JacksonImmuno Research, 1:500, West Grove, PA) for 2 hr at room temperature, washed again in PBS, mounted onto slides, and coverslipped with mounting medium with DAPI (Vector). Images of stained sections (4 equivalent sections per animal) were captured on Olympus DP71 and Leica DM5500B epifluorescent microscopes. Sections were stained at the same time and images of the mPFC (40x magnification) were taken with the same exposure time. Using Image-J (NIH), an intensity threshold was applied to each image to include the positive labeling while minimizing the inclusion of non-specific, background staining in sections. Immunoreactive cells were counted by an experimenter blinded to the treatment condition.

Electrophysiology

Animals were sacrificed immediately after the post-test 2. Coronal slices containing the PL-mPFC were cut (300 μ m thick) using a vibratome (VT1200S, Leica Microsystems) and placed into a bath of oxygenated ice-cold sucrose solution (in mM: 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 0.5 CaCl₂, 7.0 MgSO₄, 213 sucrose, pH 7.4). Slices were transferred to oxygenated artificial cerebrospinal fluid (aCSF; in mM: 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgSO₄, 26 NaHCO₃, and 10 dextrose, pH 7.4) at 35 °C for 1 h and then held at room temperature until used for recording. Slices were transferred into a recording chamber mounted on an Olympus upright microscope (BX51), where they were bathed in oxygenated aCSF. Neurons were visualized with optics of infrared differential interference contrast (IR-DIC) and a digital video camera system. Whole-cell recordings in brain slices were performed as previously described (Li *et al*, 2016; Xing *et al*, 2016). Briefly, EPSCs were recorded using patch electrodes with an open tip resistance of 5–7 M Ω (internal solution, in mM: 110 D-gluconic acid, 110 CsOH, 10 CsCl₂, 1 EGTA, 1 CaCl₂, 5 QX-314, 1 ATP-Mg, 10 HEPES, at pH 7.4 adjusted with CsOH). All experiments were conducted with Axon MultiClamp 700B amplifier (Molecular Devices) and data were acquired using pCLAMP 9.2 software. In voltage-clamp experiments, evoked excitatory postsynaptic currents (EPSCs) in layer 5 pyramidal neurons were elicited by stimulating layer 2/3 with a single pulse (0.1 ms, 10–100 μ A, 10 s inter-stimulus interval) through a bipolar electrode and recorded in aCSF containing the GABA_A receptor antagonist picrotoxin (50 μ M). The peak amplitudes of EPSCs recorded at –60 mV were directly measured to represent AMPA receptor-mediated current because the opening of NMDA receptor channel at this potential was negligible. We also recorded EPSCs in the same neuron using the same stimulation intensity while holding at +60 mV.

Data Analysis

For physiological recording, data were analyzed using Clampfit 10.2 (Molecular Devices). Only the connections giving stable EPSCs without rundown for at least 5 min were considered for further analysis of drug and stimulus effects. For AMPA, the evoked EPSC amplitudes were measured by averaging 30 traces from the onset to peak of EPSCs; for NMDA, the evoked EPSC amplitudes were measured at 4 different time points (25 ms, 50 ms, 75 ms and 100 ms after the peak of the EPSCs) after average. Measuring different time points allows us to determine NMDA receptor subunit-mediated EPSC component. Specifically, the decay time of NR2A-mediated current is usually within 50 ms while decay time of NR2B-mediated current is analyzed 50 ms after the EPSC peak at +60 mV (Cull-Candy *et al*, 2001; Wang *et al*, 2008).

For behavioral tests, 6–10 animals each group were used. The CPP scores refer to (the time spent in the cocaine-paired chamber during the post-test session) – (time spent in the cocaine-paired chamber during the pre-test session). For the mice who received no cocaine injection, either white or black chamber is defined as the “drug”-paired chamber, and the calculation time for pre-test and post-test are from the same chamber. One-way ANOVA or two-way ANOVA analyses were performed using IBM SPSS statistics 24. For results with significant interaction effects in two-way ANOVA, simple effect test was conducted for further analysis. For results without significant interaction effects, Bonferroni’s post-hoc test

or Student's t-test was further conducted as needed. To determine differences between two groups, a Student's t-test was performed. All values were expressed as mean $\pm$ SEM. Correlation analyses were conducted using Pearson's simple linear correlations (Pearson r) with IBM SPSS statistics. The significance level was set at $P < 0.05$.

Results

Cocaine increased KDM6B protein levels specifically in the mPFC in early withdrawal

To examine a possible role for KDM6B in cocaine withdrawal, we first examined the post-withdrawal expression of KDM6B in the mPFC. For 4 consecutive days, mice were injected intraperitoneally (i.p.) with cocaine (20 mg/kg) or saline, and sacrificed at six varying time points (2 hr, 24 hr, 3, 7, 14, and 30 days) following the last cocaine injection (Fig. 1A). As there is no difference across saline groups at each time point, representative Western blots only show the 2 hr group (Fig. 1B). Statistical analyses (Supplementary Results 1) showed that KDM6B was significantly increased at withdrawal day (WD) 3, WD 7, and WD 14 compared with their corresponding saline groups (Fig. 1B). These results show that KDM6B protein levels remained constant after cocaine injection until WD 3, after which it reached its highest level at WD 7, and then returned to saline levels at WD 30. These findings suggested that KDM6B is preferentially activated by early drug withdrawal (before WD 30).

Next, we tested if this increase also occurred in other brain regions which are closely related to drug-induced memory and have direct interaction with mPFC among the reward circuitry. Mice received 4 consecutive days of i.p. injection of cocaine or saline and were sacrificed after 7 days of withdrawal (Fig. 1C) as WD 7 had the highest expression of KDM6B in the mPFC. Statistical analyses (Supplementary Results 2) showed a marked mPFC-specific increase in the expression of KDM6B, but not in the NAc or hippocampus (Fig. 1D). These findings suggest that the increase of KDM6B protein is both temporal and brain-region specific.

Thus far, we have demonstrated an overall increase of KDM6B protein in the mPFC. However, different subregions of the mPFC in rodents are highly specialized and individually receive distinct inputs and exert top-down control over behaviors through varied outputs. For instance, the prelimbic PFC (PL) promotes drug-induced memory (Ma *et al*, 2014) whereas the infralimbic PFC (IL) is believed to attenuate the original memory and promote extinction of drug reward memory (Augur *et al*, 2016; Huang *et al*, 2018; Otis *et al*, 2014).

Hence, to distinguish the expression difference in the subregions of the mPFC, we conducted immunostaining and examined KDM6B-positive neurons on WD 7 (Fig. 2A). Quantitative analyses showed a significant increase of KDM6B-positive neurons in the PL region after cocaine exposure compared with saline groups ($t_{14} = 1.628$, $p < 0.05$, t -test; Fig. 2B-C&F). Further, cocaine increased cell numbers in both Layer 2/3 and Layer 5/6 (Supplementary Results 3 & Table S1 PL).

In contrast to the increase of KDM6B-positive cell number in the PL, cocaine treatment did not change the cell number in the IL region ($t_{10} = 0.3537$, $p > 0.05$, t -test; Fig. 2D-E&G).

Further laminar quantification of the KDM6B-positive cells in the IL indicated no significant effect of cocaine treatment (Supplementary Results 3 & Table S1 IL). These data suggested that the KDM6B protein expression was specifically increased in the PL rather than IL. These findings suggest that the KDM6B is related to drug memory given the critical role of PL in drug-associated memories.

KDM6B inhibition had no effect on memory retrieval but decreased cocaine-associated memory reconsolidation

To identify the function of KDM6B, we used its selective inhibitor (GSK-J4, Supplementary Discussion 1) and performed CPP (Supplementary Discussion 2) to measure its effect on drug-induced memory. Briefly, systemic injections of GSK-J4, which were aimed to induce erasure of the cocaine-conditioned memories, were administered before (to target retrieval) or after (to target reconsolidation) of post-test 1, but not the post-test 2 (Figure S1). After 4 days of conditioning, mice received 2 post-tests on WD 5 and WD 7 (Fig. 3A). As KDM6B was significantly increased from WD 3, GSK-J4 was administered daily during WD 3–7 (Donas *et al*, 2016). GSK-J4 injection had no effect on the post-test 1 (Supplementary Results 4), suggesting that activation of KDM6B was not required for retrieval of cocaine-induced memory. However, GSK-J4 significantly attenuated the conditioned memory during the post-test 2, while the inhibitor itself had no effect on the CPP score (Supplementary Results 4, Fig. 3B). These findings indicate that KDM6B activity is required during cocaine-conditioned memory reconsolidation (after retrieval).

We next examined the potential effects of GSK-J4 on basal locomotor activity and found no significant effect on total locomotion during post-test 2 (Fig. 3C and Supplementary Results 5). These results corroborate our findings in Fig. 3B, further demonstrating that KDM6B inhibition during reconsolidation is able to attenuate memory strength without influencing exploration in the test apparatus.

To exclude the possibility that the attenuation of drug-induced memory is due to extensive inhibition of GSK-J4 (5 injections), we next injected the inhibitor only after retrieval (Fig. 3D). Consistently, this injection regimen also significantly decreased the cocaine-induced CPP during the post-test 2 (Supplementary Results 6), suggesting that KDM6B inhibition specifically blocked the reconsolidation (Fig. 3E).

KDM6B inhibition attenuates cocaine-primed reinstatement

Activation of prefrontal cortical glutamatergic neurons critically contributes to cocaine-primed reinstatement (McFarland *et al*, 2003), so we next examined the potential effect of KDM6B inhibition on cocaine-induced reinstatement. After 4 consecutive days of cocaine conditioning, the post-test 1 and post-test 2 were similarly performed as described above (Fig. 3F). We found that 3 injections of GSK-J4 had no effect on post-test 1, which was indicated by insignificant effects of treatment (saline, priming cocaine) ($F_{(1,21)} = 1.516$, $p = 0.2293$), inhibitor ($F_{(1,21)} = 0.0110$, $p = 0.9172$), nor significant interaction between the two factors ($F_{(1,21)} = 0.0860$, $p = 0.7716$, Fig. 3G post-test 1). Two-way ANOVA analysis on post-test 2 indicated significant effect of inhibitor ($F_{(1,21)} = 11.02$, $p = 0.0031$), but not treatment ($F_{(1,21)} = 0.0711$, $p = 0.7922$) or interaction between the two factors ($F_{(1,21)} =$

0.3131, $p = 0.5814$). T test showed that GSK-J4 injection significantly decreased the conditioned memory during the post-test 2 ($p < 0.05$, cocaine+vehicle+saline vs cocaine+GSK-J4+saline; and $p < 0.05$, cocaine+vehicle+cocaine vs cocaine+GSK-J4+cocaine, Fig. 3G post-test 2). For the seven days following post-test 2, mice were left undisturbed with no behavioral testing or cocaine exposure. On WD 14, mice were treated with a saline or cocaine priming (10 mg/kg) injection. Two-way ANOVA analysis indicated significant effects of treatment (saline, priming cocaine) ($F_{(1,21)} = 9.326$, $p = 0.0060$) and inhibitor ($F_{(1,21)} = 7.379$, $p = 0.0129$), and significant interaction between the two factors ($F_{(1,21)} = 5.865$, $p = 0.0246$). Simple effect test showed that cocaine priming dramatically increased CPP compared to mice without cocaine injection ($p < 0.01$, cocaine+vehicle+saline vs cocaine+vehicle+cocaine) and GSK-J4 injection (during reconsolidation) significantly attenuated the preference score during post-test 3 ($p < 0.01$, cocaine+vehicle+cocaine vs cocaine+ GSK-4+cocaine, Fig. 3G post-test 3).

As our findings and previous study consistently reported that early drug intervention not only disrupted CPP memory right after injection, but also attenuated long-term memory (Maze *et al*, 2010), next we performed correlative analysis of CPP scores between post-test 2 and post-test 3. Our results showed a strong positive correlation between memory reconsolidation and reinstatement within cocaine+vehicle+cocaine group ($r = 0.6053$, $p = 0.0130$, Fig. S3A), but not cocaine+GSK+cocaine group ($r = -0.3052$, $p = 0.3615$, Fig. S3B). These results indicated that cocaine-induced long-term memory is dependent on early reconsolidation.

KDM6B inhibition with systemic GSK-J4 injection following cocaine-CPP test targets H3K27me3 and exhibits brain-region specificity

Our results thus far have demonstrated the significant increase of KDM6B after cocaine withdrawal and inhibition of its activity dramatically attenuated cocaine-induced memory. After CPP post-test 2, we immediately sacrificed all animals and collected brain tissues. Notably, the increase of KDM6B in the mPFC is similarly observed after the conditioning and CPP test (data not shown to avoid redundancy), suggesting that the KDM6B change is caused by cocaine withdrawal, independent of behavior. To further explore the mechanisms underlying how GSK-J4 disrupted cocaine-associated memories, we examined the repressive histone mark H3K27me3 which is the substrate of KDM6B (Fig. 4A). Two-way ANOVA comparing the protein levels among the 4 groups revealed significant effects of both cocaine and inhibitor but not interaction between cocaine and inhibitor effect ($F_{(1, 24)} = 6.576$, $p = 0.0170$, inhibitor factor; $F_{(1, 24)} = 11.86$, $p = 0.0021$, cocaine factor; $F_{(1, 24)} = 3.868$, $p = 0.0609$, interaction). T-test showed that H3K27me3 was significantly decreased after cocaine withdrawal ($p < 0.01$). Further, when we injected GSK-J4 after withdrawal, it dramatically blocked the cocaine-induced decrease ($p < 0.05$) (Fig. 4B). This inhibitory effect of GSK-J4 is unlikely the general effect on H3K27me3 because chronic injection of GSK-J4 did not alter basal H3K27me3 levels in saline-treated mice ($p > 0.05$) (Fig. 4B). These findings suggest that KDM6B may regulate the cocaine-induced memory by demethylating H3K27me3.

However, previous studies also reported a weaker effect of GSK-J4 on other KDMs such as KDM5B and KDM5C (Heinemann *et al*, 2014; Kruidenier *et al*, 2012), raising a concern of GSK-J4's specificity in inhibiting KDM6B. As both KDM5B and KDM5C catalyze the removal of methyl groups from trimethylated lysine 4 of histone H3 (H3K4me3) (Klein *et al*, 2014; Rondinelli *et al*, 2015), we also measured histone mark H3K4me3 (Fig. 4B). Two-way ANOVA comparing the protein levels among the 4 groups revealed no significant effects of cocaine, inhibitor, or interaction between cocaine and inhibitor effect ($F_{(1, 20)} = 0.3394$, $p = 0.5683$, inhibitor factor; $F_{(1, 20)} = 1.643$, $p = 0.2181$, cocaine factor; $F_{(1, 20)} = 0.1497$, $p = 0.7039$, interaction). This finding suggested that GSK-J4 might exhibit a specific inhibitory effect on KDM6B/H3K27me3 pathway.

To determine whether the effects of systemic GSK-J4 are acting at other cocaine-reward related brain regions, we also measured H3K27me3 in NAc and hippocampus. Two-way ANOVA comparing the protein levels among the 4 groups revealed no significant effects of cocaine, inhibitor, or interaction between cocaine and inhibitor effect ($F_{(1, 14)} = 0.1185$, $p = 0.7358$, inhibitor factor; $F_{(1, 14)} = 0.135$, $p = 0.7188$, cocaine factor; $F_{(1, 14)} = 0.2746$, $p = 0.6085$, interaction) in the NAc; similarly, there was no significant effects ($F_{(1, 15)} = 1.184$, $p = 0.2937$, inhibitor factor; $F_{(1, 15)} = 1.807$, $p = 0.1989$, cocaine factor; $F_{(1, 15)} = 0.09799$, $p = 0.7586$, interaction) in the hippocampus (Fig. 4C). These findings localized the role of GSK-J4 function specifically to the mPFC.

The increase of KDM6B coincides with the increase of NR2A, but not NR2B subunit during early cocaine withdrawal

Cocaine-induced synaptic plasticity in the mPFC is highly associated with the strength of drug-associated memories (Otis and Mueller, 2017). Our results show that the increase of KDM6B occurs primarily in the early phase of withdrawal, a time point characterized with an increase of NMDA receptor-containing silent synapses in the mPFC and NAc (Graziane *et al*, 2016; Ma *et al*, 2014). Therefore, using the same treatments (Fig. 5A), we next examined changes in NMDA receptor subunits, including NR2A and NR2B, which are most dynamic throughout cocaine withdrawal. We chose mice (age 8–12 week) to ensure that the development *per se* has no effect on the NMDA receptor (Monaco *et al*, 2015), and this was also demonstrated by Western blotting results from saline injection group (Fig. 5B). Statistical analyses (Supplementary Results 7) showed that NR2A protein increase was barely detectable at 24 hr, and its amount started to increase from WD 3, a level that persisted until WD 30. By comparison, NR2B protein increases were detectable at 24 hr, and progressively increased until WD 14, after which it declined to saline levels (Fig. 5B).

These results indicated that dynamic changes in NR2A coincided with KDM6B during early withdrawal, which suggested that KDM6B affects cortical synaptic plasticity via regulating NR2A.

KDM6B inhibition during reconsolidation specifically reversed cocaine-induced increase of NR2A protein and NR2A-mediated currents in the mPFC

Considering that the KDM6B regulation is highly gene-specific, we then quantified the glutamatergic receptor subunit expression after the post-test 2. During the early phase of

cocaine withdrawal, we did not observe significant changes in GluR1, GluR2 or NR1 subunit in mPFC whole cell lysates (Fig. 6A; Supplementary Results 8 & 9 and Discussion notes 3).

Two-way ANOVA analysis comparing protein levels of NR2A showed a significant effect of cocaine and inhibitor ($F_{(1, 25)} = 6.564$, $p = 0.0168$, cocaine factor; $F_{(1, 25)} = 5.515$, $p = 0.0271$, inhibitor factor) without an effect on the interaction ($F_{(1, 25)} = 2.506$, $p = 0.1260$). T-test showed that KDM6B inhibition specifically blocked the NR2A increase ($p < 0.05$, cocaine+GSK-J4 vs cocaine+vehicle), but it had no effect on the NR2A levels of cocaine naïve animals ($p > 0.05$, saline+GSK-J4 vs saline+vehicle). These findings suggested that GSK-J4 specifically blocked cocaine-induced NR2A protein increase (Fig. 6B). To determine whether this inhibitory effect of GSK-J4 on the cocaine-induced increase of NR2A is correlated with GSK-J4-mediated disruption of memory reconsolidation, we performed correlative analysis between the decrease of NR2A protein expression level and the decrease of CPP memory strength. Our results showed a strong correlation between the two parameters ($r = 0.6371$, $p = 0.0481$, Fig. S4).

In contrast, two-way ANOVA analysis comparing protein levels of NR2B showed a significant effect of cocaine ($F_{(1, 20)} = 16.99$, $p = 0.0005$), without an effect on the inhibitor factor or interaction between cocaine and inhibitor ($F_{(1, 20)} = 0.2509$, $p = 0.6219$, inhibitor factor; $F_{(1, 20)} = 0.04739$, $p = 0.8299$, interaction). T-test showed that KDM6B inhibition showed no significant effect on the cocaine-induced NR2B increase ($p > 0.05$, cocaine +GSK-J4 vs cocaine+vehicle). These findings suggested that GSK-J4 had no effect on the NR2B protein expression (Fig. 6B).

Thus far, our studies have demonstrated that the KDM6B modulated the NMDA receptor protein expression following cocaine exposure. To characterize the synaptic function of NMDAR changes during cocaine-mediated memory reconsolidation, mice were sacrificed immediately after the behavior on WD 7 (Fig. 7A) for patch-clamp recordings (Fig. 7B&C). We recorded evoked EPSCs in layer 5 pyramidal neurons of the PL region by using a single pulse stimulation of layer 2/3 in a voltage-clamp mode in the presence of picrotoxin, with the first evoked current amplitude at a range between 50–100 pA in a holding potential of -60 mV. In the same cell, we then recorded evoked EPSCs with a holding potential of $+60$ mV (Fig. 7B). This allowed us to compare the NMDA/AMPA ratio, the AMPA-EPSC amplitude, and the NMDA-EPSC amplitude among 3 groups of animals (Snyder *et al*, 2013; Wang *et al*, 2008). One-way ANOVA analysis indicated a significant effect of group ($F_{(2,14)} = 8.111$, $p = 0.0046$) and *post hoc* analyses showed significantly higher NMDA/AMPA ratio in neurons from cocaine+vehicle group compared with neurons from saline+vehicle ($p < 0.05$) and cocaine+GSK-J4 group ($p < 0.05$, Fig. 7D). In contrast, we found that neither cocaine nor GSK-J4 had an effect on AMPA receptor currents, as one-way ANOVA revealed no effect on evoked AMPA-mediated EPSCs ($F_{(2,41)} = 0.0287$, $p = 0.9717$, Fig. 7E).

Next, we measured NMDA receptor transmission at 50 ms after the peak of evoked EPSCs at $+60$ mV. One-way ANOVA analysis indicated a significant effect of group ($F_{(2,33)} = 6.414$, $p = 0.0044$) and *post hoc* analyses showed significantly higher amplitude in neurons from cocaine+vehicle group compared with neurons from saline+vehicle ($p < 0.05$) and

cocaine+GSK-J4 group ($p < 0.05$, Fig. 7F). These results suggested that GSK-J4 following cocaine exposure specifically modulated NMDA receptor-mediated currents, but not AMPA receptor-mediated currents.

To further determine which NMDAR subunit contributes to the inhibitory effect of GSK-J4, we measured the evoked EPSCs at different time points as NR2A-mediated current usually decays within 50 ms whereas NR2B-mediated current decays much slower (Cull-Candy *et al*, 2001; Wang *et al*, 2008). Two-way ANOVA analysis showed a significant effect of treatment ($F_{(2, 135)} = 8.755$, $p = 0.0003$) and time ($F_{(3, 135)} = 10.41$, $p < 0.0001$), without significant interaction effect ($F_{(6, 135)} = 0.6218$, $p = 0.7126$, Fig. 7G). One-way ANOVA analysis showed a significant effect of treatment ($F_{(2, 38)} = 5.034$, $p = 0.0115$) at 25 ms and ($F_{(2, 33)} = 6.414$, $p = 0.0044$) at 50 ms. Bonferroni's *post-hoc* test showed that GSK-J4 significantly decreased cocaine-induced amplitude increase at both 25 ms and 50 ms after the peak ($p < 0.05$), but not 75 ms and 100 ms ($p > 0.05$). As both NR2A and NR2B make a contribution during 25–50 ms while only NR2B is involved 50 ms after the peak, it is reasonable to speculate that the inhibitory effect of GSK-J4 following cocaine exposure is via modulating NR2A subunits.

Discussion

In this study, we identified that the H3K27me3 demethylase, KDM6B, was increased specifically in the mPFC with concomitant protein decrease of H3K27me3 during the early phase of cocaine withdrawal. Importantly, GSK-J4, a selective KDM6B inhibitor, attenuated cocaine-conditioned memory. Furthermore, GSK-J4 not only specifically prevented the decrease of H3K27me3 but also reversed cocaine-induced increase of NR2A expression and function in the mPFC. Thus, our data demonstrated a key role of KDM6B in drug-associated memory in the forebrain, which may be through modulation of NR2A expression and function by demethylation of H3K27me3, providing a novel molecular link between addictive drugs and epigenetic modification in the mPFC (Fig. S2).

KDM6B is temporally and brain-region specifically increased during early cocaine withdrawal

KDM6B is demonstrated to be critically involved in the neurogenesis and development processes. For instance, embryonic stem cells repress developmental genes by utilizing H3K27me3, and this methylation is removed in a tissue- and cell-specific manner during differentiation and KDM6B is directly involved in this embryogenesis (Burchfield *et al*, 2015). Furthermore, the adult subventricular zone (SVZ) neural stem cells (NSC) also require the KDM6B to regulate the chromatin state via either gene promoters or neural transcriptional enhancers, thereby activating neurogenic gene expression (Park *et al*, 2014). Apart from its critical role in development and neurogenesis, KDM6B has also been found in neural plasticity. However, this fundamental neurogenesis-related enzyme has never been highlighted in drugs of abuse. A prevailing view in the addiction field is that drugs of abuse engage normal developmental mechanisms which render the adult differentiated neurons highly plastic, thus driving stereotyped and stable memories. Based on this scenario and the

emerging evidence that the mRNA was increased after cocaine withdrawal (Li *et al*, 2017; Liu *et al*, 2018; Sadakierska-Chudy *et al*, 2017), KDM6B might be one of those candidates.

As our results showed, KDM6B is indeed increased specifically in the mPFC after repeated cocaine exposure, which is consistent with the microarray analysis and RNA-seq data (Li *et al*, 2017; Sadakierska-Chudy *et al*, 2017). Our histology results further demonstrated that the protein level increased (cell number) with subregion specificity (PL vs IL) (Fig. 2). This brain region-specific upregulation began from WD 3 until WD 14, which coincided with NMDA receptor increase initiation (Fig. 5B). Interestingly, a recent study showed that the mRNA of KDM6B is increased transiently after retrieval test on the first day of cocaine withdrawal in the dorsal hippocampus, suggesting the involvement of KDM6B in memory reconsolidation (Liu *et al*, 2018). This finding is very interesting, as the temporal sequence of KDM6B upregulation in hippocampus (first day after withdrawal) versus mPFC (3 days to 14 days after withdrawal) reinforced the system consolidation theory that memories are initially stored in the hippocampus (recent memory) and, over time, slowly consolidated and reconsolidated into the neocortex permanently (remote memory) (Kitamura *et al*, 2017). Thus, the temporal context in which KDM6B modulates NMDA receptor subunits may be restricted to a critical time window during the early phase of cocaine withdrawal. These data, therefore, identify a treatment time window and KDM6B inhibitor, GSK-J4, as a promising candidate for targeted clinical interventions in cocaine use disorder.

The epigenetic marker H3K27me3 is associated with transcriptional repression. In agreement with previous reports showing that KDM6B specifically demethylates H3K27me3 (Agger *et al*, 2007; Burchfield *et al*, 2015; Hong *et al*, 2007; Jones *et al*, 2018), we observed that cocaine also decreased this repression mark (H3K27me3), rather than H3K4me3, during early withdrawal. Blocking KDM6B with GSK-J4 specifically increased the H3K27me3 expression (Fig. 4), indicating that KDM6B functions in H3K27me3-dependent manner in the context of cocaine withdrawal.

Previous studies demonstrated that histone modifications are directly involved in cocaine-dependent expression of specific genes, thus affecting the behavioral responses (Malvaez *et al*, 2010). For example, H4 hyperacetylation at the *cFos* promoter occurred after single cocaine injection, whereas chronic, but not acute, cocaine-induced H3 hyperacetylation at the *BDNF* and *Cdk5* promoters (Kumar *et al*, 2005), suggesting that histone modification is gene-, context- or regimen-specific. This could explain the discrepancy between our study, which shows that 4 consecutive days of noncontingent cocaine decreased H3K27me3 at WD 7, and previous PCR results (Sadakierska-Chudy *et al*, 2017) that did not observe changes in H3K27me3 at 12–14 days of cocaine exposure. Of course, the different time points measured (3 days vs 7 days after withdrawal), animal species and dosage regimen may also contribute to this inconsistency.

The early phase of drug withdrawal is characterized by dynamic changes of NMDA receptors which shape the drug-induced memory. Previous studies extensively reported the NR2B molecular pathways in the context of drug addiction and development, leaving the other NMDA receptor subunit, such as NR2A largely unexplored. Here we reported that both NR2A protein expression and function were enhanced, coincident with the increase of

KDM6B in mPFC during early cocaine withdrawal. Notably, inhibition of KDM6B not only reversed the decrease of histone suppressive mark H3K27me3 but also attenuated the elevated NR2A expression and function following cocaine exposure. These pronounced effects strongly suggest that H3K27me3-mediated epigenetic modulation is involved in regulating NR2A. However, how exactly H3K27me3 regulates NR2A is still unclear. One prediction is that H3K27me3 directly targets the promotor of *Grin2a* to manipulate the NR2A gene expression. Our recent study reported a H3K27me3 enrichment in NMDA receptor subunits in the mPFC of naïve animals by using chromatin immunoprecipitation (CHIP) assay (Gulchina *et al*, 2017). Regarding cocaine withdrawal, future CHIP assay might reveal this possibility. Alternatively, a previous study also reported that KDM6B may partly promote NMDA receptor gene expression by regulating BDNF (Wijayatunge *et al*, 2018). Therefore, H3K27me3 may target other genes to indirectly modulate NR2A expression and function by affecting NR2A protein synthesis/ degradation and/or phosphorylation.

Notably, we observed that when GSK-J4 was administered in saline-injected mice, there was no clear effect on H3K27me3 expression. This finding is consistent with a previous study which reported that systemic GSK-J4 treatment significantly reduced the growth of mouse brainstem tumors and extended animal survival, with increased cell H3K27me3 positivity. In contrast to K27M tumors, wild-type H3.3-expressing cells showed no response to GSK-J4 treatment (Hashizume *et al*, 2014). Similarly, another study showed that systemic administration of an HDAC inhibitor facilitated CPP extinction only in mice exposed to cocaine-paired chambers, not for saline-conditioned chambers (Malvaez *et al*, 2010). Since histone methylation is a dynamic process, GSK-J4-induced inhibition in KDM6B might be offset by its corresponding lysine methyltransferase (KMTs), leading to a globally balanced steady-state H3K27me3 in the saline injected animals. However, repeated cocaine exposure dramatically upregulated the protein expression of KDM6B (Fig. 1), and as a consequence, the homeostatic regulation was disrupted, thus leading to the decreased H3K27me3. At the same time, increased KDM6B potentially provides more modulation sites, therefore more vulnerable to GSK-J4 treatment, thereby promoting the recovery of H3K27me3 expression. However, we cannot rule out the possible activity changes of KMTs, but H3K27me3 changes may provide some indirect evidence that KDM6B outweighs KMTs in the mPFC. The well-kept balance of H3K27me3 in saline only animals also explained why there was no effect of GSK-J4 on behavior.

On the contrary to NR2A, the NR2B subunit is upregulated 24 hours after last cocaine injection, earlier than the KDM6B increase at WD 3, suggesting that *Grin2b* gene is not a major target of KDM6B/H3K27me3 during early cocaine withdrawal. It is, however, puzzling that the KDM6B declined to saline levels after WD 30 whereas NR2A levels remain high after WD 30, suggesting that KDM6B is only responsible for activating NR2A subunit production during early withdrawal. Further studies are needed to determine whether this inconsistency reflects major mechanistic differences between the early trigger or initiation of NR2A and its later maintenance, as this may potentially represent a switch in synaptic plasticity that converts silent synapses from the early phase of withdrawal into functional ones in the late phase of withdrawal.

Role of NR2A in the mPFC in reconsolidation of cocaine conditioned memory and reinstatement

Previous studies have shown that specific glutamate receptors are modulated independently in different memory processes. Multiple observations reveal that NR2A- and NR2B-containing NMDARs in the basolateral amygdala (BLA) have dissociable roles in memory restabilization and destabilization, respectively, while AMPA receptors are only required for memory retrieval (Milton *et al*, 2013). Additionally, both BLA and PFC are crucial for consolidation, retrieval or expression of Pavlovian fear memory (Kitamura *et al*, 2017). Although mounting evidence has established that basic machinery underlying normal synaptic plasticity are subjected to cocaine-induced modifications within specific pathways (Huang *et al*, 2009), the direct link between cocaine-conditioned memory and the specific molecular substrates (e.g. NMDA subunits) remains unclear. This gap was bridged by our correlative analysis between the decreased CPP memory strength and the decreased NR2A protein expression by GSK-J4 injection (Fig. S4). Besides, this result is reminiscent of another cocaine study, in which decreased cocaine-induced locomotor sensitization in CREB-binding protein knockout mice is correlated with the decreased histone acetylation (Levine *et al*, 2005). Taken together, our working hypothesis that GSK-J4 modulates cocaine-conditioned memory via epigenetic regulation on NR2A is in agreement with these previous findings.

It is noteworthy that our histology data suggested that the KDM6B is specifically increased in the prelimbic area rather than infralimbic area. Extensive evidence demonstrated that prelimbic cortex is critical for drug-induced memory (Otis *et al*, 2017), as opposed to infralimbic cortex which promotes extinction reward memory. This may imply that KDM6B pathway is related to cocaine memory. Indeed, our results demonstrated the role of cocaine-induced epigenetic remodeling leading to the alteration of reconsolidation and reinstatement of cocaine CPP memory. Inhibition of KDM6B did not affect the memory retrieval. First, this indicates that the development- or neurogenesis-related enzyme, *per se*, does not convey intrinsic pro- or anti-addiction information. Second, as KDM6B specifically targets on NR2A, it has no effect on the AMPA receptors (Fig. 6), which may account for the unaffected retrieval of cocaine-induced memory. As reconsolidation can only occur when a memory is retrieved (Nader, 2003), when GSK-J4 was injected after the retrieval, it dramatically blocked the conditioned memory (Fig. 3B). This was further confirmed when GSK-J4 was only injected during the reconsolidation (Fig. 3E), thus excluding the possibility of memory impairment induced by repeated inhibitor injections.

As silent synapse-related pathological change is amenable to neurogenesis-related pharmacotherapy (KDM6B) on memory reconsolidation, we hypothesized that blocking memory reconsolidation will also affect the reinstatement of drug memory at the later time point. Indeed, we found that KDM6B inhibition during the reconsolidation dramatically attenuated cocaine-induced reinstatement (Fig. 3G). Thus, it is plausible that the cocaine-induced long-term memory is dependent on the early reconsolidation. This prediction is consistent with the findings that histone deacetylase (HDAC) inhibitors facilitate extinction and attenuate reinstatement of cocaine-induced conditioned place preference (Malvaez *et al*, 2010; Miller and Marshall, 2005). The correlation between early reconsolidation and

reinstatement is further strengthened by our correlative analysis between post-test 2 and post-test 3 (Fig. S3A) and a previous human study in which erasing or disruption of memory reconsolidation abolished a memory trace, therefore blocking long-term memory reinstatement (Agren et al, 2012).

GSK-J4 has shown much potential in the clinic for cancer treatment (Dai *et al*, 2017; Grasso *et al*, 2015; Hashizume *et al*, 2014; Li *et al*, 2018; Ntziachristos *et al*, 2014; Yan *et al*, 2017). This drives us to mimic clinic application on animals with i.p. injection. Although systemic administration may affect peripheral and many brain regions, GSK-J4's effects on CPP behavior should mainly be via inhibition of KDM6B in the mPFC for the following reasons. First, previous *in vivo* brain study has shown that GSK-J4 is blood-brain barrier permeable and takes effect in the brain with systemic injection in mice (Hashizume *et al*, 2014), and cocaine-induced increase of KDM6B was only observed in the mPFC region but not in the NAc or hippocampus. Second, systemic administration of GSK-J4 has no effects on the basal level of H3K27me3 but can prevent the cocaine-induced decrease of H3K27me3. Besides, we observed that GSK-J4 affects H3K27me3 only in the mPFC but not in the NAc and hippocampus (Fig. 4C), same as cocaine-induced upregulation of KDM6B only in the mPFC (Fig. 1D). Consistently, systemic administration of GSK-J4 specifically reversed cocaine-induced increase of NR2A protein expression and NR2A-mediated currents in layer 5 pyramidal neurons in the PL region of the mPFC, indicating that GSK-J4 normalized cortical synaptic plasticity which may greatly contribute to drug-associated memory. Taken together, our data strongly suggested that systemic GSK-J4 sufficiently disrupted cocaine-induced memory and synaptic plasticity mainly through inhibiting KDM6B in the mPFC.

In summary, we demonstrated that KDM6B is involved in regulating cocaine-associated memory via modulating cortical synaptic plasticity, therefore, KDM6B may be a novel target for the development of tactics to treat cocaine abuse. In the future, it is interesting to further explore how KDM6B epigenetically modulates NR2A. In addition, as segregated cell populations are intermingled in the mPFC and different drugs may bias specific cell types, future studies may focus on addressing and disentangling the cell-type and/or pathway-specific contribution (e.g., dopamine D1 vs D2 receptors) to cocaine abuse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We would like to thank all the members of our laboratory for help and stimulating discussions, especially to Dr. Bo Xing for very kind suggestions. This study was supported by the National Nature Science Foundation of China (NSFC 81471820, 81172903) to C.X. Yan, Commonwealth Universal Research Enhancement (CURE) Program to Y.C. Li, NIH R01D A031900 to R.A. España, as well as R01MH085666 and R21MH110678 to W.J. Gao.

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Highlights

1. KDM6B protein is selectively increased in the mPFC during cocaine withdrawal.
2. Inhibiting KDM6B decreases cocaine-conditioned memory reconsolidation.
3. Inhibiting KDM6B attenuates cocaine-primed reinstatement.
4. Inhibiting KDM6B specifically blocks the NR2A expression and function.

Fig. 1. KDM6B protein levels are specifically increased in the mPFC independent of behavior after repeated cocaine exposure.

(A) A timeline illustrating the experimental design for cocaine or saline treatment prior to sacrifice at 6 varying time points. Cocaine (20 mg/kg i.p.) was injected daily in the home cage without behavioral testing during days 1 to 4. Underlined numbers represent the days animals were sacrificed. (B) Representative Western blots and summary graph show the KDM6B protein levels in whole cell fractions at 2 hr, 24 hr, WD 3, WD 7, and WD 14 after saline- and cocaine-exposure. Dashed square highlights the dynamic changes of KDM6B during early withdrawal. ** $p < 0.01$ and *** $p < 0.001$ vs corresponding saline group. (C) A

timeline illustrating the experimental design for cocaine or saline treatment prior to sacrifice at WD 7. Cocaine (20 mg/kg i.p.) was injected daily in the homecage without behavioral testing during days 1 to 4. Underlined numbers represent the days animals were sacrificed. (D) Representative Western blots and summary histograms showing the KDM6B protein levels in whole cell fractions from the mPFC, NAc, and hippocampus at WD 7 after saline- and cocaine-exposure. *** $p < 0.001$ vs. corresponding saline group. $n = 4\text{--}6$ mice/group.

Fig. 2. Cocaine induces a specific increase of KDM6B in the PL but not the IL region of the mPFC.

(A) A timeline illustrating the experimental design for the treatment of mice with cocaine or saline prior to sacrifice on WD 7. Cocaine (20 mg/kg) was daily i.p. injected at homecage without behavior during days 1 to 4. Underlined numbers represent the days animals were sacrificed. (B) Approximate dissection site and image taken area (green rectangular) in the PL. (C) Representative images of immunostaining for KDM6B (in green) and counterstaining with DAPI (in blue) in the PL of the mPFC 7 days after cocaine or saline

treatment. Scale bars, 20 μm . **(D)** Approximate dissection site and image taken area (green rectangular) in the IL. **(E)** Representative images of immunostaining for KDM6B (in green) and DAPI (in blue) in the IL of mPFC 7 days after cocaine or saline treatment. Scale bars, 20 μm . **(F)** and **(G)** Summary histograms showing the number of KDM6B-positive neurons in the PL/IL regions. Cg1: cingulate cortex area 1; fmi: forceps minor of the corpus callosum; M1: primary motor cortex; M2: secondary motor cortex; PL: prelimbic cortex; IL: infralimbic cortex. $*p < 0.05$ vs corresponding saline group. $n = 4$ mice/group.

Fig. 3. KDM6B inhibitor (GSK-J4) treatment had no effect on memory retrieval (post-test 1), but decreased cocaine-associated memory after reconsolidation (post-test 2) and attenuated cocaine-primed reinstatement.

(A) A timeline illustrating the experimental design for GSK-J4 or saline treatment the induction of CPP. CPP was induced in C57BL/6 mice by daily cocaine injections (20 mg/kg i.p.) during days 1 to 4. GSK-J4 (0.5 mg/kg i.p.) was administered daily on WD 3–7. On WD 5 and WD 7, GSK-J4 was injected 30 min before each test. Numbers in bold represent pre-test or post-test. (B) Histogram revealing that GSK-J4 injections had no effect on post-test 1, but prevented CPP expression during post-test 2. (C) Histogram revealing that GSK-

J4 injections had no effect on locomotion over 15 min during post-test 2. S+V: Saline +Vehicle; S+G: Saline+GSK-J4; C+V: Cocaine+Vehicle; C+G: Cocaine+GSK-J4. $*p < 0.05$ vs corresponding Saline+Vehicle group; $^{\wedge}p < 0.05$ vs corresponding Saline+GSK-J4 group. **(D)** A timeline illustrating the experimental design for GSK-J4 or saline treatment after induction of CPP. GSK-J4 (0.5 mg/kg i.p.) was administered daily immediately after retrieval (post-test 1) on WD 5, on WD 6 and 30 min before post-test 2 on WD 7. Numbers in bold represent pre-test or post-test. **(E)** Histogram revealing that GSK-J4 injections, but not vehicle injections, prevented CPP expression during post-test 2 (WD 7). $***p < 0.001$ vs corresponding Saline+Vehicle group; $^{\wedge\wedge}p < 0.001$ vs corresponding Saline+GSK-J4 group; $^{\#}p < 0.05$ vs corresponding Cocaine+Vehicle group. **(F)** A timeline illustrating the experimental design for GSK-J4 or saline treatment after induction of CPP. Numbers in bold represent pre-test or post-test. **(G)** Histogram revealing that GSK-J4-treated mice exhibited significantly attenuated both conditioned memory after reconsolidation (post-test 2) and cocaine-primed reinstatement compared with vehicle-treated mice (post-test 3). $*p < 0.05$, $**p < 0.01$ vs Cocaine+Vehicle+Saline group; $^{\wedge}p < 0.001$ vs Cocaine +GSK-J4+Saline group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Cocaine+Vehicle+Cocaine group. $n = 6-10$ mice/group.

Fig. 4. Systemic GSK-J4 injection following cocaine-CPP test targets H3K27me3 and exhibits brain-region specificity

(A) A timeline illustrating the experimental design for saline or cocaine treatment prior to sacrifice after post-test 2 (WD 7). Numbers in bold represent pre-test or post-test and underlined numbers represent the days animals were sacrificed. (B) Representative Western blots and summary histograms showing the H3K27me3 and H3K4me3 protein levels in histone fractions from mPFC after post-test 2 of different treatment groups. (C) Representative Western blots and summary histograms showing the H3K27me3 protein

levels in histone fractions from NAc and hippocampus after post-test 2 of different treatment groups. S+V: Saline+Vehicle; S+G: Saline+GSK-J4; C+V: Cocaine+Vehicle; C+G: Cocaine+GSK-J4. $**p < 0.01$ vs corresponding Saline+Vehicle group; $^{\wedge}p < 0.01$ vs corresponding Saline+GSK-J4 group; $\#p < 0.05$ vs corresponding Cocaine+Vehicle group. n = 4–5 mice/group.

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Fig. 5. KDM6B increase coincides with the increase in NR2A, but not NR2B during the early cocaine withdrawal in mPFC.

(A) A timeline illustrating the experimental design for cocaine or saline treatment prior to sacrifice at 6 varying time points. Cocaine (20 mg/kg i.p.) was injected in the homecage during days 1 to 4. Underlined numbers represent the days animals were sacrificed. (B) Representative Western blots and summary graphs showing NR2A and NR2B protein levels in whole cell fractions at different time points after saline- and cocaine-exposure. Dashed rectangular highlight the dynamic changes of NR2A and NR2B during early withdrawal. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs corresponding saline group. $n = 4-6$ mice/group.

Fig. 6. KDM6B inhibitor GSK-J4 specifically blocks the NR2A increase after post-test 2 in the mPFC.

(A) A timeline illustrating the experimental design for saline or cocaine treatment prior to sacrifice after post-test 2 (WD 7). Numbers in bold represent pre-test or post-test and underlined numbers represent the days animals were sacrificed. (B) Representative Western blots and summary histograms showing the different receptor subunits in whole cell fractions from mPFC of different treatment groups. S+V: Saline+Vehicle; S+G: Saline+GSK-J4; C+V: Cocaine+Vehicle; C+G: Cocaine+GSK-J4. * $p < 0.05$ vs corresponding Saline+Vehicle group; ^ $p < 0.05$ vs corresponding Saline+GSK-J4 group; # $p < 0.05$ vs corresponding Cocaine+Vehicle group. $n = 4-6$ mice/group.

Fig. 7. KDM6B inhibitor specifically blocks the NR2A-induced transmission increase, but not other AMPA or NMDA receptor subunits in the PL region of the mPFC.

(A) A timeline illustrating the experimental design for saline or cocaine treatment prior to sacrifice after post-test 2 (WD 7). Numbers in bold represent pre-test or post-test and underlined numbers represent the days animals were sacrificed. (B) Coronal section illustrating patch-clamp recordings of stimulation-evoked synaptic currents in the PL-mPFC. (C) Representative traces of evoked EPSCs at -60 and +60 mV from PL of different treatment groups. (D) Summary histograms show that neurons from the cocaine+vehicle

group had significantly higher NMDA/AMPA ratio as compared with neurons from saline +vehicle and cocaine+GSK-J4 group. (E) Neither cocaine nor GSK-J4 had an effect on AMPA receptor currents. (F) Neurons from the cocaine+vehicle group had a significantly higher amplitude of NMDA as compared with neurons from saline+vehicle and cocaine +GSK-J4 group. (G) GSK-J4 inhibited cocaine-induced NMDA-EPSC amplitude change at both 25 – 50 ms, but not at 75 and 100 ms after the EPSC peak at +60mV. S+V: Saline +Vehicle; C+V: Cocaine+Vehicle; C+G: Cocaine+GSK-J4. * $p < 0.05$ vs corresponding Saline+Vehicle group; # $p < 0.05$ vs corresponding Cocaine+Vehicle group. n = 4 mice/group.