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Methamphetamine-Associated Memory is Regulated by a Writer and an Eraser of Permissive Histone Methylation

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Abstract

Background—Memories associated with drugs of abuse, such as methamphetamine (METH), increase relapse vulnerability to substance use disorder by triggering craving. The nucleus accumbens (NAc) is essential to these drug-associated memories, but underlying mechanisms are poorly understood. Posttranslational chromatin modifications, such as histone methylation, modulate gene transcription, thus we investigated the role of the associated epigenetic modifiers in METH-associated memory.

Methods—Conditioned place preference was used to assess the epigenetic landscape in the NAc supporting METH-associated memory (n=79). The impact of histone methylation (H3K4me2/3) on the formation and expression of METH-associated memory was determined by focal, intra NAc knockdown (KD) of a writer, the methyltransferase MLL1 (n=26), and an eraser, the histone demethylase KDM5C (n=38), of H3K4me2/3.

Results—A survey of chromatin modifications in the NAc of animals forming a METH-associated memory revealed the global induction of several modifications associated with active transcription. This correlated with a pattern of gene activation, as revealed by microarray analysis, including upregulation of *Oxtr* and *Fos*, whose promoters also had increased H3K4me3. KD of *Mll1* reduced H3K4me3, *Fos* and *Oxtr* levels and disrupted METH-associated memory. KD of *Kdm5c* resulted in hypermethylation of H3K4 and prevented the expression of METH-associated memory.

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Conclusions—The development and expression of METH-associated memory are supported by regulation of H3K4me2/3 levels by MLL1 and KDM5C, respectively, in the NAc. These data indicate that permissive histone methylation, and the associated epigenetic writers and erasers, represent potential targets for the treatment of substance abuse relapse, a psychiatric condition perpetuated by unwanted associative memories.

Keywords

epigenetics; nucleus accumbens; methyltransferase; demethylase; MLL; KDM5C

Introduction

Learned associations between the rewarding effects of a drug, such as methamphetamine (METH), and contextual stimuli present at the time of drug use are a central component of acquiring and maintaining drug seeking behavior and are capable of inducing relapse years after the initiation of abstinence, perpetuating substance use disorder (1-3). Understanding the mechanisms that underlie these unwanted memories should provide insight into effective therapies aimed at reducing context-induced relapse.

By receiving inputs from brain regions implicated in reward and memory, including the ventral tegmental area, amygdala, hippocampus (HPC) and medial prefrontal cortex, the nucleus accumbens (NAc) is uniquely positioned to mediate reward-based behaviors (4), including drug seeking triggered by associative memories. Drug-induced transcriptional activation in the NAc is thought to be key to the behavioral and neurophysiological changes that occur with drug intake (4, 5). Further, the NAc has been implicated in many forms of learning, including contextual fear, spatial memory, instrumental learning and Pavlovian reward memory (6). However, learning-induced transcriptional changes within the NAc have not been examined (7, 8). Given that *de novo* transcription is required for memory formation (9) and that the NAc is a hub for reward, memory and drug seeking, mechanisms contributing to memory-induced transcriptional changes in the NAc may provide insight into approaches aimed at disrupting METH-associated memories.

Epigenetic modifications modulate transcriptional activity without altering the DNA sequence (10) and drugs of abuse have been shown to induce posttranslational modification of histones (H), including acetylation (Ac) and methylation (me) (5, 11). Histone acetylation is associated with transcriptional activation. However, methylation has been implicated in both repression and activation, depending on the specific lysine residue (K) that is modified and the number of methyl moieties that are attached (10). Methylation is regulated by enzymes that add moieties (“writers”, methyltransferases [HMTs]) or remove them (“erasers”, demethylases [KDMs]) (10, 12). Associative memories are supported by changes in both transcriptionally permissive (H3K4me3) and repressive (H3K9me2) methylation (13-15), suggesting a potential for therapeutic disruption of drug-associated memories by targeting chromatin modifying enzymes. MLL1 (Mixed Lineage, Leukemia) has been identified as an HMT for the permissive methylation that occurs at lysine 4 (H3K4me2/me3) and is required for adult neurogenesis, synaptic plasticity and HPC-dependent memory formation. It is also involved in prefrontal GABAergic dysfunction associated with

schizophrenia and the mechanisms of cortical spreading depression (13, 16-19). De novo mutations in *Mll* have also recently been associated with Wiedemann-Steiner syndrome, a disorder marked by hypertrichosis cubiti and Intellectual Disability (20). This particular “writer” can drive mono-, di- and tri-methylation at H3K4 (10). These same histone residues can be demethylated by members of the KDM1 and KDM5 families of demethylases (10). Inhibition of the eraser KDM1A/LSD1, which targets mono- and di-methylation, disrupts HPC-dependent memory formation (15). The KDM5 family's function in the intact brain, on the other hand, has not yet been studied. However, *Kdm5c/Smcx* mutations, which decrease demethylase activity, have been found in male patients diagnosed with X-linked Intellectual Disability (XL-ID) and short-term memory deficits have been reported in carrier females (21-27). Additionally, a role for this demethylase in neuronal survival and dendritic development has been reported in primary neuronal culture (28). Like all members of the KDM5 family, KDM5C catalyzes the demethylation of di- and tri-methylated H3K4 (10).

To investigate the role of H3K4 methylation modifiers in METH-associated contextual memory, we employed conditioned place preference (CPP), in which Pavlovian associations are formed between a specific context (conditioned stimulus, CS+) and the rewarding effects of METH (29). As activation of gene expression occurs with exposure to drugs of abuse and during memory storage, we aimed to test the hypothesis that modifiers of histone modifications supportive of active transcription (e.g. H3K4me2/me3) are involved in METH-associated memory.

Methods and Materials

Animals

8-12 week old male C57Bl/6 mice (Jackson Laboratories) were housed in groups of four on a 12 hr light/dark cycle with *ad libitum* access to food and water. All experiments were performed during the light part of the diurnal cycle. Housing, animal care and experimental procedures were consistent with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

siRNA-mediated focal knockdown of *Mll1* and *Kdm5c*

KD of *Mll1* or *Kdm5c* was first assayed in naïve mice 2 and 5 days after intra-NAc injection of each siRNA (ON-TARGETplus SMART pool - mouse *Mll1*, *Kdm5c*, or non-targeting pool; Dharmacon; see Table S1 for target sequences) using JetSI transfection reagent (Polyplus Transfection) (30, 31). Mice were anesthetized with 1.0-1.5% isoflurane and placed into a stereotaxic apparatus (Kopf). The appropriate siRNA-JetSI complexes were injected bilaterally into the NAc (1µl at 200nl/min; A/P: +1.6mm, M/L: +/-2.6mm from bregma and D/V: -4.8mm from skull at a 20° angle (32)) using an automated injection pump through a 10µl syringe and 33G stainless steel needle. The syringe was left in place for 5min to allow for diffusion of the siRNA-JetSI complexes. Coordinates were first validated with 500nl of a GFP-expressing adeno-associated virus (GFP-AAV). Coronal sections collected 1 week later showed GFP expression spread across 1mm of the A/P axis of the NAc, affecting both the core and medial shell subregions; expression was not detected in the dorsal striatum. In a separate group of animals used for behavioral assessments, bilateral stainless-

steel guide cannulae (26G; Plastics One) were placed 1mm above the NAc using the same coordinates. Metacam (1.5mg/ml) was administered immediately after surgery. 5-7 days later, training began and mice were injected with *Mll1*, *Kdm5c* or control siRNA using infusion needles (Plastics One) that projected 1mm beyond the tip of the guide cannula.

Conditioned Place Preference

Animals were trained as previously described (33). For additional details, please see Supplemental Information.

RNA and Protein Extraction

For the microarray, RNA was extracted from NAc tissue punches using the AllPrep DNA/RNA Micro Kit (Qiagen). For all other experiments, NAc protein and RNA were extracted using the AMBION® PARIS kit (Life Technologies).

Gene Expression Analysis Microarray

100ng of total RNA from each CPP sample (7-8 samples per group) was pooled, such that RNA from each animal in a given group was equally represented in the appropriate pooled sample, resulting in 3 samples of 1µg each for the array: Sal CPP, METH HC and METH CPP. Transcriptome analysis was performed with the GeneChip Mouse Gene 1.0 ST Array (Affimetrix) by the Scripps Florida Genomics Core. Briefly, in vitro transcription was performed to produce labeled cRNA, which was then fragmented (~100bp), added to the chip, hybridized overnight, washed, stained and scanned. Data were normalized using the MAS5 algorithm; signal intensities lower than 100 a.u. were considered not expressed. Data for the METH CPP group were expressed as fold change relative to Sal CPP and METH HC control groups; hit genes were identified by fold changes > 1.6, compared to both control groups.

qRT-PCR

Validation by qRT-PCR was performed using the individual samples obtained prior to pooling a portion for the microarray. The iScript One-Step qRT-PCR Kit (BioRad) was used with mouse TaqMan expression assays (see Table S1; Life Technologies) and 20ng of RNA. All samples were run in triplicate. Data was analyzed using the $\Delta\Delta C_t$ method.

Histone extraction and Western blotting

Histones were extracted using the EpiQuik® Total Histone Extraction kit (Epigentek). 2µg of extracted histones or 40µg of total protein were resolved on 15 or 7% SDS-PAGE gels, respectively, and transferred onto PVDF membranes, then blocked for 1h at RT with 5% BSA (for histones) or 5% non-fat milk (for total protein) in TBS-T (50mM Tris, 150mM NaCl, 0.1% Tween20). See Table S2 for antibodies and concentrations. Membranes were washed with TBS-T and incubated with HRP-coupled secondary antibodies (anti-rabbit IgG, 1:1000; anti-mouse IgG, 1:1000; Vector). Protein expression was assessed by chemoluminescence (Pierce® ECL, Thermo Scientific) and exposure to autoradiography film (Denville Scientific). ImageJ software was used to quantify band intensities. Relative

protein expression was calculated by normalizing the integrated band density values to total H4 or GAPDH.

Chromatin immunoprecipitation (ChIP) with qRT-PCR

ChIP was performed using an EpiQuik® Tissue Chromatin Immunoprecipitation Fast kit (Epigentek) and a ChIP grade rabbit anti-H3K4me3 antibody (Abcam). Normal IgG was used as a negative control. qRT-PCR was performed with SsoAdvanced™ SYBR® Green Supermix kit (BioRad) on purified DNA samples obtained from input, anti-H3K4me3 IP and normal IgG IP samples using primers designed against the promoter regions of Fos and Oxt (see Table S1 for primer sequences). IP values were expressed relative to each sample's input signal.

Statistical analysis

One-way and repeated measure ANOVAs, one-sample t-tests and Wilcoxon Signed-Rank tests were used to analyze all data sets, with Bonferroni post hoc tests for significant effects.

Results

Formation of a METH-associated memory involves global and promoter-specific changes in marks of active histone methylation in the NAc

An understanding of the epigenetic and transcriptional changes induced by METH CPP was first determined, in order to guide our selection of the most appropriate class of epigenetic writers and erasers to explore in METH-associated memory. A typical CPP protocol involves 3 pairings between the unconditioned stimulus (US; METH) and CS+ (context). Through this training, animals form a Pavlovian association between the rewarding effects of METH and the environmental context in which it is administered and later show a preference for that environment. To investigate the molecular mechanisms occurring during the initial stages of formation of this conditioned response to METH, we analyzed changes in the NAc occurring after the first CS-US pairing (Fig. 1A). Analysis at this time point reflects mechanisms involved in the initial encoding of an association between the US and CS, prior to behavioral manifestation of the preference. To specifically identify changes associated with METH CPP formation, two key control groups were included – Saline CPP to exclude changes associated with exposure to the novel environment and METH home cage (HC) to exclude changes associated with exposure to METH alone (Fig. 1A).

We first explored the potential for epigenetic regulation at the time of learning by measuring global levels of several transcriptionally active (H3K4me2/me3, pan-AcK H2-3 and pan-AcK H4) and repressive (H3K9me2 and H3K27me2) chromatin modifications in the NAc. METH CPP was accompanied by an increase in active histone methylation modifications (H3K4me2: $F_{(2,21)}=9.237$, H3K4me3: $F_{(2,21)}=10.752$; $P<0.001$) and pan-AcK H2-3 ($F_{(2,15)}=5.97$, $P<0.05$), but not pan-AcK H4. This is consistent with a previous report demonstrating increased acetylation of H3, but not H4, in the HPC with contextual associative learning and in the NAc with cocaine administration (34, 35). Methylation of residues associated with repressed transcription were unchanged or decreased (H3K9me2: $F_{(2,15)}=0.224$; H3K27me2: $F_{(2,20)}=4.252$, $P>0.05$; Fig. 1B-C).

Using the same design depicted in Figure 1A, we next performed an exploratory microarray to identify the general transcriptional profile of the NAc during the formation of a METH-associated memory. Results suggest that close to 100 genes are upregulated by METH-associated contextual learning (Fig. 2A), with no genes downregulated below the 1.6-fold cut-off (Table S3). Further analysis revealed the upregulated genes fall into classes associated with synaptic growth and structure, memory, transcription, translation, chromatin modification and development. We were particularly interested in genes with established roles in memory and/or addiction. Of these, we selected two to serve as a proxy for the epigenetic changes likely occurring on a number of genes during the associative process, *Cebpd* (CCAAT/enhancer binding protein delta) and *Oxtr* (oxytocin receptor). We also selected *Fos*, an immediate early gene and transcription factor whose expression has previously been shown to be increased in the NAc by CPP for cocaine and METH, and is a commonly used marker of neural activation (36-38).

Using qRT-PCR, we confirmed that the expression of *Fos* and *Oxtr* mRNAs, but not *Cebpd*, was increased in the METH CPP group (*Fos*: $F_{(2,18)}=15.541$, $P<0.001$; *Oxtr*: $F_{(2,18)}=6.512$, $P<0.01$; *Cebpd*: $F_{(2,18)}=2.877$, $P>0.05$; Fig. 2B). *Fos* expression was also increased in the METH HC group compared to Sal CPP ($P<0.05$), yet *Fos* mRNA levels in the METH CPP group were still greater than the group that received METH in their home cage ($P<0.05$; Fig. 2B). Given the repressive effect of oxytocin in the NAc on METH CPP (39), the enhanced expression of *Oxtr* in the NAc may represent an endogenous, homeostatic mechanism aimed at counteracting the impact of METH. Future studies will be needed to explore this intriguing possibility.

We next investigated the chromatin state of *Oxtr* and *Fos*, focusing on transcriptionally permissive H3K4me3 in the promoter of these genes for several reasons. Despite being one of the more persistent histone modifications, methylation has received little attention in the memory field (10, 40, 41). Furthermore, trimethylation of H3K4 occurs concomitantly with active transcription, while dimethylation can be present at poised, inactive genes (42, 43). Finally, the writers and erasers for this modification appear to participate in cognition, yet function in the adult brain is poorly understood. Consistent with increased *Fos* in the METH HC and METH CPP groups, ChIP-qPCR analysis revealed a trend toward increased H3K4me3 levels at the *Fos* promoter in both groups ($F_{(2,29)}=2.41$, $P=0.108$, Fig. 2C). At the *Oxtr* promoter, levels of H3K4me3 were specifically increased by METH CPP training ($F_{(2,29)}=4.483$, $P<0.05$, Fig. 2C), consistent with its transcriptional profile (Fig. 2B). These results provide evidence that increased methylation of H3K4 may indeed be an important epigenetic modification involved in supporting METH-context associations, warranting further investigation of its writers and erasers.

The epigenetic writer of H3K4me, Mll1, is necessary in the NAc for METH-associated memory

We next examined modifiers of permissive methylation, focusing first on the role of a writer of H3K4 methylation, MLL1, in the modulation of METH-associated memory. *Mll1* was specifically upregulated in the NAc 1 hour after training and downregulated in animals that received METH in the homecage, as compared to saline controls ($F_{(2,14)}=9.52$, $P<0.005$,

Fig. 3A). Interestingly, *Mlll* mRNA levels were reduced in METH HC mice, while H3K4me3 levels were upregulated at the *Fos* promoter in this same control group (Fig. 2C). This suggests that other HMTs involved in H3K4 methylation (e.g. MLL2, SETD1A, SETD1B) may be upregulated and/or related KDMs downregulated by non associative administration of METH.

To determine the behavioral importance of this increase in *Mlll*, we employed siRNA-mediated focal KD of the gene within the NAc. As can be seen from injection of a GFP-AAV, stereotaxic coordinates targeted the NAc core with spread limited to the shell (Fig. 3B). Using naïve adult mice, we next confirmed effective KD of *Mlll* mRNA *in vivo* two and five days after intra-NAc siRNA injection, as compared to a non-targeting, control siRNA (2 days: $t_3 = 4.54$, $P < 0.05$, 5 days: $t_3 = -3.63$, $P < 0.05$; Fig. 3C). Consistent with MLL1's role as an H3K4 methyltransferase, its knockdown resulted in a 50% reduction in H3K4me3 as well ($t_7 = 2.53$, $P < 0.05$; Fig. 3D). Finally, we found that *Mlll* KD dramatically reduced the transcript levels of *Fos* and *Oxtr* within the NAc (*Fos*: $F_{(1,9)} = 20.38$, $P < 0.005$; *Oxtr*: $F_{(1,9)} = 10.00$, $P < 0.05$; Fig. 3E), suggesting that MLL1-mediated H3K4 methylation contributes to their transcriptional regulation.

To determine the role of NAc MLL1 in the formation of METH-associated memory, *Mlll* was knocked down prior to training (Fig. 4A). Animals that received intra-NAc infusions of the *Mlll* siRNA failed to develop a preference for the METH-paired side of the CPP apparatus (Fig. 4B; Control siRNA: $N = 13$, $Z = -2.34$, $P < 0.05$; *Mlll* siRNA: $N = 13$, $Z = -1.15$, $P > 0.05$). After testing, reduction of *Mlll* mRNA was confirmed ($F_{(1,14)} = 4.83$, $P < 0.05$; Fig. 4C).

The epigenetic eraser of H3K4me, Kdm5c, is the most abundant H3K4 demethylase in the NAc and is necessary for expression of an established METH-associated memory

Given the loss of function achieved through KD of the H3K4me writer MLL1 (Fig. 4), we next explored the potential for a gain of function (memory enhancement) through manipulation of an H3K4me eraser. We focused on KDM1A and the KDM5 family (KDM5A D, also known as RBP2, PLU-1, SMCX and SMCY, respectively), because of their suggested roles in cognition (15, 22-27, 44).

Very little is known about the role of KDM5 demethylases in the mammalian brain (28) and their expression pattern remains uncharacterized, with the exception of two earlier studies that compared whole brain levels of *Kdm5c* and *Kdm5d* (24, 45). Thus, we first determined the abundance of *Kdm5a-d* and *Kdm1a* within the NAc of naïve, adult male mice. While transcript for all demethylases was detected in the NAc, the XL-ID gene, *Kdm5c*, was expressed at relatively high levels, as its expression was approximately 20% of *Gapdh*, an abundant housekeeping gene (Fig. 5A). The other *Kdm5* family members and *Kdm1a* were expressed at levels 16-100 times less than *Gapdh* (Fig. 5A). We then compared *Kdm5c* mRNA expression levels across several brain regions relevant to substance abuse and memory and found them to be comparable (Fig. 5B).

Kdm5c levels were equal to controls in the NAc 1 hour after training (Fig. 6A; $P > 0.05$). However, this did not preclude the possibility that a loss of demethylase activity might lead

to a gain of memory function through an accumulation of H3K4 methylation. Therefore, using naïve adult mice, we next confirmed that intra-NAc injection of *Kdm5c* siRNA effectively knocked down the demethylase's mRNA *in vivo* 2 days later ($F_{(1,14)}=13.23$, $P < 0.005$; Fig. 6B). Interestingly, transcript levels had returned to baseline by 5 days post-siRNA, indicating a fairly rapid turnover rate for *Kdm5c* ($P > 0.05$; Fig. 6B). Because so little is known about this demethylase in the adult brain and because the siRNA had a modest effect on the gene's mRNA levels, we also confirmed KD at the protein level ($F_{(1,12)}=16.59$, $P < 0.005$; Fig. 6C). Consistent with reports from cell culture and peripheral tissue, KDM5C loss also led to an accumulation of H3K4me3 ($F_{(1,10)}=5.99$; $P < 0.05$; Fig. 6D), extending its role as an H3K4 demethylase to the brain and indicating that KDM5C contributes to the active maintenance of H3K4me3 in the NAc (28, 46-50).

To determine the role of NAc KDM5C in the formation of METH-associated memory, *Kdm5c* was knocked down prior to training by two siRNA infusions to ensure suppression of the demethylase for the entire training period (Fig. 7A). Control and *Kdm5c* siRNA-treated groups developed an equal preference for the METH paired side of the CPP apparatus (Fig. 7B; Control siRNA: $N=8$, $Z=-2.24$, $P < 0.05$; *Kdm5c* siRNA: $N=10$, $Z=-1.99$, $P < 0.05$), with *Kdm5c* KD showing no memory-enhancing effect. After the behavioral task, we observed that KDM5C and H3K4me3 levels had returned to baseline by the time of expression testing (KDM5C: $F_{(1,13)}=0.02$, $P > 0.05$; H3K4me3: $F_{(1,12)}=0.79$, $P > 0.05$, Fig. 7C), again indicating rapid recovery of KDM5C levels after siRNA treatment. Importantly, both locomotion and velocity were normal in the *Kdm5c* siRNA-treated mice during training (Fig. S1), indicating that a lack of memory enhancement could not be attributed to a change in the degree of METH-induced locomotor activation.

We next explored the potential for post-training manipulation of KDM5C to affect expression of METH-associated memory, as disruption of established drug-associated memories is an emerging approach to the treatment of substance abuse (1, 2). To achieve the necessary temporal control over KDM5C, we infused *Kdm5c* siRNA into the NAc one day after the first METH CPP test ($P > 0.05$; Fig. 7D-E). At the post-siRNA injection test (Test 2), *Kdm5c* KD mice no longer showed a preference for the METH-paired chamber (Test $\times$ siRNA interaction $F_{(1,20)}=5.061$, $P < 0.05$; Test 2 control vs. *Kdm5c* siRNA $P < 0.005$; Fig. 7E). Suspecting that this effect may have been due to a rapid acceleration of extinction (51, 52), we examined place preference expression during the first 5 minutes of the test, as accelerated extinction would be defined by an initial preference that rapidly dissipated during the 15 minute test period. This lack of a preference for the METH-paired compartment in *Kdm5c* siRNA animals was apparent even in the first 5 minutes ($F_{(1,21)}=6.90$, $P < 0.05$; Fig. S2), suggesting that a lack of CPP expression in Test 2 was not due to accelerated extinction. Following behavioral testing, reduction of KDM5C protein and hypermethylation of H3K4 by *Kdm5c* siRNA were confirmed (KDM5C: $F_{(1,12)}=7.24$, $P < 0.05$; H3K4me3: $F_{(1,18)}=4.85$, $P < 0.05$; Fig. 7F).

Discussion

METH-associated memory formation is accompanied by the induction of a NAc transcriptional program linked to permissive histone modifications. Consistent with this, we

found that loss of the H3K4 HMT, *Mll1*, within the NAc impaired METH-associated memory, while post-consolidation loss of *Kdm5c* disrupted the expression, but not formation, of the same memory. This indicates that a balance of histone methylation must be maintained in the NAc to successfully support memory.

The upregulation of several epigenetic marks associated with active transcription correlated with the increase in expression of close to 100 genes by METH context associative learning. While our study does not fully address this possibility, these chromatin modifications are likely a driving influence in the transcriptional changes reported, as suggested by the impact of *Mll1* KD on baseline *Fos* and *Oxtr* expression. However, these epigenetic changes are also expected to place a number of genes in a transcriptionally primed state, such that the threshold for a given gene to respond to future stimulation (53), such as subsequent CPP training trials, is altered. Therefore, these chromatin modifications may influence subsequent gene transcription, enabling a context to produce a behavioral outcome (a place preference or context-induced drug seeking) in the absence of reinforcement (METH). Additional mechanistic studies will be needed to test such a hypothesis.

An emerging theme for drug-associated memory is that inhibition of repressive epigenetic modifying enzymes (G9a, DNMT3a, HDAC5) in the NAc enhances the acquisition of CPP (35, 51, 54-57). The converse appears to be true as well, as the activating histone-modifying enzyme CBP is necessary for cocaine CPP to be acquired (35). This can now be extended to include the activating HMT, MLL1, as loss of the enzyme disrupted METH CPP. Interestingly, KDM5C belongs to the former category of repressors of gene expression (28, 46). However, unlike the above mentioned examples, KDM5C KD in the NAc impaired METH CPP only after conditioning had taken place. This result is especially intriguing, as KD of KDM5C and dysregulation of H3K4me3 levels occurred after the memory had stabilized. Given the timing of the manipulation, a blockade of reconsolidation is unlikely to account for the effect (51, 58-61). Another possibility is accelerated extinction (within the Test 2 session), but our time-bin analysis of the preference during Test 2 (Fig. S2) revealed that *Kdm5c* KD prevented expression of the place preference from the beginning of the test. Rather, the result predicts that this KDM controls the maintenance or ability to retrieve memory by regulating stability of the trace, motivation for drug seeking, and/or ability to access the memory. KDM5C has been implicated in the suppression of REST (RE1-Silencing Transcription Factor) (46), a transcriptional repressor. A number of memory-related genes have RE1 binding sites, including the NMDA receptor subunits NR1, NR2A and NR2B. NR1 expression is required for the long-term maintenance of memory and NR2A is required for memory retrieval (62, 63). Thus, loss of KDM5C may disrupt established memory through an accumulation of H3K4 methylation that drives REST transcription, leading to a repression of key components of the NMDA receptor. Regardless of the exact mechanism, these results represent a new potential target for disrupting context-induced METH seeking behavior.

Although our results and those of others indicate that *Kdm5c* continues to be expressed in the adult brain (24, 45), virtually nothing is known about its functional role in this context. Our demonstration that *in vivo* KDM5C KD results in enhanced levels of H3K4me3 indicates that the protein continues to actively function as a demethylase in the adult brain

and may be the predominant KDM in the NAc. Elevation of H3K4me3 levels with KDM5C KD also indicates the presence of active HMTs in NAc cells, as methyl groups accrue on the H3K4 residue in its absence, even under baseline conditions (Fig. 6D). Thus the levels of this epigenetic mark appear to be regulated in mature neurons by a dynamic interplay between H3K4 HMTs, such as MLL, and KDMs. Such dynamic control of H3K4me3 levels in the adult brain suggests that this residue can rapidly respond to environmental cues in the control of gene expression. Importantly, in addition to the disruption of METH-associated memory following pre-training loss of MLL, knockdown of KDM5C disrupted an already established association, representing the first evidence for a behavioral regulatory role of any member of the KDM5 family of histone demethylases in the adult brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Induction of posttranslational histone modifications associated with active gene expression in the NAc during the formation of a METH-associated memory. **(A)** Schematic of experimental design. **(B)** Histone modifications associated with transcriptional activation (H3K4me2, me3, pan-AcK H2-3, pan-AcK H4) or repression (H3K9me2, H3K27me2) were measured in the NAc by Western blot and normalized to total H4. **(C)** Representative images of Western blots. * $P < 0.05$, error bars represent SEM.

Figure 2.

Formation of a METH-associated memory is associated with transcriptional activation in the NAc. **(A)** Using the same experimental design depicted in Fig. 1A, microarray analysis in the NAc revealed the upregulation of 91 genes, relative to control groups (Sal CPP, METH HC). **(B)** From the group of 91 genes selectively upregulated in the METH CPP group, *Cebpd*, *Fos* and *Oxtr* were selected for qRT-PCR validation. **(C)** Schematic of the region of the *Fos* and *Oxtr* promoters amplified from genomic DNA samples obtained from H3K4me3 chromatin immunoprecipitation (upper panel). Promoter-specific H3K4me3 at

Fos and *Oxtr* was measured by ChIP-qRT-PCR in the NAc of METH CPP and control mice (lower panel). * $P < 0.05$, error bars represent SEM.

Figure 3.

The H3K4 histone methyltransferase, MLL1, is increased during METH CPP acquisition and its loss results in H3K4 demethylation. **(A)** Using the same experimental design depicted in Fig. 1A, *Mll1* expression was measured by qRT-PCR. **(B)** Representative coronal section of a mouse brain injected in the NAc core with an adeno-associated virus (AAV1) expressing the enhanced Green Fluorescent Protein (eGFP). The same brain coordinates were used for subsequent siRNA delivery. **(C)** *Mll1* levels in the NAc two and five days after intra-NAc siRNA injection. **(D)** Intra-NAc KD of *Mll1* resulted in demethylation of H3K4 in the NAc. **(E)** Effect of *Mll1* KD on *Fos* and *Oxt* mRNA levels. * $P < 0.05$, error bars represent SEM.

Figure 4.

NAc MLL1 is required for METH-associated memory. **(A)** Schematic of experimental design. **(B)** Effect of focal KD of *Mll1* in the NAc on METH-associated memory. **(C)** *Mll1* KD in the NAc was confirmed after behavioral testing. * $P < 0.05$, error bars represent SEM.

Figure 5.

Kdm5c is the most abundant H3K4 demethylase in the NAc. **(A)** Expression of the KDM5 (*Kdm5a*, *Kdm5b*, *Kdm5c* and *Kdm5d*) and KDM1 (*Kdm1a*) families of H3K4 demethylases was assayed in the NAc by qRT-PCR and expressed relative to *Gapdh* expression. **(B)** *Kdm5c* mRNA expression was also measured across several brain regions relevant to psychostimulants and memory (NAc, dStr, mPFC, AMY and anterior HPC). Error bars represent SEM.

Figure 6.

Loss of *Kdm5c* in the NAc results in H3K4 hypermethylation. **(A)** Using the same experimental design depicted in Fig. 1A, *Mlll* expression was measured by qRT-PCR. **(B)** *Kdm5c* levels in the NAc two and five days after intra-NAc siRNA injection. **(C)** KDM5C protein levels in the NAc two days after intra-NAc siRNA injection. **(D)** Intra-NAc KD of *Kdm5c* resulted in hypermethylation of H3K4 in the NAc. * $P < 0.05$, error bars represent SEM.

Figure 7.

Focal knockdown of *Kdm5c* in the NAc after consolidation disrupts METH-associated contextual memory. (A) Schematic of experimental design for B-C. (B) Pre-training intra-NAc *Kdm5c* siRNA had no effect on METH-associated memory. (C) KDM5C protein and H3K4me3 levels in the NAc were measured after behavioral testing. Lower panel contains representative images of Western blots for KDM5C (~170kDa), H3K4me3 (~17kDa) and GAPDH (~36kDa). (D) Schematic of experimental design for E-F. (E) METH-associated memory was assayed before (Test 1) and after (Test 2) *Kdm5c* siRNA infusion. (F) KDM5C

protein and H3K4me3 levels in the NAc were measured after behavioral testing. Lower panels contain representative Western blot images of KDM5C, H3K4me3 and GAPDH. * $P < 0.05$, error bars indicate SEM.