Class I HDAC Inhibition Blocks Cocaine-Induced Plasticity Through Targeted Changes in Histone Methylation

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Abstract

Induction of histone acetylation in the nucleus accumbens (NAc), a key brain reward region, promotes cocaine-induced alterations in gene expression. Histone deacetylases (HDACs) tightly regulate the acetylation of histone tails, but little is known about the functional specificity of different HDAC isoforms in the development and maintenance of cocaine-induced plasticity, and prior studies of HDAC inhibitors report conflicting effects on cocaine-elicited behavioral adaptations. Here, we demonstrate that specific and prolonged blockade of HDAC1 in NAc of mice increased global levels of histone acetylation, but also induced repressive histone methylation and antagonized cocaine-induced changes in behavior, an effect mediated in part via a chromatin-mediated suppression of $GABA_A$ receptor subunit expression and inhibitory tone on NAc neurons. Our findings suggest a novel mechanism by which prolonged and selective HDAC inhibition can alter behavioral and molecular adaptations to cocaine and inform the development of novel therapeutics for cocaine addiction.

Drug addiction is a chronic, debilitating psychiatric disorder characterized by high rates of relapse. Recent studies suggest that post-translational modifications (PTMs) of histones in

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nucleus accumbens (NAc), an important neural substrate for the addicting actions of drugs of abuse, mediate long-lasting transcriptional and behavioral changes in response to cocaine or other psychostimulants. For example, repeated psychostimulant administration increases global levels of histone acetylation and decreases global levels of histone methylation (which are normally associated with gene activation and repression, respectively) in NAc[1–8].

Histone deacetylases (HDACs) are a family of enzymes capable of repressing gene expression by removing acetyl groups from histone substrates[9]. Studies investigating the effects of pan-HDAC inhibition on psychostimulant-induced behavioral plasticity have yielded conflicting results, with some studies reporting that systemic or intra-NAc HDAC inhibition enhances the behavioral effects of cocaine or amphetamine, and other studies reporting changes in the opposite direction[1,3–5,10–15]. These discrepant findings suggest layers of complexity that have not been adequately considered to date, which might include different affinities of various HDAC inhibitors for different HDAC isoforms, highly specific biological actions of different HDAC isoforms in regulating psychostimulant responses, and time-dependent effects of HDAC inhibition in brain.

We addressed these possibilities in the present study in several ways. While previous work has targeted a combination of Class I and II HDACs, Class III HDACs, or specific Class II HDAC isoforms[1,3–5,10–16], no study to date has systematically examined the role of nuclear-specific Class I HDAC isoforms in the behavioral effects of drugs of abuse. We thus induced local knockouts of HDAC1, 2, or 3 in NAc of adult floxed mice via viral expression of Cre recombinase in this brain region, and found that only prolonged knockdown of HDAC1 significantly suppressed cocaine-induced behavioral plasticity. While acute HDAC inhibition enhances the behavioral effects of cocaine or amphetamine[1,3,4,13,14], studies suggest that more chronic regimens block psychostimulant-induced plasticity[3,5,11,12]. We found that continuous infusion of the selective pharmacological HDAC inhibitor, *N*-(2-aminophenyl)-4-[*N*-(pyridin-3-yl-methoxycarbonyl) aminomethyl]benzamide (MS-275), directly into NAc over a broad time course attenuated cocaine's behavioral effects and triggered other chromatin modifications in the NAc known to oppose cocaine-induced plasticity. We show that prolonged HDAC inhibition paired with repeated cocaine selectively induced a form of repressive histone methylation, dimethylation of Lys9 of histone H3 (H3K9me2), in NAc, which, through the repression of $GABA_A$ receptor subunits and inhibitory tone on NAc medium spiny neurons, contributed to blockade of cocaine-induced plasticity. Together, these findings demonstrate cross talk among different types of histone modifications in the adult brain and identify a novel molecular mechanism that controls an individual's sensitivity to psychostimulant exposure.

RESULTS

Regulation of Cocaine's Behavioral Effects by HDAC1 in NAc

Most HDAC activity is catalyzed by Class I HDACs. To investigate the role of each of the three main Class I HDACs most highly expressed in brain, HDACs 1-3[17], in cocaine action, we engineered selective knockdowns of these enzymes from NAc by injecting a viral vector expressing Cre into this brain region of adult mice homozygous for floxed alleles of each enzyme isoform[18,19] (Supplementary Fig. 1). We then tested the animals in a cocaine

locomotor sensitization paradigm, a widely used model of cocaine-induced behavioral plasticity[20,21]. We found that the local knockdown of HDAC1 significantly reduced cocaine locomotor sensitization, but had no effect on responses to initial cocaine doses (Fig. 1a). In contrast, local knockdown of HDAC2 or HDAC3 had no effect on cocaine-induced behaviors (Fig. 1c and e). Together, these findings establish a specific role for HDAC1, compared with HDACs 2 and 3, in NAc in mediating behavioral responses to cocaine. Selective knockdown of each of the three HDAC isoforms was confirmed by qPCR (Fig. 1b, d, f). Interestingly, these knockdowns influenced the expression of other HDACs in NAc. Local knockdown of HDAC1 decreased expression of *HDAC2* and *SIRT1* (a Class III HDAC) and increased expression of *HDAC5* (a Class II HDAC); both *SIRT1* and *HDAC5* have previously been implicated in cocaine's behavioral effects[5,16]. In contrast, knockdown of HDACs 2 or 3 led to compensatory increases in other Class I and II HDACs.

We next tested whether direct infusion of the selective HDAC inhibitor, MS-275, had similar effects. While MS-275 targets all three major Class I HDACs, HDACs 1-3, it exhibits by far the highest *in vitro* affinity for HDAC1: EC_{50} (nM) = 181±62 (HDAC1), 1155±134 (HDAC2), and 2311±803 (HDAC3)[22]. *In vivo* selectivity of MS-275 has not been examined. Mice were implanted with osmotic minipumps for continuous and direct infusion of MS-275 into NAc. Five days after surgery, mice were subjected to our cocaine locomotor sensitization paradigm (10 mg/kg). Similar to local knockdown of HDAC1, chronic infusion of MS-275 into the NAc blocked cocaine-induced locomotor sensitization with no effect on baseline mobility or responses to initial cocaine doses (Fig. 2a).

HDAC Regulation of Repressive Histone Methylation in NAc

Repeated cocaine administration has been shown to induce a global increase in histone acetylation and a global decrease in repressive H3K9 methylation in NAc, both of which promote transcriptional activation and behavioral responses to cocaine[1,2,5,6,23]. To validate MS-275's pharmacological activity, and to probe its effects on several types of cocaine-regulated histone PTMs, we quantified levels of acetylated and methylated forms of H3 in the NAc under the site of MS-275 infusion 24 hours after repeated cocaine (12 days of continuous MS-275, 7 daily doses of cocaine 20 mg/kg). As shown previously[24], intra-NAc infusion of MS-275 increased global levels of acetylated H3 at Lys14 (H3K14ac) (Fig. 2b) and repeated cocaine increased global levels of acetylated H3 at Lys9 (H3K9ac) (Fig. 2c). Neither treatment altered total protein levels of H3 (Fig. 2b). To date, virtually nothing is known about the catalytic selectivity of HDAC1 or other HDACs for various H3 acetylation sites (e.g., K9 vs. K14); the apparent different effects of cocaine and MS-275 on H3K14 and K9 acetylation might relate to the surrounding chromatin landscape, as K14's neighboring amino acids, unlike those of K9, are not modified. While MS-275 treatment alone did not alter levels of either the repressive euchromatic mark H3K9me2 or the heterochromatic trimethylation of H3 at Lys9 (H3K9me3), both were elevated when MS-275 was paired with repeated cocaine (Fig. 2d and Supplementary Fig. 2a). We did not see a global decrease in either of these marks following repeated cocaine alone, as we have published previously[6,23]. However, this prior work was performed on non-operated (surgery-naïve) mice, and we found that the use of cannulae and intra-NAc infusions alone increased global H3K9me2 levels in NAc (Supplementary Fig. 2b), which may have complicated our ability to detect

cocaine-induced decreases. Given that these measures are taken on a global scale, the absence of a cocaine-induced decrease in global H3K9me2 does not preclude altered targeting of this mark to specific gene promoters in response to cocaine. The effect of intra-NAc infusion of MS-275 on histone PTMs in this brain region was specific to modifications normally altered by repeated cocaine. Thus, neither repeated cocaine administration nor MS-275 treatment produced changes in global levels of a major activating form of histone methylation, H3 Lys4 trimethylation (H3K4me3), or of another repressive histone methylation, H3 Lys27 trimethylation (H3K27me3) (Supplementary Fig. 2c and d).

In contrast to the effects seen with prolonged and continuous infusion of MS-275 into the NAc, acute infusions just prior to daily cocaine treatment had no significant effect on locomotor activity or sensitization (Supplementary Fig. 3a), indicating that chronic knockdown or blockade of HDAC1 is required for attenuation of cocaine's behavioral effects. Furthermore, while acute MS-275 paired with cocaine treatment increased global levels of H3K9ac, global levels of H3K9me2 remained unchanged (Supplementary Fig. 3b and c), suggesting that the increase in H3K9me2 following chronic MS-275 treatment is important for blocking sensitized locomotor responses to cocaine. Together, these findings highlight a striking chromatin signature that occurs only in the combined presence of chronic HDAC inhibition by MS-275 plus repeated exposure to cocaine.

To gain insight into the mechanism underlying the selective increase in H3K9me2 and H3K9me3 in NAc in response to local infusion of MS-275 plus repeated cocaine, we next profiled lysine methyltransferases (KMTs) known to catalyze these repressive histone marks: G9a, G9a-like protein (GLP), and suppressor of variegation 3–9 homolog 1 (SUV39H1). As previously shown[6], 24 hours after repeated cocaine exposure, *G9a* expression was significantly downregulated in NAc, with no effect seen on *GLP* or *SUV39H1* (Fig 2e). However, in striking contrast, expression of both *G9a* and *SUV39H1* was significantly <u>elevated</u> in NAc of mice receiving MS-275 plus repeated cocaine (Fig. 2e), consistent with the increased global levels of H3K9me2 and H3K9me3 seen uniquely under these conditions. Given that MS-275 plus repeated cocaine increased global levels of AcH3, we hypothesized that transcriptional activation of *G9a* and *SUV39H1* following MS-275 treatment with repeated cocaine might be mediated through targeted binding of AcH3 at the promoters of these KMTs. Using quantitative chromatin immunoprecipitation (qChIP) with an antibody to H3K9ac, as we wanted to examine a promoter-enriched AcH3 mark[24], we found a significant increase in H3K9ac binding at the promoters of all three H3K9 KMTs, *G9a*, *GLP*, and *SUV39H1*, following chronic MS-275 treatment with repeated cocaine (Fig. 2f). These data thus reveal a novel interplay between activating and repressive chromatin remodeling: in the context of MS-275 mediated hyperacetylation, repeated exposure to cocaine induces a targeted increase in repressive histone methylation that may serve to dampen hyperactive patterns of gene expression.

To understand how HDAC1 might play a unique role in mediating cocaine-induced decreases in H3K9 KMT gene expression, we used qChIP to probe the binding of the three main Class I HDAC isoforms expressed in NAc, HDACs 1, 2 and 3, to KMT gene promoters in NAc 4 hours after repeated cocaine. We found a significant increase in HDAC1 binding at the promoters of both *G9a* and *GLP* (Fig 3a), but unaltered binding of

both HDACs 2 and 3 at these same promoters (Fig 3b and c). A peptide competition assay with western blotting revealed that the antibodies used for this qChIP experiment are specific for their respective targets (data not shown). These data suggest that repeated cocaine induces the selective binding of HDAC1 to the *G9a* and *GLP* promoters, associated with their repression, and provide one mechanism by which HDAC1 uniquely regulates H3K9 KMT gene expression in response to repeated cocaine.

GABAergic Targets For MS-275-Cocaine Action

Since cocaine-induced decreases in repressive H3K9me2 in NAc are an important mediator of behavioral responses to cocaine[6], we hypothesized that the induction of H3K9me2 following MS-275 treatment with repeated cocaine observed here might be one key mechanism by which chronic MS-275 administration blocks cocaine-regulated behavioral sensitization. To identify possible gene targets for MS-275-induced transcriptional repression, we profiled gene families known to be upregulated by cocaine and important for cocaine-induced behavioral plasticity. Previous genome-wide promoter analyses using ChIP coupled to promoter microarrays (ChIP-chip) identify several GABA$_A$ receptor subunits as targets for increased transcription factor binding following repeated cocaine[5]. More recent studies demonstrate cocaine-induced increases in the frequency of GABA$_A$ receptor-mediated miniature inhibitory post-synaptic currents (mIPSCs) in striatonigral medium spiny neurons in whole striatum and a requirement for α2 subunit-containing GABA$_A$ receptors for the induction of behavioral sensitization to cocaine[25,26]. We therefore focused on regulation of GABA$_A$ receptor subunits in our experimental paradigm.

We found that repeated cocaine significantly induced expression of multiple GABA$_A$ receptor subunit genes in NAc and that cocaine-mediated increases in GABA$_A$-α1, -α3, and -β2 subunit genes (*GABRA1*, *GABRA2* and *GABRB2*) were blocked by chronic intra-NAc MS-275 infusion (Fig. 4a). MS-275infusion by itself caused an induction in GABA$_A$ receptor subunit gene expression similar to that of cocaine alone, again demonstrating a unique interaction upon combined exposure to MS-275 plus cocaine. We then probed GABA$_A$-α1 to verify whether its altered pattern in gene expression occurred at the protein level. Indeed, GABA$_A$-α1 protein levels displayed qualitatively similar patterns of expression across treatment conditions (Supplementary Fig. 4a). These data suggest that GABA$_A$ signaling in NAc may be an important mediator of cocaine-induced locomotor activation. We therefore infused bicuculline, a competitive GABA$_A$ receptor antagonist, into NAc and tested animals in the cocaine locomotor sensitization paradigm. We found that daily infusion of bicuculline blocked the locomotor-activating effects of cocaine (Supplementary Fig. 4b).

To test whether the transcriptional changes in GABA$_A$ receptor subunits correlated with altered binding of acetylated and methylated H3 at specific gene promoters, we performed qChIP with antibodies to H3K9ac or H3K9me2. We found that, while MS-275 treatment with repeated cocaine caused a significant increase in H3K9ac binding at GABA$_A$ subunit gene promoters, there was a much more prominent increase in repressive H3K9me2 enrichment, suggesting a reversal of cocaine-induced transcriptional de-repression with MS-275 (Fig. 4b and c). We further found that repeated cocaine administration decreased

H3K9me2 binding at many GABA$_A$ subunit gene promoters in NAc, consistent with cocaine's known suppression of this mark in this brain region and with the cocaine-induced patterns of GABA$_A$ subunit gene activation reported here (Fig. 4c).

To determine whether the reversal of cocaine-mediated increases in GABA$_A$ receptor subunit expression by MS-275 is functional, we recorded spontaneous inhibitory postsynaptic currents (IPSCs) from medium spiny neurons in the NAc shell of acute brain slices prepared 12 days after minipump implantation and 24 hours after repeated cocaine or saline. We found no difference in the amplitude of these events between the four conditions (vehicle + saline, vehicle + cocaine, MS-275 + saline, and MS-275 + cocaine; Fig. 4d). However, spontaneous IPSC frequency was significantly increased in animals receiving either cocaine or MS-275 infusions alone, and this increase was completely reversed in animals exposed to the two treatments in combination. The lack of change in amplitude coupled with an alteration in frequency suggests that cocaine or MS-275 induce an increase in the number of functional inhibitory synapses, without affecting individual synaptic strength, consistent with previous findings[25].

DISCUSSION

Our results provide the first evidence for a selective role of the nuclear specific Class I HDAC, HDAC1, in mediating cocaine-induced behavioral plasticity and suggest a novel interplay among activating and repressive histone PTMs in the transcriptional regulation of cocaine-induced molecular adaptations. We show that local knockout of HDAC1, as well as chronic and continuous infusion of MS-275, a pharmacological inhibitor highly selective *in vitro* for HDAC1, in NAc suppressed cocaine-induced locomotor sensitization. This effect was not seen with local knockout of other Class I isoforms, HDACs 2 and 3. Knockout of these different HDACs resulted in distinct patterns of altered expression of other HDACs, but only knockdown of HDAC1 affected expression of isoforms previously implicated in behavioral responses to cocaine. We further show that both repeated cocaine and chronic HDAC inhibition alone increased global levels of activating histone acetylation in the NAc but that, in combination, these treatments induced repressive histone methylation through H3K9ac binding at the promoters of specific KMTs and the subsequent induction of these enzymes. Analysis of promoter occupancy 4 hours after repeated cocaine revealed the selective association of HDAC1 with the *G9a* and *GLP* promoters in NAc, suggesting a mechanism for HDAC1's unique effects on repressive histone methylation and cocaine action. Further studies are needed to understand the molecular steps by which HDAC1, but not other Class I HDACs, is recruited to these KMT promoters. Finally, we identify GABA$_A$ receptor subunits as functional targets for both cocaine-induced decreases in H3K9me2 and cocaine plus MS-275 increases in this repressive methyl mark. Together, our findings suggest a dynamic interplay among different types of histone modifications in the adult brain, identify HDAC1 as a novel target for drug-induced plasticity, and reveal a role for, and mechanism by which, GABA$_A$ receptors in NAc are altered to mediate physiological changes important for the behavioral effects of cocaine.

Several studies to date have identified altered patterns of histone acetylation in NAc as important mediators of psychostimulant-induced behavioral and molecular plasticity.

HDACs tightly regulate histone acetylation and both non-selective HDAC inhibition and manipulations of specific Class II or III isoforms can alter psychostimulant-induced adaptations[1,3–5,10–16]. Class I HDACs differ from other classes of HDACs in that they are nuclear specific, demonstrating very little nuclear-cytoplasmic shuttling, and evidence suggests highly specific biological actions of the different isoforms within this class[27,28]. Recent reports have revealed that Class I HDAC isoforms 1–3 regulate memory and mediate responses to antipsychotic drugs[29–32], suggesting a potentially distinct role, as yet unexplored, for such enzymes in drug addiction as well. The selective role for HDAC1 reported here may seem surprising given evidence of regulatory cross talk between HDACs 1-3, such that loss of HDAC1 in embryonic stem cells causes a compensatory upregulation of HDACs 2 and 3[33]. However, such a compensatory mechanism is thought to be incomplete[33]. Moreover, we show a very different pattern of compensation in adult NAc. Local knockdown of HDAC1 led to decreased *HDAC2* and *SIRT1* expression, which is in agreement with previous findings that loss of HDAC1 leads to an overall reduction in total HDAC activity in non-nervous tissues[33]. Interestingly, HDAC1 knockdown also caused an upregulation in *HDAC5* expression. Previous findings have implicated both SIRT1 and HDAC5 in mediating behavioral responses to cocaine. *SIRT1* is upregulated in NAc following repeated exposure to cocaine and SIRT inhibition decreases behavioral responses to the drug[5]. Conversely, nuclear levels of HDAC5 are downregulated in NAc by repeated cocaine and loss of HDAC5 results in cocaine hypersensitivity[16]. While further work is required to determine the extent to which such compensatory regulation of SIRT1 and HDAC5 might contribute to the behavioral effects of HDAC1 knockdown, our observation of selective HDAC1 binding to H3K9 KMTs suggests an important direct role for HDAC1. As noted, evidence for distinct functions of Class I HDAC isoforms in the adult mouse brain has been reported recently in a study comparing HDAC1 and 2 contributions to cognitive processes. Although HDACs 1 and 2 form functional heterodimers and are often found in the same protein complexes[14], memory formation was impaired with overexpression of HDAC2 but not HDAC1 in the hippocampus[29]. Our findings show an opposite dissociation of function and implicate HDAC1 in NAc in cocaine-induced plasticity. These results thereby provide new insight into the selective roles of Class I HDACs in the adult mouse brain.

The effects of pharmacological inhibition of HDACs on psychostimulant-induced plasticity appear to depend on the timecourse of HDAC inhibition. Studies employing co-administration procedures in which inhibitors are given acutely, just prior to psychostimulant administration, report heightened behavioral responses to the drug[1,3,4,13,14]. In contrast, experimental paradigms like the one employed here, in which HDAC inhibitors are administered more chronically, for several days prior to psychostimulant exposure, show inhibited expression[3] or decreased acquisition of behavioral adaptations to drug[5,11,12]. The clustering of seemingly discrepant results based on experimental methodologies is interesting in light of our present findings. Both HDAC inhibitors and psychostimulants increase global levels of histone acetylation in NAc. Thus, when co-administered acutely, these drugs may have synergistic effects, leading to heightened transcriptional activation of psychostimulant-regulated target genes. In contrast, when a psychostimulant is given in the context of prolonged, HDAC inhibitor-induced hyperacetylation, homeostatic processes may

direct AcH3 binding to the promoters of genes (e.g., *G9a*) responsible for inducing chromatin condensation and gene repression (e.g., via H3K9me2) in order to dampen already heightened transcriptional activation. Our present findings thus demonstrate clear cross talk among histone PTMs and suggest that decreased behavioral sensitivity to psychostimulants following prolonged HDAC inhibition might be mediated through decreased activity of HDAC1 at H3K9 KMT promoters and subsequent increases in H3K9me2 and gene repression. The same complexity has been reported previously with local knockdown of HDAC5 in the NAc[16]. While many genes were induced, just as many were repressed, and among the induced genes were transcriptional repressors such as *SUV39H1*.

Previous studies examining chromatin remodeling in NAc underlying drug-induced behavioral plasticity have focused solely on histone PTMs known to occur on active, euchromatic regions of the genome (e.g. H3K14ac, H3K9ac, H3K9me2, H3K4me3). More recent evidence suggests that cocaine exposure additionally causes a de-repression of previously silenced chromatin (heterochromatin) in NAc: repeated cocaine reduced global levels of H3K9me3, a histone PTM that is associated specifically with silenced, non-coding regions of the genome, which mediated a decrease in total heterochromatin levels in NAc[23]. Our present findings, that chronic MS-275 plus repeated cocaine increase H3K9me3 and expression of *SUV39H1*, the enzyme that catalyzes this modification, suggest that regulation of non-coding regions of the genome may also be important for MS-275's suppression of cocaine-induced behavioral activation.

Numerous studies to date have implicated glutamatergic plasticity in the brain's reward circuitry in the development and maintenance of addictive-like behaviors. Studies have shown that altered glutamate receptor subunit levels and altered glutamate transmission in NAc are important molecular and physiological changes underlying the development of cocaine-induced behavioral sensitization[34–39]. Further evidence suggests that glutamate receptor subunits are targets for the transcription factors FosB and phospho-CREB, and that their genes display promoter hyperacetylation, providing one mechanism for the sustained changes in glutamatergic transmission in NAc in response to repeated cocaine[5,14,39,40]. More recent studies have begun to reveal a role for $GABA_A$ receptor subunits in NAc in mediating cocaine-induced behavioral adaptations. Repeated cocaine increases the frequency of $GABA_A$ receptor-mediated mIPSCs in striatonigral medium spiny neurons, and $\alpha2$ subunit-containing $GABA_A$ receptors are required for the induction of cocaine behavioral sensitization[25,26]. Furthermore, ChIP-chip analyses have identified several $GABA_A$ receptor subunits as direct, physiological targets for increased FosB and phospho-CREB binding following repeated cocaine[5]. Here, we provide further evidence for GABAergic plasticity in NAc as a potential mediator of cocaine behavioral plasticity and identify chromatin remodeling as a regulator of cocaine's effect on $GABA_A$ receptor subunit expression. Although $GABA_A$ receptor subunit expression, as well as inhibitory tone on NAc medium spiny neurons, was increased by either cocaine or MS-275 alone, only the cocaine-induced increase was associated with a behavioral phenotype. The absence of enhanced locomotor activity following MS-275 treatment alone suggests that, although increases in $GABA_A$ subunit expression and functional synaptic changes are necessary for

the expression of cocaine-induced locomotor activation, they are not sufficient. Several studies to date have identified putative target genes for cocaine-induced promoter hyperacetylation and transcriptional upregulation[1,5,14]. Only recently has cocaine-induced decreases in repressive H3K9me2 promoter binding been linked to the transcriptional upregulation of genes important in mediating behavioral adaptations to cocaine[6,41]. Our data provide evidence linking cocaine-induced increases in GABA$_A$ receptor subunit expression to decreased binding of H3K9me2 at these gene promoters. Although combined treatment of MS-275 and repeated cocaine did cause a significant increase in H3K9ac binding at GABA$_A$ subunit gene promoters, we found a much more prominent increase in repressive H3K9me2 enrichment, suggesting that the normalization of GABA$_A$ subunit expression and inhibitory tone on NAc medium spiny neurons under these conditions was mainly driven by a reversal of cocaine-induced transcriptional de-repression.

The finding that MS-275 with repeated cocaine increased both H3ac and H3me2 on Lys9 residues at GABA$_A$ subunit gene promoters deserves further comment. The NAc is composed primarily of two distinct subpopulations of medium spiny projection neurons, those expressing dopamine D1- and those expressing dopamine D2-receptors, and these different neuronal populations exhibit different molecular adaptations in response to repeated cocaine[42–44]. In the present study, we did not distinguish between these different neuronal subtypes, thus it is possible that the increases in H3ac and H3me2 at the same Lys residue reported here are occurring in different neuronal populations. Future experiments are required to address this and alternative possibilities.

The interaction between cocaine and MS-275 reported here is noteworthy. Either cocaine or MS-275 treatment alone caused global increases in H3 acetylation and increases in GABA$_A$ subunit gene expression, but when combined, these treatments caused increases in global repressive H3K9me2, most likely driven by a loss of HDAC1 and a subsequent gain in H3ac at H3K9 KMT promoters, that prevented cocaine-induced increases in GABA$_A$ subunit gene expression and inhibitory tone in NAc (Supplementary Fig. 5). The results highlight a unique mode of biological regulation that provides further insight into mechanisms of chromatin regulation in the adult brain. Results of the present study also specifically inform the mechanisms underlying the prolonged actions of HDAC inhibitors in NAc and broaden current knowledge of molecular and chromatin endpoints for novel addiction treatments.

METHODS

Animals and treatments

Animals were housed in a colony room maintained at a constant temperature (23°C) on a 12 hour light/dark cycle (lights on from 0700 to 1900 hour) with *ad libitum* access to food and water. Eight- to 10-week-old male C57BL6/J mice (Jackson Laboratory, Bar Harbor, ME, USA) were used in all experiments unless stated otherwise. Animals were housed 5 per cage and habituated in our facility 1 week before experimentation. Members of the same cage were randomly assigned to different experimental groups for behavioral studies. All mouse procedures were approved and performed in accordance with guidelines stipulated by the Institutional Animal Care and Use Committee at Mount Sinai.

To induce focal deletion of specific Class I HDACs in NAc neurons, we used mutant mice homozygous for a floxed HDAC1, 2 or 3 allele (backcrossed to C57BL/6J mice) which have been described elsewhere[18,19]. Mice were sterotaxically-injected with herpes simplex virus (HSV) vectors expressing GFP or Cre-GFP (*HDAC1*$^{fl/fl}$ and *HDAC2*$^{fl/fl}$) or adeno-associated virus (AAV) vectors expressing GFP or Cre-GFP (*HDAC3*$^{fl/fl}$) in the NAc between the age of 8 and 10 weeks. AAV vectors were used for HDAC3, as they were required to obtain a degree of knockdown equivalent to that seen for HDACs 1 and 2. Both vectors target neurons only and infect ~1 mm^3 of brain[6] (see Supplemental Fig. 1). The efficiency of Cre-mediated recombination was quantified by quantitative PCR (qPCR) at the end of behavioral testing. For experiments administering the pharmacological inhibitor *N*-(2-aminophenyl)-4-[*N*-(pyridin-3-yl-methoxycarbonyl) aminomethyl]benzamide (MS-275, 100 μM; provided by Dr. Stephen Haggarty, the Broad Institute), mice were stereotaxically implanted with subcutaneous Alzet mini-pumps (model 1002; Durect) fastened to bilateral guide cannulae targeting the NAc. Twelve hours prior to implantation, mini-pumps were activated to initiate continuous delivery (0.25 μl/hour over 14 days) of either vehicle (5 hydroxypropyl β-cyclodextrin; Trappsol, CTD) or drug. All mice were singly housed post-surgery.

For Western blotting, qPCR, chromatin immunoprecipitation (ChIP), and electrophysiology, mice receiving continuous delivery of either vehicle or MS-275 for 5 days were started on daily injections of either saline (7 treatments saline, i.p.) or cocaine (7 treatments 20 mg/kg cocaine-HCl, i.p.). Mice were used 24 hours after the final drug treatment and 12 days of continuous vehicle or MS-275 infusion. For HDAC ChIPs, non-surgery mice received daily injections of either saline (7 treatments saline, i.p.) or cocaine (7 treatments 20 mg/kg, i.p.) and were used 4 hours after the final drug treatment. Note that we used a higher dose of cocaine for these molecular studies compared with our behavioral testing, based on established precedents in the literature where such higher doses are often required to detect statistically significant findings.

Stereotaxic surgery

Mice anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) were positioned in a small-animal stereotax and the cranial surface was exposed. Bilateral infusions of virus (0.5 μl HSV or AAV) into the NAc were administered at a rate of 0.1 μl/min using 33 gauge syringe needles positioned at a 10° angle (AP + 1.6; ML + 1.5; DV − 4.4 relative to Bregma). Mice receiving HSV or AAV infusions were given 6 and 20 days of recovery following surgery, respectively. These timepoints were selected based on the onset of maximal viral-mediated transgene expression for the two vectors and to mimic the timecourse of MS-275 treatment prior to behavioral testing. For MS-275 infusions, two mini-pumps were positioned subcutaneously on the mouse's back. Two small cranial holes were made above the NAc and bilateral cannulae (28 gauge stainless steel) were lowered at a position of AP + 1.4; ML + 1.0; DV −4.0 from bregma. Mice were given 5 days recovery, with continuous MS-275 or vehicle infusion, before beginning the cocaine locomotor assay described below. All cannulae placements were verified at the time animals were killed through visualization of the cannulae tracks; only animals with tracks terminating in the NAc were included in the study (>90% of all animals tested).

For bicuculline (Tocris, Ellisville, MO) and acute MS-275 infusions, bilateral guide cannulae (26-gauge, 5.0 mm length) were introduced 2.5 mm above the NAc at a position of AP +1.6; ML + 1.0; DV − 3.4 from bregma. To prevent obstruction of the cannulae, at the end of surgery, dummy cannulae were inserted into the guide cannulae. Mice were given 1 week recovery before beginning the cocaine locomotor assay described below. After behavioral testing, injection sites were confirmed by direct visualization as noted above. In the case of the acute MS-275 experiment 15-gauge NAc punches were collected for Western blot analysis 30 minutes following the end behavioral testing.

Locomotor activity assay

Mice were injected with saline or cocaine (i.p.) at the same time each day and placed in standard rat cages located inside a Photobeam Activity System (San Diego Instruments, San Diego, CA). On day 0, mice were acclimated to the novel environment for 30 minutes and then given a saline injection for baseline activity measures. On days 1–5 mice were given injections of cocaine (10 mg/kg). Horizontal ambulations in the x and y plane were measured for 30 minutes following all injections.

For bicuculline and acute MS-275 experiments, daily drug (bicuculline dissolved in 0.9% saline or MS-275, 100μM, dissolved in 5 hydroxypropyl β-cyclodextrin) or vehicle infusions (10 ng/0.5 μl/side over 2.5 minutes) into the NAc were made through an inner cannula (33-gauge) attached to a Hamilton syringe 15 minutes prior to behavioral testing. The experimenter was not blinded for these experiments. Instances of faulty behavioral tracking were removed from the analyses.

RNA isolation and qPCR

Bilateral 15-gauge NAc punches were homogenized in Trizol and processed according to the manufacturer's instructions. RNA was purified with RNAesy Micro columns (QIAGEN) and spectroscopy confirmed that the RNA had 260/280 and 260/230 ratios >1.8. RNA was reversed transcribed into cDNA using iScript cDNA synthesis (Bio-Rad). qPCR was performed using ~2.5 ng of cDNA for each reaction plus primers and SYBR Green. Each reaction was run in duplicate and analyzed following the ΔΔCt method as previously described using glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) as a normalization control[16]. See Supplemental Table S1 for mRNA primer sequences. The experimenter was not blinded for these experiments.

Western blot analysis

15-gauge NAc punches were homogenized in 30 μl of 1 M HEPES lysis buffer (1% SDS) containing protease and phosphatase inhibitors using an ultrasonic processor. Protein concentrations were determined using a DC protein assay and 10–20 μg samples of total protein were electrophoresed on 18% Tris-HCl polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane, blocked for 1 hour in 5% BSA, and incubated overnight at 4°C with either anti-AcH3K14 (Millipore, 1:1,000), anti-AcH3K9 (Abcam, 1:1,000), anti-H3K9me2 (Abcam, 1:500), anti-H3K9me3 (Millipore, 1:500), anti-H3K4me3 (Millipore, 1:500), anti-H3K27me3 (Millipore, 1:500), anti-GAPDH (Cell Signaling, 1:60,000) or anti-total histone H3 (Abcam, 1:5,000). Membranes were then incubated with

peroxidase-labeled secondary antibodies (1:15,000-1:60,000 depending on the primary antibody used) and bands were visualized using SuperSignal West Dura substrate. Bands were quantified with NIH Image J Software and normalized to GAPDH to control for equal loading. The experimenter was not blinded for these experiments.

Chromatin immunoprecipitation (ChIP)

Fresh NAc punches were prepared for ChIP as previously described[5,45] with minor modifications. Briefly, 2 15-gauge NAc punches per animal (4 animals pooled per sample) were collected, cross-linked with 1% formaldehyde and quenched with 2 M glycine before freezing at −80°C. Prior to sample sonication, IgG magnetic beads (Invitrogen; sheep anti-rabbit/mouse) were incubated with either anti-AcH3K9 (rabbit polyclonal ChIP grade, Abcam), anti-H3K9me2 (mouse monoclonal ChIP grade, Abcam), anti-HDAC1 (mouse monoclonal ChIP grade, Abcam), anti-HDAC2 (mouse monoclonal ChIP grade, Abcam) or anti-HDAC3 (mouse monoclonal ChIP grade, Abcam) antibodies overnight at 4°C under constant rotation in block solution. Tissue sonication and chromatin shearing were carried out as previously described[5]. Average DNA fragment size in the range of 200–1,000 bps was confirmed using Agilent 2100 Bioanalyzer. Following sonication, equal concentrations of chromatin were transferred to new tubes and ~5% of the final products were saved to serve as input controls. Following washing and resuspension of the antibody/bead conjugates, antibody/bead mixtures (~7.0 μg antibody/sample) were added to each chromatin sample and incubated for ~16 hours under constant rotation at 4°C. Samples were further washed and reverse cross-linked at 65°C overnight and DNA purified using a PCR purification kit (QIAGEN). Following DNA purification, samples were used for qPCR analysis and normalized to their appropriate input controls as previously described[5]. Normal mouse IgG immunoprecipitations were performed to control for appropriate enrichment of signal amplification. See Supplemental Table S1 for promoter primer sequences. The experimenter was not blinded for these experiments. The anti-HDAC1, -2, and -3 antibodies used here for ChIP have been utilized in prior studies[30,32,33]. While we demonstrated the specificity of each antibody with peptide blocking experiments and western blotting (see main text), another important control lacking in the field is demonstrating selective immunoprecipitation of each HDAC isoform by its respective antibody. Until such controls become feasible technically, data for HDAC ChIPs must be viewed with some caution.

Electrophysiology

Coronal NAc slices (250 μm thick) were cut in ice-cold sucrose artificial CSF (ACSF) (in mM): 254 sucrose, 3 KCl, 1.25 NaH$_2$PO4, 10 D-glucose, 24 NaHCO$_3$, 2 CaCl$_2$, and 2 MgSO$_4$, pH 7.35 (oxygenated with 95% O$_2$ and 5% CO$_2$, 295–305 mOsm). After a 1 h recovery at 37°C in ACSF (254 mM sucrose replaced by 128 mM NaCl), electrophysiological recordings were performed at 30–32°C in ACSF containing 1 mM kynurenic acid to block EPSPs and 1.5 μM tetrodotoxin to block action potentials. Patch pipettes (3–5 MΩ resistance) for whole-cell recordings were filled with an internal solution containing the following (in mM): 130 cesium chloride, 10 HEPES, 10 phosphocreatine, 10 EGTA, 2 ATP-Mg, 0.5 GTP, and 1 QX-314 [2(triethylamino)-N-(2,6-dimethylphenyl) acetamine] (a Na$^+$ channel blocker), pH 7.4 (280–290 mOsm). The shell of the NAc was identified under visual guidance using infrared differential interference contrast video

microscopy with a 40× water-immersion objective (Olympus BX51-WI). Whole-cell voltage-clamp recordings were performed with a computer-controlled amplifier (MultiClamp 700B), digitized (Digidata 1440), and acquired with Axoscope 10.1 (Molecular Devices) at a sampling rate of 10 kHz. Only cells with resting membrane potential of −72 to −82 mV and that displayed inward rectification were included for analysis. The frequency and amplitude of spontaneous IPSCs were analyzed using Minianalysis software (Synaptosoft). These experiments employed a blinding procedure.

Statistical analysis

Three-way ANOVAs were used to calculate statistics for locomotor activity of $HDAC1^{fl/fl}$, $HDAC2^{fl/fl}$, $HDAC3^{fl/fl}$, and MS-275 treated mice and their controls. Behavioral data for these experiments were transformed to square root as in all of these datasets the means were proportional to the variance as opposed to the standard deviation. One- and two-way ANOVAs were performed to determine significance for all comparisons involving MS-275 and treatment, including Western blotting, qPCR, ChIP, and electrophysiology experiments. Student's t tests were performed to determine significance for all comparisons involving HDAC chromatin immunoprecipitation with corrections for multiple comparisons. The Grubbs' test was employed when appropriate to identify outliers. All experiments were carried out 1–3 times and in instances of repeated experiments data replication was observed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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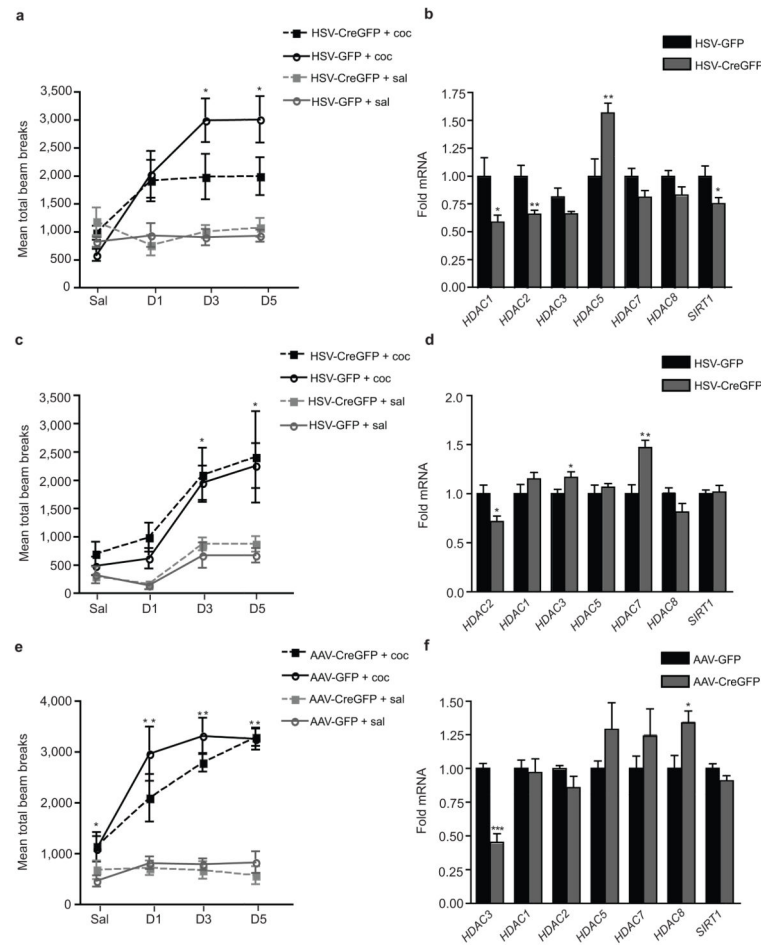
Figure 1.
HDAC1 in NAc regulates locomotor responses to cocaine. (**a**, **c**, **e**) Cocaine (coc, 10 mg/kg) locomotor sensitization in floxed- (**a**) HDAC1 and (**c**) HDAC2 mice injected with HSV-CreGFP or HSV-GFP, and (**e**) HDAC3 mice injected with AAV-CreGFP or AAV-GFP. All data are presented as mean ± s.e.m. (**a**, **c**, **e**) A significant main effect (three-way ANOVA on square-root transformed data) of day (**a,** $F_{3,90} = 11.257$, $P < 0.0005$; **c,** $F_{3,36} = 26.040$, $P < 0.0001$; **e,** $F_{3,57} = 19.479$, $P < 0.001$) and drug (**c,** $F_{1,12} = 24.238$, $P < 0.0001$) and significant interactions between day and virus (**a,** $F_{3,90} = 3.007$, $P = 0.034$) and day and drug (**a,** $F_{3,90} = 11.741$, $P < 0.0005$; **e,** $F_{3,57} = 11.976$, $P < 0.001$) as well as a trend towards a day by drug by virus interaction (**a,** $F_{3,90} = 2.173$, $P = 0.097$) were observed. $*P < 0.04$ and $**P < 0.001$, Bonferroni *post hoc* tests. (**b**, **d, f**) qPCR validation of (**b**) HDAC1, (**d**) HDAC2, and (**f**) HDAC3 knockdown and mRNA expression of other HDACs in the NAc of *HDAC1^{fl/fl}*, *HDAC2^{fl/fl}* and *HDAC3^{fl/fl}* mice. $*P < 0.05$, $**P < 0.01$ and $***P < 0.0001$, student's *t* test. (For behavioral experiments: N = 4–6/group (saline condition); N = 6–11/group (cocaine condition). For qPCR validation: N = 6–8/group).
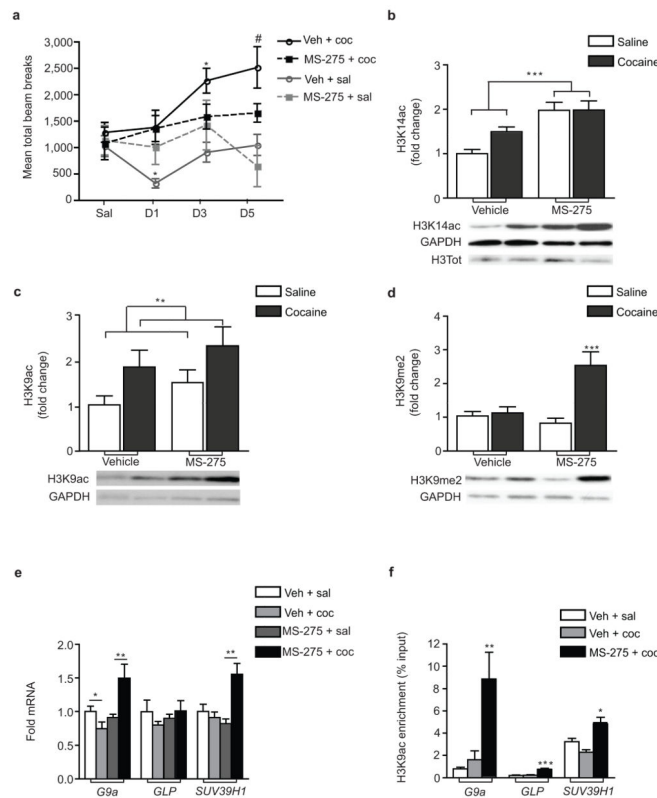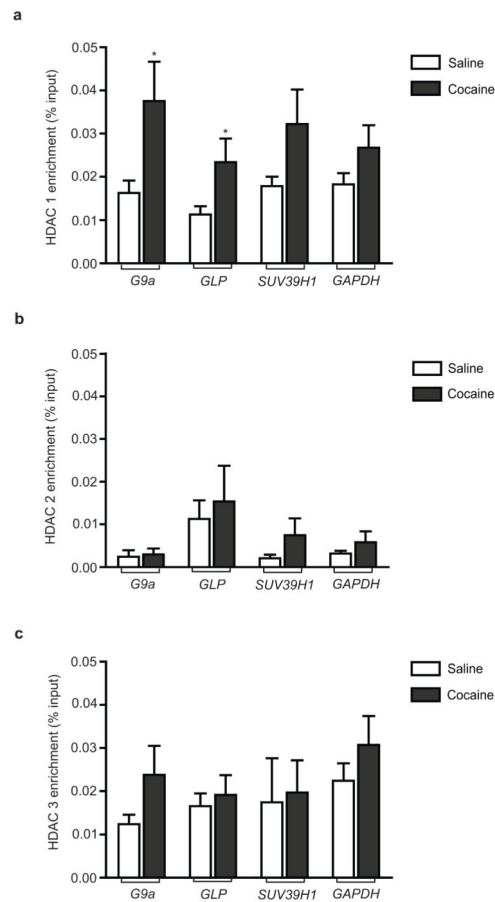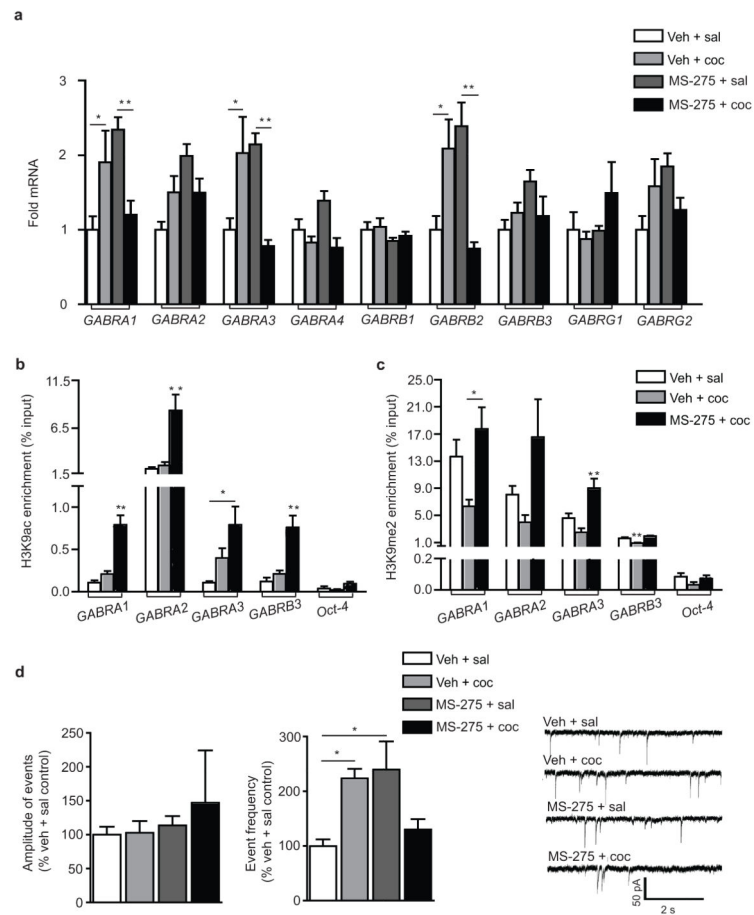
Figure 2.
Chronic MS-275 infusion into NAc blocks locomotor responses to cocaine and alters repressive histone methylation. (**a**) Cocaine (10 mg/kg) locomotor sensitization in animals receiving continuous intra-NAc infusion of MS-275 or vehicle (Veh) (100 μM). A significant main effect (three-way ANOVA on square-root transformed data followed by Bonferroni *post hoc* tests) of day ($F_{3,54} = 3.004$, $P = 0.037$) and significant interactions between day and drug ($F_{3,54} = 4.578$, $P = 0.006$) and day and treatment ($F_{3,54} = 3.101$, $P = 0.034$) were observed. Day by drug by treatment interaction ($F_{3,54} = 1.607$, $P = 0.19$). (N = 5 or 6/group (vehicle treatment); N = 6 or 7/group (MS-275 treatment). (**b**–**d**) Global levels of H3 acetylation and methylation in NAc after 12 days of continuous treatment with MS-275 (100 μM) and 24 hour after repeated cocaine (20 mg/kg, seven daily doses). All data represented as normalized values to GAPDH (**b**) A significant main effect (two-way ANOVA followed by Bonferroni *post hoc* tests) of treatment (H3K14ac, $F_{1,22} = 23.20$, ***$P < 0.001$), (**c**) a main effect of drug (H3K9ac, $F_{1,24} = 6.252$, **$P < 0.02$), and (**d**) a significant interaction between drug and treatment (H3K9me2, $F_{1,18} = 15.23$, $P = 0.001$) were observed (N = 5–8/group). Full-length blots are presented in Supplemental Figure 6. (**e** and **f**) mRNA expression of H3K9 KMTs and quantitative H3K9ac ChIP in NAc after 12 days of continuous treatment with MS-275 (100 μM) and 24 hour after repeated cocaine (20 mg/kg). (**e**) A significant interaction (two-way ANOVA followed by Bonferroni *post hoc* and planned student's *t* tests) between treatment and drug (*G9a*, $F_{1,27} = 11.11$, $P = 0.0025$; *SUV39H1*- $F_{1,27} = 12.82$, $P = 0.0013$) was observed (N = 7 or 8/group). (**f**) A significant effect (one-way ANOVA followed by Bonferroni *post hoc* tests) of MS-275 with repeated

cocaine on H3K9ac binding to H3K9 KMT promoters ($G9a$, $F_{2,16} = 8.749$, $P = 0.0034$; GLP, $F_{2,16} = 21.01$, $P < 0.0001$; $SUV39H1$, $F_{2,16} = 9.108$, $P = 0.0029$ was observed (N = 5 or 6/group). [#]$P = 0.14$ (trend), *$P < 0.05$ and **$P < 0.02$, ***$P < 0.001$. All data are represented as mean ± s.e.m.

Figure 3.
HDAC binding to KMT gene promoters after repeated cocaine. (**a**–**c**) Quantitative ChIP for HDAC1, 2, and 3 in NAc from animals treated with repeated cocaine at 4 hours after the last injection. Significance determined using Student's *t* tests. (**a**) HDAC1 (*GAPDH*, $t_{13} = 1.520$, $P = 0.1524$; *G9a*, $t_{14} = 2.227$, *P 0.05; *GLP*, $t_{14} = 2.090$, *P 0.05; *SUV39H1*, $t_{12} = 1.735$, $P = 0.108$) (N = 7 or 8/group). (**b**) HDAC2 (*GAPDH*, $t_{14} = 0.9857$, $P = 0.341$), (*G9a*, $t_{12} = 0.2604$, $P = 0.799$), (*GLP*, $t_{11} = 0.3611$, $P = 0.7249$), (*SUV39H1*, $t_{14} = 1.343$, $P = 0.2006$) (N = 5–8/group). (**c**) HDAC3 (*GAPDH*, $t_{12} = 1.061$, $P = 0.3098$), (*G9a*, $t_{12} = 1.624$, $P = 0.1303$), (*GLP*, $t_{12} = 0.4761$, $P = 0.6245$), (*SUV39H1*, $t_{12} = .1775$, $P = 0.8621$) (N = 7/group). All data are presented as percent input enrichment.

Figure 4.
Chronic MS-275 infusion prevents cocaine-induced changes in GABA$_A$ receptor subunit expression and GABAergic tone in NAc. (**a**) mRNA expression of GABA$_A$ receptor subunits (N = 5–8/group) in NAc after 12 days of continuous treatment with MS-275 (100 μM) and 24 hours after repeated cocaine (20 mg/kg). A significant interaction (two-way ANOVA followed by Bonferonni *post hoc* tests) between treatment and drug for many subunits was observed (*GABRA1*, $F_{1,27} = 16.62$, $P = 0.004$; *GABRA2*, $F_{1,27} = 8.658$, $P = 0.0066$; *GABRA3*, $F_{1,26} = 21.83$, $P < 0.0001$; *GABRB2*, $F_{1,22} = 21.84$, $P < 0.0001$; *GABRG2*, $F_{1,20} = 6.458$, $P = 0.0194$). (**b, c**) Quantitative H3K9ac and H3K9me2 ChIP in NAc from mice treated with repeated cocaine or chronic MS-275 infusion into NAc with repeated cocaine at 24 hours. Data are presented as percent input enrichment (N = 5 or 6/group). (**b**) A significant effect (one-way ANOVA followed by Bonferonni *post hoc* tests) of MS-275 with repeated cocaine on H3K9ac binding at GABA$_A$ receptor subunit promoters (*GABRA1*, $F_{2,16} = 26.02$, $P < 0.0001$; *GABRA2*, $F_{2,16} = 11.37$, $P = 0.0012$; *GABRA3*, $F_{2,14} = 5.841$, $P = 0.0169$; *GABRB3*, $F_{2,16} = 14.39$, $P = 0.0004$; *Oct-4*, $F_{2,12} = 3.038$, $P = 0.0932$) was observed. (**c**) A significant difference (one-way ANOVA followed by Bonferonni *post hoc* tests) in H3K9me2 binding at GABA$_A$ receptor subunit promoters (*GABRA1*, $F_{2,16} = 4.902$, $P = 0.0243$; *GABRA2*, $F_{2,15} = 3.910$, $P = 0.0468$; *GABRA3*, $F_{2,16} = 10.77$, $P = 0.0015$; *GABRB3*, $F_{2,15} = 14.83$, $P = 0.0004$; *Oct-4*, $F_{2,13} = 1.365$, $P = 0.2954$) was observed. (**d**)

IPSCs from NAc medium spiny neurons from mice treated with chronic saline or cocaine, and exposed to intra-NAc infusion of vehicle or MS-275 (N = 5–8/group). A significant interaction (two-way ANOVA followed by Bonferonni *post hoc* tests) between treatment and drug was observed for the frequency of spontaneous IPSCs ($F_{1,23} = 14.43$, $P = 0.0009$). *$P < 0.05$ and **$P < 0.01$. All data presented as mean ± s.e.m.