Behavioral/Cognitive

HDAC3 Is a Negative Regulator of Cocaine-Context-Associated Memory Formation

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Cocaine-induced neuroplasticity mediated by histone acetylating and deacetylating enzymes may contribute to addiction-like behaviors. For example, overexpression of histone deacetylases (HDACs) 4 or 5 in the nucleus accumbens suppresses cocaine-induced conditioned place preference (CPP) acquisition in mice. HDAC4 and HDAC5 are known to interact with HDAC3, but the role of HDAC3 in cocaine-induced behaviors has never been examined. In this study, we address the hypothesis that HDAC3 is a negative regulator of cocaine-context-associated memory formation in mice. We examined the role of HDAC3 during the conditioning phase of CPP, when the mouse has the opportunity to form an associative memory between the cocaine-paired context and the subjective effects of cocaine. To address this hypothesis, *Hdac3*$^{flox/flox}$ and *Hdac3*$^{+/+}$ mice (generated from a C57BL/6 background) were infused into the nucleus accumbens with adeno-associated virus expressing Cre recombinase to create focal, homozygous *Hdac3* deletions. *Hdac3*$^{flox/flox}$ mice exhibit significantly enhanced CPP acquisition, which is correlated with increased gene expression during the consolidation phase of acquisition. Increased gene expression of *c-Fos* and *Nr4a2* is correlated with decreased HDAC3 occupancy and increased histone H4 lysine 8 acetylation at their promoters. The results from this study demonstrate that HDAC3 negatively regulates cocaine-induced CPP acquisition.

Introduction

Drugs of abuse strengthen associations between drug-context-associated cues and the drug's reinforcing effects (Everitt and Robbins, 2005; Levine et al., 2005; Hyman et al., 2006). It is hypothesized that similar molecular mechanisms responsible for long-term memory formation also participate in the formation of long-term, cocaine-context-associated memories (Nestler, 2002; Hyman, 2005; Everitt et al., 2008). An underlying molecular mechanism of both cocaine-induced neuroplasticity associated with addiction (Kumar et al., 2005; Renthal et al., 2007; McClung and Nestler, 2008; Malvaez et al., 2011; Benekareddy et al., 2012; for review, see Rogge and Wood, 2012) and long-term memory formation (Swank and Sweatt, 2001; Levenson et al., 2004; Fischer et al., 2007; Vecsey et al., 2007; for review, see Peixoto and Abel, 2012) is histone acetylation, a form of chromatin modification.

Histone acetylation modulates histone-DNA interactions via histone-acetyltransferases (HATs), which usually facilitate transcription, and histone deacetylases (HDACs), which usually repress transcription (Kouzarides, 2007). In the hippocampus, the HAT CREB-binding protein is a critical, positive regulator of long-term memory formation (Alarcón et al., 2004; Korzus et al., 2004; Wood et al., 2005; Barrett et al., 2011). In the nucleus accumbens (NAc), CREB-binding protein was recently found to mediate acute cocaine-induced histone acetylation, gene expression, and conditioned place preference (CPP) acquisition (Malvaez et al., 2011).

Like HATs, HDACs are also involved in both long-term memory formation and CPP acquisition. In the NAc, HDAC4 and HDAC5 are negative regulators of CPP acquisition (Kumar et al., 2005; Renthal et al., 2007; Taniguchi et al., 2012). Similarly, HDAC2 and HDAC3 are negative regulators of memory formation (Guan et al., 2009; McQuown et al., 2011). Interestingly, HDAC3, which is enriched in the mouse NAc (Renthal et al., 2007), functions *in vitro* with HDAC4 or HDAC5 in multiprotein transcriptional repressor complexes (Fischle et al., 2002, Karagianni and Wong, 2007; Lahm et al., 2007). Therefore, HDAC3, in association with HDAC4 and/or HDAC5 in the NAc, may be involved in CPP acquisition.

Considering that HDAC3 is a negative regulator of memory formation (McQuown et al., 2011), we hypothesized that HDAC3 negatively regulates cocaine-context-associated memory formation (as tested by CPP, a model of cocaine-context-associated memory; Cunningham et al., 2006). More specifically, we predict that cocaine exposure during the conditioning phase of CPP relieves HDAC3-mediated repression of genes necessary for the contextual association that leads to acquisition. In support of the idea that HDACs prevent strengthening of associative memories during CPP conditioning, Taniguchi et al. (2012) observed that viral overexpression of a mutant, nuclear sequestered

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form of HDAC5 in the mouse NAc suppresses cocaine-induced CPP acquisition only when transduced before CPP conditioning, but not when transduced after CPP conditioning. This suggests that HDACs function to regulate transcription during the consolidation phase of CPP acquisition. We addressed the above hypothesis using *Hdac3*$^{flox/flox}$ genetically modified adult mice (Mullican et al., 2011) treated with adeno-associated virus expressing Cre recombinase (AAV-Cre) to generate NAc-specific deletions of *Hdac3* and examine the effect on histone acetylation, gene expression, and cocaine-induced CPP.

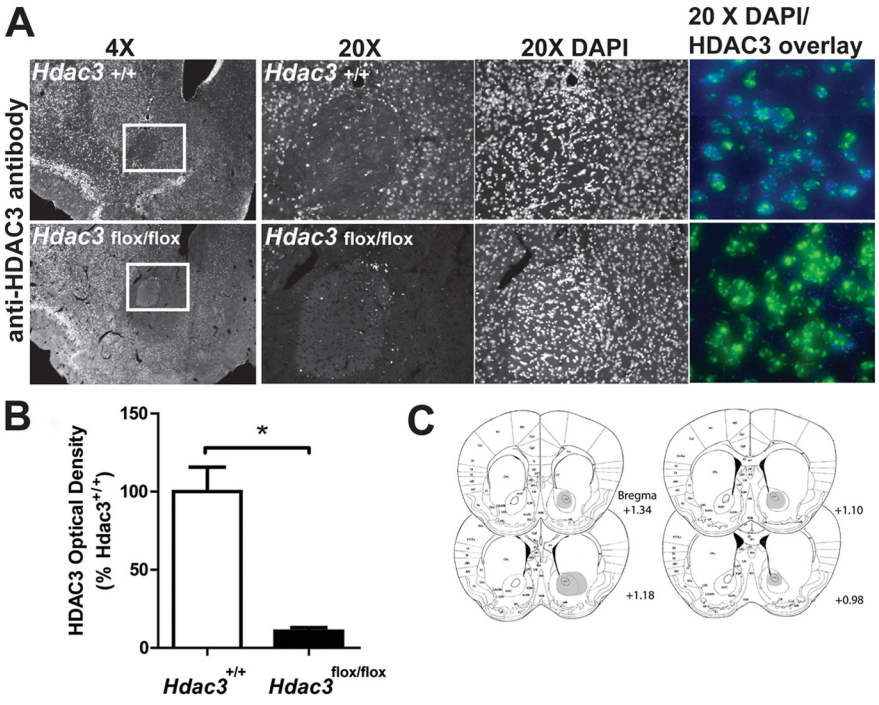

Figure 1. Intra-NAc AAV-Cre infusions generate NAc-specific deletions of HDAC3 in *Hdac3*$^{flox/flox}$, but not *Hdac3*$^{+/+}$, mice. ***A***, Focal, homozygous deletion of *Hdac3* in the NAc is shown in 4× and 20× magnified images of representative coronal hemisections from *Hdac3*$^{+/+}$ (top) and *Hdac3*$^{flox/flox}$ (bottom) mice (A/P + 1.25 relative to bregma) subjected to IHC using anti-HDAC3 specific antibodies. The NAc is boxed in white. HDAC3 immunoreactivity is unaffected in *Hdac3*$^{+/+}$ mice. Magnified confocal images (20×) of HDAC3- and DAPI-stained tissue (right, HDAC3 = blue; DAPI = green; *Hdac3*$^{+/+}$ = top; *Hdac3*$^{flox/flox}$ = bottom) confirm the presence and integrity of nuclei in both genotypes. ***B***, Quantification of anti-HDAC3 IHC demonstrates that HDAC3 is significantly less abundant in intra-NAc AAV-Cre infused *Hdac3*$^{flox/flox}$ vs *Hdac3*$^{+/+}$ mice. *Hdac3*$^{flox/flox}$, $n = 40$; *Hdac3*$^{+/+}$, $n = 39$. *$p < 0.001$. ***C***, The shaded regions of the mouse atlas images illustrate the extent of HDAC3 deletions. Although the figure is shaded in only one hemisphere, all mice used in the study harbored bilateral, NAc-specific deletions of HDAC3.

Materials and Methods

Subjects and surgical procedures

All experiments were performed in accordance with the institutional animal care and use committee at the University of California, Irvine, and were consistent with Federal guidelines. Mice of either sex were 8–12 weeks old and had access to food and water *ad libitum* in their home cages with lights maintained on a 12 h light/dark cycle. Behavioral testing was performed during the light portion of the cycle. *Hdac3*$^{flox/flox}$ and wild-type (*Hdac3*$^{+/+}$) littermate mice were maintained on a C57BL/6 background (Mullican et al., 2011). Briefly, these mice were generated at the laboratory of Dr. Mitch Lazar at the University of Pennsylvania (Philadelphia, PA) with loxP sites flanking exon 4 through exon 7 of the *Hdac3* gene, a region required for the catalytic activity of the enzyme. NAc-specific *Hdac3* deletions were generated 2 weeks before behavioral testing by infusing 0.25 μl (~1 E^{13} vector particles, titer and quality quantified by Penn Vector Core, University of Pennsylvania) of AAV2.1-Cre (AAV-Cre; Penn Vector Core) at a rate of 0.1 μl/min bilaterally into the NAc (A/P + 1.2 mm; M/L +1.0 mm; D/V −4.2 mm) of *Hdac3*$^{flox/flox}$ and *Hdac3*$^{+/+}$ mice, anesthetized with isofluorane in a digital stereotaxis (Stoelting).

Immunohistochemistry

To verify *Hdac3* deletions after CPP testing, all mice were deeply anesthetized with 0.1 ml of sodium pentobarbital injected intraperitoneally (50 mg/kg; Sigma-Aldrich) and perfused transcardially with ice-cold PBS (PBS, pH 7.4; Sigma-Aldrich) followed by ice-cold 4% paraformaldehyde (pH 7.4; Fisher Scientific) using a peristaltic perfusion pump (Fisher Scientific). Whole brain specimens were harvested and placed in 4% paraformaldehyde solution at 4°C overnight followed by incubation in 30% sucrose-ddH2O solution (Fisher Scientific) for 48 h at 4°C before sectioning. Brains were embedded in Tissue-Tek OCT (Sakura FineTek) and sectioned at −20°C. Serial coronal sections 20 μm thick were cut on a Cryostat and collected from the region corresponding to the NAc. In all sections, DAPI staining was performed to visualize nuclei. Immunohistochemistry (IHC) was performed with anti-HDAC3 IgG (1:1000; Cell Signaling Technology; Fig. 1*A*) primary antibody and goat anti-rabbit FITC secondary (Jackson ImmunoResearch). Images were acquired using an Olympus BX51 microscope with a 4× or 20× objective, CCD camera (QImaging), and QCapture Pro 6.0 software (QImaging). Confocal raster scan images were acquired with Zeiss Axiovert 200M inverted microscope using a 20× apochromatic objective using the Slide Scan module of MetaMorph (Molecular Devices). ImageJ software was used to quantify optical densities of target proteins in both hemispheres of at least three slices per animal to give a mean optical density ± SEM for that animal.

Coronal sections from a separate set of mice (those used in the quantitative RT-PCR [RT-qPCR] studies, see below) were used to examine H4K8Ac levels by IHC with anti-H4K8Ac IgG (1:1000; Abcam; Fig. 3*B*). Those slices came from flash-frozen brains of mice killed 1 h after drug injection and 30 min after conditioning (see below). The 20-μm-thick coronal sections were thaw mounted on glass slides, fixed with 4% paraformaldehyde for 10 min at room temperature, and subjected to IHC as described above.

CPP

Cocaine-induced CPP was performed as described previously (Malvaez et al., 2010). Briefly, mice were handled for 2 min/d for 3 d before the pretest. The next day, they were paired 30 min/d for 4 d (unbiased, counterbalanced protocol) with alternating intraperitoneal injections of cocaine-HCl (2.5, 5, or 10 mg/kg; Sigma-Aldrich) and 0.9% saline. Forty-eight hours after the last conditioning session, preference (difference in time spent in the cocaine-paired compartment compared with the saline-paired compartment [CPP score]) was assessed (15 min test) in all animals in a drug-free state. Preference and total distance traveled (to rule out behavioral changes in motility as a confounding factor) were tracked with MPEG videos recorded with digital video cameras mounted above the CPP chambers using EthoVision 3.1 software (Noldus Technology).

Chromatin immunoprecipitation and RT-qPCR

Hdac3$^{flox/flox}$, *Hdac3*$^{+/+}$, and C57BL/6 mice were handled, administered the pretest, and given either 5 mg/kg cocaine or saline in a paired compartment as described above for CPP. One hour after that injection (and 30 min after the end of the conditioning session), mice were killed by cervical dislocation and the brains were flash frozen by submersion into dry, ice-cold isopentane and stored at −80°C. In the case of C57BL/6 tissue, the same brains were used for RT-qPCR and chromatin immuno-

precipitation (ChIP) by collecting punches from one hemisphere for PCR and the other for ChIP in a counterbalanced fashion.

RT-qPCR. Tissue was collected as $2 \times 500\ \mu m^3$ punches from coronal sections in the area of the focal deletion in *Hdac3*$^{flox/flox}$ and comparable area in *Hdac3*$^{+/+}$ mice as confirmed by IHC from adjacent 20 μm coronal sections (see above). RNA was isolated using the RNeasy Minikit (Qiagen). cDNA was made from 50 ng of total RNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). The following primers were derived from the Roche Universal ProbeLibrary: *c-Fos* mRNA left primer, 5′-ggggcaaagtagagcagcta-3′; *c-Fos* mRNA right primer, 5′-agctccctcctccgattc-3′; probe, atggctgc; *Nr4a2* left primer, 5′-ttgcagaatatgaacatcgaca-3′; *Nr4a2* right primer, 5′-gttccttgagcccgtgtct-3′; probe, ttctcctg; *Grin3a* left primer, 5′-ggtttcacagagcattaaacacat-3′; *Grin3a* right primer, 5′-ggtccatcttctccatctgc-3′; probe, gcagccat; *Egr2* left primer, 5′-ctacccggtggaagacctc-3′; *Egr2* right primer, 5′-gtcaatgttgatcatgccatct-3′; probe, cttcccca; *Hdac4* left primer, 5′-aatcctgcccgtgtgaac-3′; *Hdac4* right primer, 5′-gtaggggccacttgcaga-3′; probe, gcccagca; *Hdac5* left primer, 5′-ccacggactcctctgcat-3′; *Hdac5* right primer, 5′-ctggggctacctccacct-3′; probe, caggagcc; *Atf3* left primer, 5′-gacagagtgcctgcacaaag-3′; *Atf3* right primer, 5′-catgtatatcaaatgctgttt-3′; probe 17 of Roche Universal Library (*c-Fos*, *Nr4a2*, *Grin3a*, *Egr2*, *Hdac4*, *Hdac5*, and *Atf3* mRNA probes are conjugated to the dye FAM); glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) mRNA left primer, 5′-atggtgaaggtcggtgtga-3′; *Gapd* mRNA right primer, 5′-aatctccactttgccactgc-3′; probe, tggcggtattgg (*Gapd* mRNA probe is conjugated to LightCycler Yellow 555). The nonoverlapping dyes and quencher on the reference gene allow for multiplexing in the Roche LightCycle 480 II machine. All values were normalized to *Gapd* expression levels. Analysis and statistics were performed using the Roche proprietary algorithms and REST 2009 software based on the Pfaffl method (Pfaffl, 2001; Pfaffl et al., 2002).

ChIP. C57BL/6 mice were injected with either 5 mg/kg cocaine or saline and confined for 30 min (as described above for RT-qPCR and CPP). Brains were collected 1 h after the drug injection and the NAc isolated from $2 \times 500\ \mu m^3$ punches taken from coronal sections containing the NAc from the contralateral hemisphere used in RT-qPCR from the same mouse. Coronal sections adjacent to the punches from the same animals were used for H4K8Ac IHC (as stated above). ChIP was performed as described previously (Malvaez et al., 2011) with a kit from Millipore. Briefly, tissue was cross-linked using 1% formaldehyde (Sigma-Aldrich), lysed, and sonicated, and chromatin was immunoprecipitated overnight with anti-HDAC3 IgG (Millipore), anti-acetylated H4 lysine 8 IgG (anti-H4K8Ac; Millipore), or anti-rabbit IgG (negative control; Millipore). The immunoprecipitate was collected using magnetic protein A beads (Millipore). After washing, chromatin was eluted from the beads and reverse cross-linked in the presence of proteinase K before column purification of DNA. *Fos* and *Nr4a2* promoter enrichment in ChIP samples was measured by quantitative real-time PCR using the Roche 480 LightCycler and SYBR Green. Primer sequences for the promoters, designed by the Primer 3 program, were as follows: *Fos* left primer, 5′-tacgacccctcaggcatac-3′; *Fos* right primer, 5′-gttttaaaggacggcagcac-3′; *Nr4a2* left primer 5′-cgggacaactgtctccactt-3′; *Nr4a2* right primer, 5′-catgtatatcaaatgctgttt-3′. Five microliters of input, anti-HDAC3 IgG, anti-H4K8Ac IgG, or anti-rabbit IgG immunoprecipitate from four separate mice from each condition were examined in duplicate. Percentage input was calculated for both the ChIP and IgG samples and then the fold enrichment was calculated as a ratio of the ChIP to IgG. An in-plate standard curve determined amplification efficiency (AE). The equation used was $AE^{\wedge(\text{Input Ct} - \text{ChIP Ct})}/AE^{\wedge(\text{Input Ct} - \text{IgG Ct})}$. Samples were then normalized to the saline condition.

Western blot analysis

C57BL/6 mice were injected with either 5 mg/kg cocaine or saline and confined for 30 min (as described above for RT-qPCR, CPP, and ChIP). Brains were collected 1 h after the drug injection and the NAc isolated from 1 mm coronal sections. Protein was isolated by homogenization on ice in tissue protein extraction reagent (T-PER; Thermo Scientific) in the presence of protease and phosphatase inhibitors. The final protein concentration was determined using the Bio-Rad protein assay and bovine serum albumin standards. Tissue samples were prepared in a standard 5× SDS/PAGE sample buffer (1 M Tris, pH 6.8, 20% v/v glycerol, 10% w/v SDS, 0.05% bromophenol blue, and 10 mm 2-β-mercapto-ethanol). Ten micrograms of protein was loaded per well and run at 120 V for 1 h on NuPage 10% Bis-Tris polyacrylamide gels (Invitrogen). Electrophoretic transfer was then performed overnight at 30 V onto a polyvinylidene difluoride membrane. Membranes were blocked for 2 h at room temperature in blocking solution (5% nonfat milk/Tris-buffered saline with Tween 20) and then incubated in primary antibodies (1:10,000 rabbit anti-HDAC3; Millipore; 1:10,000 rabbit anti-GAPDH; Millipore) with agitation overnight at 4°C. Membranes were then rinsed three times for 10 min each in blocking solution with agitation. Next, membranes were incubated for 1 h at room temperature in a 1:10,000 dilution of polyclonal goat anti-rabbit HRP secondary antibody (Millipore). Membranes were then rinsed three times for 10 min each in TBS with agitation. Supersignal Westpico Chemiluminescent substrate (Thermo Scientific) was used for chemiluminescent detection according to the manufacturer's instructions and analyzed using ImageJ software.

Statistics

Two-way ANOVAs followed by Bonferroni's *post hoc* tests (Prizm) were used to make specific comparisons when significant interactions were observed with α levels held at 0.05. Specific group comparisons were analyzed by Student's *t* tests ($\alpha = 0.05$). Data are reported as mean ± SEM.

Results

Site-specific and homozygous Hdac3 gene deletion in the adult mouse NAc

In this study, NAc-specific, homozygous deletions of *Hdac3* were generated in adult mice to investigate the *in vivo* function of HDAC3 at the molecular and behavioral levels during CPP. This method allowed us to avoid developmental and other confounds associated with traditional knock-out mice. In addition, because HDAC3 is expressed in neurons, oligodendrocytes, and glia (Broide et al., 2007; Baltan et al., 2011), the use of AAV serotype 2.1, which preferentially transduces neurons (Burger et al., 2004), allowed us to delete *Hdac3* specifically in neurons. We have previously used AAV-Cre to generate focal deletions of CREB-binding protein in the mouse NAc (Malvaez et al., 2011). The same method was used to generate site-specific deletions of *Hdac3* in the NAc (Fig. 1). Figure 1*A* shows HDAC3 expression in 4× (the NAc core is boxed in white) and 20× (magnification of the boxed region) representative images of coronal slices from intra-NAc AAV-Cre infused *Hdac3*$^{+/+}$ (top) and *Hdac3*$^{flox/flox}$ (bottom) mice after IHC with anti-HDAC3 antibody. DAPI staining (right) confirmed the presence of nuclei in the NAc of both genotypes. Quantified HDAC3 immunoreactivity in the NAc of all *Hdac3*$^{+/+}$ and *Hdac3*$^{flox/flox}$ mice used in this study is shown in Figure 1*B*. HDAC3 immunoreactivity was significantly reduced in the NAc of *Hdac3*$^{flox/flox}$ mice compared with *Hdac3*$^{+/+}$ (mean percentage of *Hdac3*$^{+/+}$ ± SEM: *Hdac3*$^{+/+}$ = 100 ± 16.972, $n = 39$; *Hdac3*$^{flox/flox}$ = 10.678 ± 2.561, $n = 40$, $t_{(79)} = 5.174$, $^*p < 0.001$).

The extent of HDAC3 deletion in *Hdac3*$^{flox/flox}$ mice is shown in Figure 1*C* ($n = 40$ mice). In this study, viral infusions and focal deletions were bilateral and *Hdac3* deletions were restricted to the NAc core and shell regions in all *Hdac3*$^{flox/flox}$ mice included in the data presented in subsequent figures. However, the figure shows only one hemisphere shaded so that the anatomical designations remain legible. These data confirm that NAc-specific *Hdac3* deletions can be generated in adult mice by intra-NAc infusions of AAV-Cre.

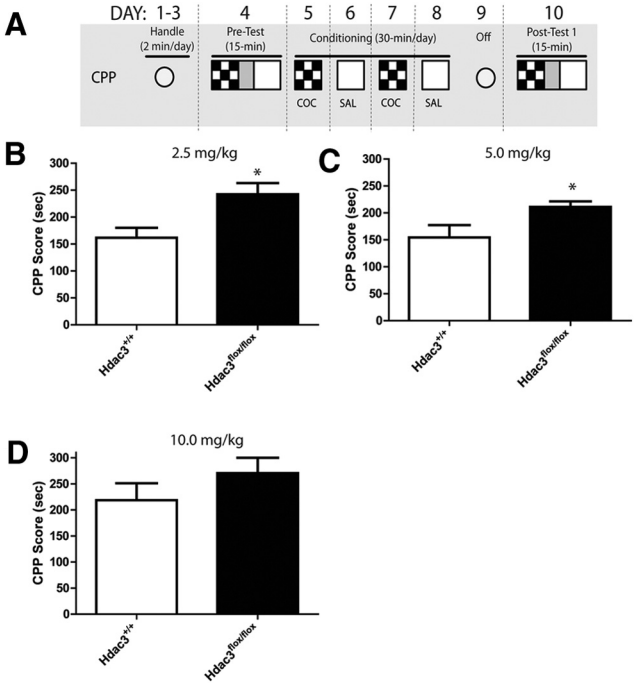

Figure 2. Cocaine-induced CPP acquisition is enhanced in *Hdac3* $^{flox/flox}$ mice compared with *Hdac3* $^{+/+}$ at low doses of cocaine. ***A***, Schematic of the CPP procedure (fully described in Materials and Methods). ***B***, ***C***, In *Hdac3* $^{flox/flox}$ mice infused intra-NAc with AAV-Cre, CPP acquisition is significantly enhanced after conditioning with 2.5 or 5 mg/kg cocaine compared with intra-NAc AAV-Cre infused *Hdac3* $^{+/+}$ littermates. For 2.5 mg/kg experiment: *Hdac3* $^{+/+}$, $n = 13$; *Hdac3* $^{flox/flox}$, $n = 9$, $^*p = 0.011$; for 5.0 mg/kg experiment: *Hdac3* $^{+/+}$, $n = 8$; *Hdac3* $^{flox/flox}$, $n = 9$, $^*p = 0.032$. ***D***, At a higher dose, no differences were seen between genotypes. For the 10 mg/kg experiment: *Hdac3* $^{+/+}$, $n = 8$; *Hdac3* $^{flox/flox}$, $n = 8$; $p = 0.688$. $^*p < 0.05$.

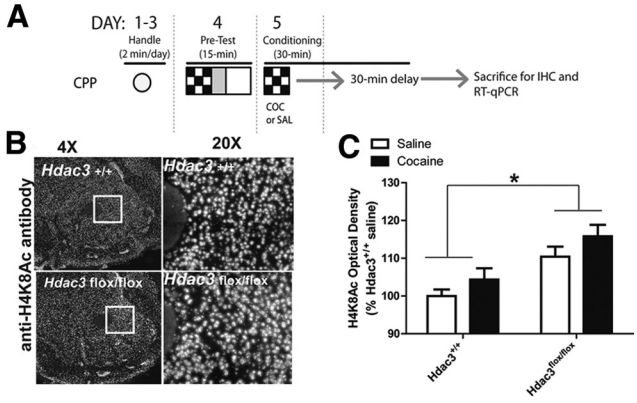

Figure 3. Acetylated H4K8, a marker of transcriptional activation, is augmented in the NAc after *Hdac3* deletion and CPP conditioning. ***A***, In this figure, data were collected from mice killed after either saline or 5 mg/kg cocaine conditioning in a single CPP trial. A schematic of the experimental design illustrates how this was done (fully described in Materials and Methods). ***B***, Representative 4× and 20× magnified images of cocaine-treated *Hdac3* $^{+/+}$ (top) and *Hdac3* $^{flox/flox}$ (bottom) coronal hemisections immunostained with anti-H4K8Ac IgG illustrate an overall increase of H4K8Ac in the NAc after *Hdac3* deletion. ***C***, Quantification of anti-H4K8Ac IHC demonstrates that H4K8Ac is more abundant in *Hdac3* $^{flox/flox}$ mice compared with *Hdac3* $^{+/+}$ littermates. *Hdac3* $^{flox/flox}$: saline, $n = 6$; cocaine, $n = 8$; *Hdac3* $^{+/+}$: saline, $n = 5$; cocaine, $n = 5$; $p = 0.024$. $^*p < 0.05$.

Focal Hdac3 deletion in the mouse NAc facilitates CPP acquisition

To investigate our hypothesis that HDAC3 is involved in the formation of cocaine-context-associated memories, *Hdac3* $^{+/+}$ and *Hdac3* $^{flox/flox}$ mice were subjected to cocaine-induced CPP to examine the effect of NAc-specific *Hdac3* deletion on acquisition/consolidation.

The schematic of the CPP procedure is shown in Figure 2*A* (fully described in Materials and Methods). In Figure 2*B*, *C*, the data show that *Hdac3* $^{flox/flox}$ mice exhibited enhanced CPP acquisition compared with *Hdac3* $^{+/+}$ mice after conditioning with low doses of cocaine (2.5 and 5.0 mg/kg cocaine, respectively), but not a higher dose (10 mg/kg; Fig. 2*D*; for 2.5 mg/kg experiment: *Hdac3* $^{+/+}$, $n = 13$; *Hdac3* $^{flox/flox}$, $n = 9$; $t_{(19)} = 2.820$, $^*p = 0.011$; for 5.0 mg/kg experiment: *Hdac3* $^{+/+}$, $n = 8$; *Hdac3* $^{flox/flox}$, $n = 9$; $t_{(15)} = 2.362$, $^*p = 0.032$). The enhanced CPP scores in *Hdac3* $^{flox/flox}$ mice were similar to acquisition scores seen in both genotypes after conditioning with the higher dose of 10 mg/kg cocaine (for 10 mg/kg experiment: *Hdac3* $^{+/+}$, $n = 8$; *Hdac3* $^{flox/flox}$, $n = 8$; $t_{(14)} = 0.409$, $p = 0.688$). *Hdac3* $^{flox/flox}$ mice exhibited ceiling levels of CPP acquisition after conditioning with low doses of cocaine (2.5 and 5 mg/kg). When the data were analyzed by factorial ANOVA (treatment × genotype), a significant effect of genotype was observed ($F_{(1,48)} = 10.84$, $^*p = 0.012$). The effect of treatment approached significance ($F_{(2,48)} = 9.20$, $p = 0.0641$). The facilitated acquisition in *Hdac3* $^{flox/flox}$ mice was most likely not due to potential performance confounds because there were no observed differences in distance traveled in a 15 min posttest (mean ± SEM: *Hdac3* $^{+/+}$ = 4219.523 ± 119.376 cm, $n = 29$; *Hdac3* $^{flox/flox}$ = 4119.422 ± 102.439 cm, $n = 26$; $t_{(53)} = 0.6292$, $p = 0.532$). These data show that NAc-specific *Hdac3* deletion enhances cocaine-induced CPP acquisition.

NAc-specific Hdac3 deletion enhances global levels of H4K8Ac in Hdac3 $^{flox/flox}$ mice concomitant with increases in c-Fos, Nr4a2, and Grin3a mRNA levels

To examine the molecular effects of *Hdac3* deletion, we examined histone acetylation and gene expression during the consolidation phase of cocaine-context-associated memory formation. Consolidation is the phase of memory during which gene expression is necessary for the encoding of a learning event into long-term memory (McGaugh, 2000). Therefore, this is the optimum time to examine molecular effects of *Hdac3* deletion that would ultimately correlate with changes in behavior observed in long-term memory tests after conditioning.

In the mouse hippocampus, an *in vivo* molecular substrate of HDAC3 is H4K8Ac (McQuown et al., 2011), which is also a marker of transcriptional activation (Kouzarides, 2007). We hypothesized that during the consolidation window in the NAc, H4K8Ac is likewise a target of HDAC3 deacetylation. To test this, a separate set of AAV-Cre infused *Hdac3* $^{+/+}$ and *Hdac3* $^{flox/flox}$ mice were killed 1 h after the first cocaine (5 mg/kg) pairing (or saline administration) to investigate HDAC3-mediated, cocaine-induced histone acetylation and gene expression during long-term associative memory formation in the CPP task (Fig. 3*A*; described in the Materials and Methods).

HDAC3-mediated histone acetylation was examined by IHC with anti-H4K8Ac antibodies (Fig. 3*B*) and quantified (Fig. 3*C*) in NAc-containing coronal slices of *Hdac3* $^{+/+}$ and *Hdac3* $^{flox/fox}$ mice with confirmed HDAC3 deletion from adjacent serial sections. The same brains were used to examine *c-Fos*, *Nr4a2*, and other mRNA levels shown in Figure 4 by taking $2 \times 500\ \mu m^3$ NAc punches (used in RT-qPCR) from the area of focal deletion identified by IHC. H4K8Ac optical density was significantly enhanced in the NAc of *Hdac3* $^{flox/flox}$ vs *Hdac3* $^{+/+}$ mice (ANOVA, significant effect of genotype, $F_{(1,14)} = 6.41$, $^*p = 0.024$; *Hdac3* $^{flox/flox}$: saline $n = 6$ mice, cocaine $n = 8$ mice; *Hdac3* $^{+/+}$: saline $n = 5$ mice, cocaine $n = 5$ mice; *Hdac3* $^{flox/flox}$ vs *Hdac3* $^{+/+}$, $p < 0.05$).

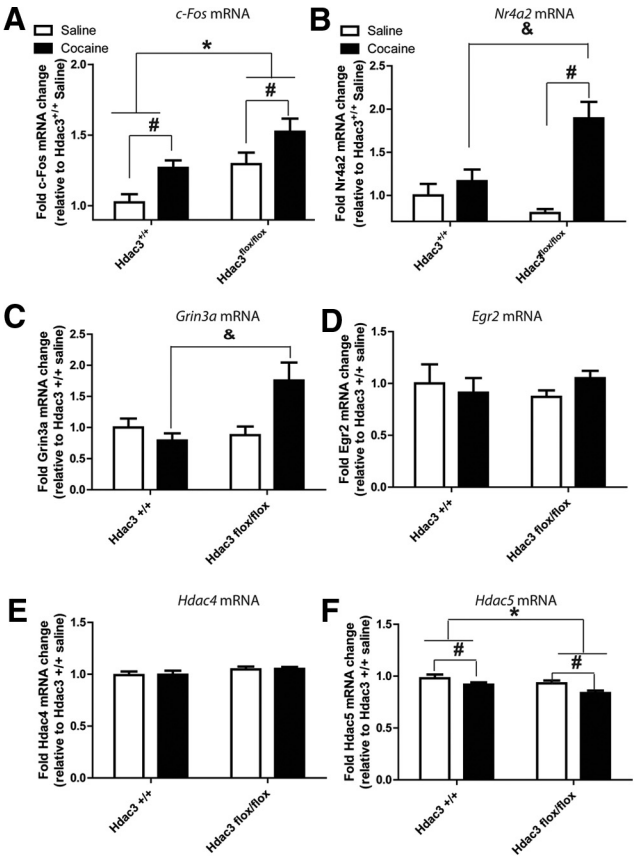

Figure 4. Changes in cocaine- and learning- and memory-associated mRNA abundances in the NAc concomitant with increased H4K8Ac. ***A***, Gene expression was analyzed from tissue punches collected from the NAc of the same brains shown in Figure 3*B* in the area of focal deletion as confirmed by IHC. *c-Fos* mRNA was increased in both genotypes after CPP conditioning with 5 mg/kg cocaine relative to conditioning with saline. A significant effect of genotype was also found by two-way ANOVA, indicating that more *c-Fos* mRNA was present in the NAc of *Hdac3*$^{flox/flox}$ mice relative to *Hdac3*$^{+/+}$ littermates. *Hdac3*$^{flox/flox}$: saline, $n = 6$; cocaine, $n = 8$; *Hdac3*$^{+/+}$: saline, $n = 5$; cocaine, $n = 5$. *Significantly different from *Hdac3*$^{+/+}$, $p < 0.05$. #significantly different from saline, $p < 0.05$. ***B***, *Nr4a2* mRNA levels from the same tissue punches used in Figure 4*B* were significantly enhanced in cocaine-conditioned, but not saline-conditioned, *Hdac3*$^{flox/flox}$ mice relative to *Hdac3*$^{+/+}$ littermates. There was a significant interaction between treatment and genotype affecting *Nr4a2* mRNA abundance in the NAc. *Hdac3*$^{flox/flox}$: saline, $n = 6$; cocaine, $n = 8$; *Hdac3*$^{+/+}$: saline, $n = 5$; cocaine, $n = 5$. #Significantly different from saline, $p < 0.05$. &Significantly different from cocaine-conditioned *Hdac3*$^{+/+}$, $p < 0.05$. ***C***, *Grin3a* mRNA levels from the same tissue punches were likewise significantly enhanced in cocaine-conditioned, but not saline-conditioned, *Hdac3*$^{flox/flox}$ mice relative to *Hdac3*$^{+/+}$ littermates. *Hdac3*$^{flox/flox}$: saline, $n = 6$; cocaine, $n = 8$; *Hdac3*$^{+/+}$: saline, $n = 5$; cocaine, $n = 5$. &Significantly different from cocaine-conditioned *Hdac3*$^{+/+}$, $p = 0.039$. ***D***, ***E***, *Egr2* and *Hdac4* mRNA levels remained unchanged by treatment or genotype. *Hdac3*$^{flox/flox}$: saline, $n = 6$; cocaine, $n = 8$; *Hdac3*$^{+/+}$: saline, $n = 5$; cocaine, $n = 5$. ***F***, *Hdac5* mRNA is decreased in both genotypes after CPP conditioning with cocaine relative to conditioning with saline. A significant effect of genotype is also observed, indicating that less *Hdac5* mRNA is present in the NAc of *Hdac3*$^{flox/flox}$ mice relative to *Hdac3*$^{+/+}$ littermates. *Hdac3*$^{flox/flox}$: saline, $n = 6$ mice; cocaine, $n = 8$ mice; *Hdac3*$^{+/+}$: saline, $n = 5$; cocaine, $n = 5$. *Significantly different from *Hdac3*$^{+/+}$, $p < 0.05$. #Significantly different from saline, $p < 0.05$.

No effect of treatment was observed ($F_{(1,14)} = 1.465$, $p = 0.2461$). Therefore, histone H4 lysine 8 (H4K8) was more acetylated in the NAc of mice with *Hdac3* deletions. Outside of the region of deletion, in the dorsal striatum, H4K8Ac levels did not differ between groups, as determined by ANOVA (effect of genotype, $F_{(1,20)} = 0.12$, $p = 0.868$; effect of treatment, $F_{(1,20)} = 3.63$, $p = 0.360$; *Hdac3*$^{flox/flox}$: saline $n = 6$ mice, cocaine $n = 8$ mice; *Hdac3*$^{+/+}$: saline $n = 5$ mice, cocaine $n = 5$ mice).

In NAc punches taken from within the region of HDAC3 deletion, 5 mg/kg cocaine-induced *Fos* gene expression was examined by RT-qPCR. In both genotypes, *c-Fos* mRNA is significantly enhanced after cocaine administration and a single CPP conditioning trial (Fig. 4*A*; ANOVA, significant effect of cocaine treatment $F_{(1,56)} = 12.12$, *$p = 0.003$; *Hdac3*$^{flox/flox}$: saline $n = 6$ mice, cocaine $n = 8$ mice; *Hdac3*$^{+/+}$: saline $n = 5$, cocaine $n = 5$; *Hdac3*$^{flox/flox}$ > *Hdac3*$^{+/+}$, $p < 0.05$). There is also a significant effect of genotype on *c-Fos* mRNA levels (ANOVA, $F_{(1,56)} = 15.07$, $p = 0.001$; *Hdac3*$^{flox/flox}$ > *Hdac3*$^{+/+}$, $p < 0.05$). These data demonstrate that cocaine conditioning enhances c-Fos mRNA abundance in both genotypes compared with saline conditioning and that *Hdac3* deletion results in an overall enhancement of *c-Fos* mRNA in *Hdac3*$^{flox/flox}$ vs *Hdac3*$^{+/+}$ mice.

In the same NAc punches used to investigate *Fos* expression, *Nr4a2* mRNA abundance was also found to be increased after 5 mg/kg cocaine administration and a single CPP conditioning trial (Fig. 4*B*). In the case of *Nr4a2*, however, cocaine enhanced mRNA expression only in *Hdac3*$^{flox/flox}$ mice treated with cocaine, but not in cocaine-treated *Hdac3*$^{+/+}$ littermates (ANOVA, significant effect of treatment $F_{(1,38)} = 22.31$, $p < 0.001$; *Hdac3*$^{flox/flox}$: saline $n = 6$ mice, cocaine $n = 8$ mice; *Hdac3*$^{+/+}$: saline $n = 5$, cocaine $n = 5$; *Hdac3*$^{flox/flox}$ saline < *Hdac3*$^{flox/flox}$ cocaine, $p < 0.001$; *Hdac3*$^{+/+}$ saline vs *Hdac3*$^{+/+}$ cocaine, $p =$ n.s.). Although a trend was observed for the effect of genotype on *Nr4a2* mRNA abundance (ANOVA, effect of genotype, $F_{(1,38)} = 3.86$, $p = 0.10$), there was a significant interaction between genotype and treatment (ANOVA, significant interaction $F_{(1,38)} = 12.15$, $p = 0.005$; *Hdac3*$^{flox/flox}$ > *Hdac3*$^{+/+}$, $p < 0.05$). These data strongly suggest that in the NAc, cocaine-mediated *Nr4a2* mRNA expression is regulated by HDAC3 during CPP conditioning.

In those same NAc punches, the mRNA levels of other genes implicated in cocaine-induced neuroplasticity were also examined; namely *Grin3a* (Fig. 4*C*), *Egr2* (Fig. 4*D*), *Atf3* (data not shown), *Hdac4* (Fig. 4*E*), and *Hdac5* (Fig. 4*F*). *Grin3a* mRNA encodes an NMDA receptor subunit shown to be important for learning and memory and synaptic plasticity mechanisms (Larsen et al., 2011). Although *Grin3a* itself has not been examined with regard to cocaine-induced neuroplasticity, *Grin3a* mRNA expression is significantly reduced in the human peripheral blood lymphocytes of opioid addicts (Roozafzoon et al., 2010). Furthermore, NMDA receptors play a critical role in the consolidation of cocaine-context-associated memories (Alaghband and Marshall, 2012; Carmack et al., 2013). Like *Nr4a2* mRNA, cocaine enhanced *Grin3a* mRNA expression only in *Hdac3*$^{flox/flox}$ mice treated with cocaine, but not in cocaine-treated *Hdac3*$^{+/+}$ littermates (Fig. 4*C*; ANOVA, significant interaction between genotype and treatment, $F_{(1,17)} = 18.03$, $p = 0.029$; *Hdac3*$^{flox/flox}$: saline $n = 6$ mice, cocaine $n = 8$ mice; *Hdac3*$^{+/+}$: saline $n = 5$, cocaine $n = 5$). Although a trend was observed for the effect of treatment on *Grin3a* mRNA abundance (ANOVA, effect of treatment, $F_{(1,17)} = 10.81$, $p = 0.083$), there was no effect of genotype ($F_{(1,17)} = 6.80$, $p = 0.1622$). These data suggest that in the NAc, cocaine-mediated *Grin3a* mRNA expression is regulated by HDAC3, and possibly H4K8Ac, during CPP conditioning.

After conditioning with either saline or cocaine, no significant effects were observed by ANOVA between treatment or genotype groups for *Egr2* (Fig. 4*D*). *Egr2*, like *Fos*, is an immediate early gene involved in cocaine-induced neuroplasticity (Freeman et al., 2010). *Atf3* mRNA is translated into a CREB superfamily transcription factor regulated by psychostimulant administration

and is implicated in drug-induced neuroplasticity (Green et al., 2008). In this study, *Atf3* mRNA (data not shown) was significantly less abundant after conditioning with cocaine in both genotypes (ANOVA, effect of treatment $F_{(1,20)} = 20.08$, $^*p = 0.036$). No effect of genotype was observed (ANOVA, effect of genotype $F_{(1,20)} = 0.00$, $^*p = 0.978$).

Although *Hdac4* mRNA remained unchanged in all of the groups tested (Fig. 4*E*), a significant effect of genotype and treatment, but no interaction, was observed for *Hdac5* mRNA (Fig. 4*F*; ANOVA, significant effect of genotype $F_{(1,19)} = 24.12$, $^*p = 0.012$; ANOVA significant effect of cocaine treatment $F_{(1,19)} = 16.56$, $^*p = 0.032$; $Hdac3^{flox/flox}$: saline $n = 6$ mice, cocaine $n = 8$ mice; $Hdac3^{+/+}$: saline $n = 5$, cocaine $n = 5$; $Hdac3^{flox/flox} < Hdac3^{+/+}$, $p < 0.05$). These latter data indicate that loss of HDAC3 in the mouse NAc results in less abundance of *Hdac5* mRNA after CPP conditioning with either saline or cocaine.

A single CPP conditioning trial with cocaine enhanced the levels of H4K8Ac at the Fos and Nr4a2 promoters in the NAc, which was associated with a decrease in HDAC3 association with the Fos and Nr4a2 promoters. Although cocaine did not cause a global, NAc-wide increase in the levels of H4K8Ac after cocaine treatment in $Hdac3^{+/+}$ mice (Fig. 3*C*), the global amount of H4K8Ac does not accurately represent the acetylation status of promoters that regulate genes involved in neuroplastic and behavioral responses to cocaine. Therefore, ChIP assays were performed to examine the levels of H4K8Ac at the *Fos* and *Nr4a2* promoters 1 h after the first cocaine (5 mg/kg) or saline injection during CPP conditioning (Fig. 5*A*). Because there is no HDAC3 to ChIP in the $Hdac3^{flox/flox}$ mouse NAc, C57BL/6 mice were used. The C57BL/6 mice were treated with 5 mg/kg cocaine or saline in parallel and identically to the $Hdac3^{flox/flox}$ and $Hdac3^{+/+}$ mice examined in the IHC and RT-qPCR studies shown in Figure 3 and Figure 4 (and illustrated in Fig. 5*A*).

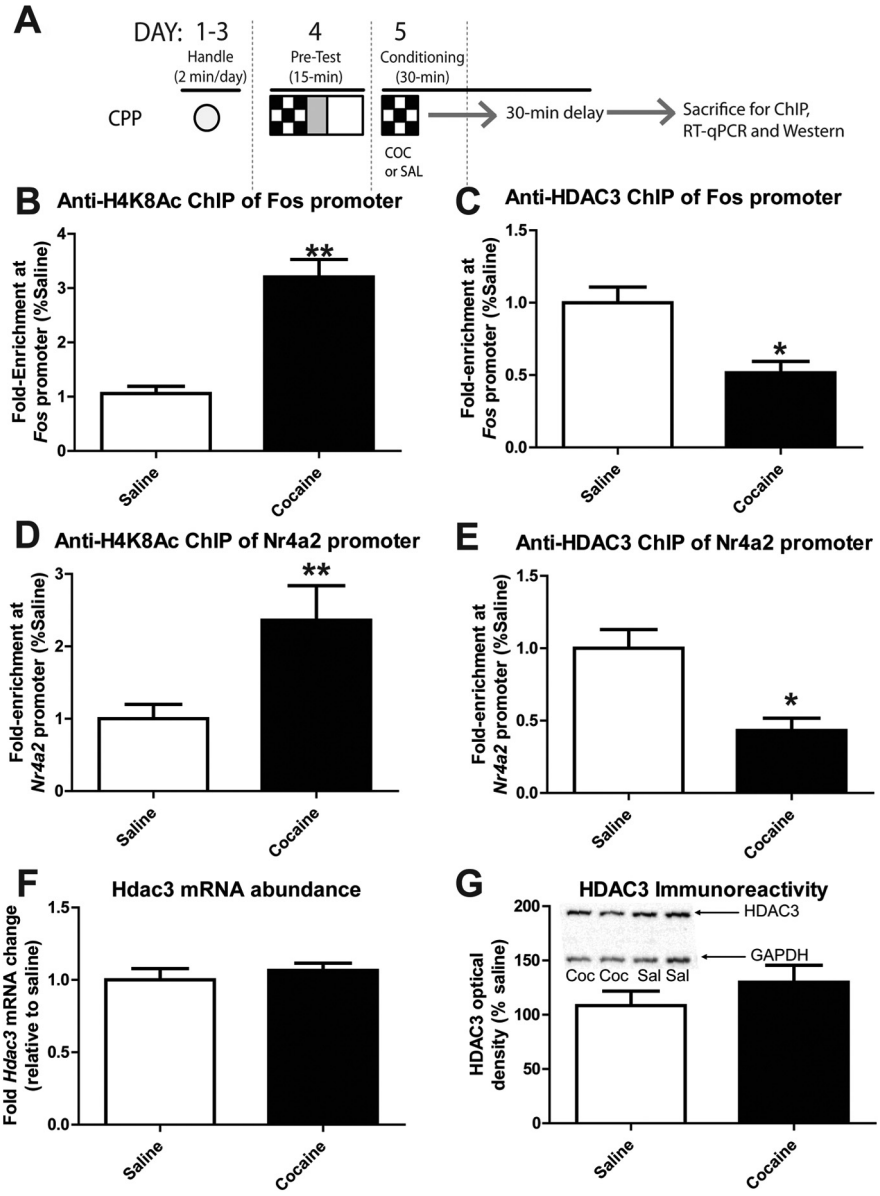

Figure 5. H4K8Ac and HDAC3 at the *Fos* and *Nr4a2* promoters in the C57BL/6 mouse NAc are affected by conditioning with cocaine. ***A***, Schematic of the experimental design illustrating how C57BL/6 mice were treated with 5 mg/kg cocaine or saline and killed exactly as the mice used for Figure 3 and Figure 4 (fully described in Materials and Methods). ***B***, ChIP with anti-H4K8Ac antibodies isolated significantly more *Fos* promoter from the NAc of cocaine-conditioned mice compared with saline-conditioned mice. $^{**}p < 0.001$. ***C***, ChIP with anti-HDAC3 antibodies isolated significantly less *Fos* promoter from the NAc of cocaine-conditioned mice compared with saline-conditioned mice. $^*p < 0.05$. ***D***, Anti-H4K8Ac ChIP of the *Nr4a2* promoter isolated more of the promoter from the NAc of cocaine-conditioned mice compared with saline-conditioned mice. $^{**}p < 0.001$. ***E***, Anti-HDAC3 ChIP isolated less *Nr4a2* promoter from the NAc after conditioning with cocaine compared with saline. $^*p < 0.05$. For all ChIP studies: saline, $n = 6$; cocaine, $n = 6$. ***F***, *Hdac3* mRNA levels in the NAc remained unchanged after cocaine conditioning. Saline, $n = 5$; cocaine, $n = 5$; $p = 0.4927$. ***G***, HDAC3 immunoreactivity from NAc proteins subjected to Western blot analyses also remains unchanged after conditioning with cocaine compared with saline. A representative image of two mice per group is shown within the graph. Saline, $n = 8$; cocaine, $n = 8$; $p = 0.308$.

In the NAc punches collected from saline- and 5 mg/kg cocaine-treated mice that underwent a single CPP conditioning trial, cocaine treatment significantly enhanced the amount of *Fos* promoter enriched by anti-H4K8Ac ChIP, indicating that there was significantly increased acetylation of H4K8 at the *Fos* promoter after cocaine treatment during a single CPP conditioning trial compared with saline treatment and conditioning (Fig. 5*B*, $t_{(10)} = 6.174$, $^*p < 0.001$; saline $n = 6$ mice, cocaine $n = 6$ mice).

We next examined the occupancy of HDAC3 at the *Fos* promoter 1 h after cocaine treatment and a single CPP conditioning trial. ChIP was performed with anti-HDAC3 antibodies. These experiments were designed to address the questions of whether HDAC3 associates with the *Fos* promoter during conditioning and, if so, does cocaine affect that association? Using the same chromatin samples used in Figure 5*B*, anti-HDAC3 ChIP enriched significantly more *Fos* promoter DNA than IgG ChIP alone. As predicted, cocaine treatment significantly reduced the amount of *Fos* promoter enriched by anti-HDAC3 ChIP, indicating that much less HDAC3 was associated with the *Fos* promoter after cocaine treatment and a single CPP conditioning trial com-

pared with saline treatment and conditioning (Fig. 5*C*; $t_{(10)} = 3.62$, $^{*}p = 0.005$; saline $n = 6$, cocaine $n = 6$). These data indicate that one mechanism by which cocaine may enhance *c-Fos* mRNA expression during CPP conditioning is by relieving HDAC3-mediated transcriptional repression.

Similar ChIP results were obtained from the same NAc punches when H4K8 acetylation and HDAC3 promoter occupancy were examined at the *Nr4a2* promoter. CPP conditioning with 5 mg/kg cocaine significantly enhanced the amount of *Nr4a2* promoter enriched by anti-H4K8Ac ChIP (Fig. 5*D*; $t_{(10)} = 2.64$, $^{*}p = 0.039$; saline $n = 6$, cocaine $n = 6$). Furthermore, CPP conditioning with cocaine significantly reduced the amount of *Nr4a2* promoter enriched by anti-HDAC3 ChIP (Fig. 5*E*; $t_{(10)} = 3.49$, $^{*}p = 0.007$; saline $n = 6$, cocaine $n = 6$). These data indicate that significantly less HDAC3 was associated with the *Nr4a2* promoter after cocaine treatment and a single CPP conditioning trial compared with saline treatment.

Finally, *Hdac3* mRNA levels were examined in the same cocaine- and saline-treated C57BL/6 mice to determine whether cocaine or conditioning regulates *Hdac3* mRNA expression. In those mice, cocaine administration before CPP conditioning had no effect on *Hdac3* mRNA abundance in the NAc relative to saline-conditioned mice (Fig. 5*F*; $t_{(8)} = 0.719$, $p = 0.4927$; saline $n = 5$ mice, cocaine $n = 5$ mice). In a separate set of identically treated C57BL/6 mice, HDAC3 protein levels were examined by Western blot analysis after conditioning with either saline or 5 mg/kg cocaine. As with *Hdac3* mRNA abundance, HDAC3 protein levels were unchanged by CPP conditioning with 5 mg/kg cocaine compared with conditioning with saline (Fig. 5*G*; $t_{(16)} = 1.055$, $p = 0.3072$; saline $n = 8$ mice, cocaine $n = 8$ mice). Therefore, cocaine-induced changes in HDAC3 promoter occupancy and H4K8 acetylation at the *Fos* and *Nr4a2* promoters are not due simply to a decrease in *Hdac3* mRNA or protein levels.

Discussion

One unique aspect of this study is that HDAC3-mediated promoter acetylation and gene expression were examined during the consolidation phase of cocaine CPP conditioning, which is a temporal window within which molecular events that are required for long-term memory formation, including gene expression, occur. The data presented in this study demonstrate that HDAC3 associates with the *Fos* and *Nr4a2* promoters in the absence of cocaine, maintaining low levels of promoter H4K8Ac, and thus suppressing transcription. When cocaine, but not saline, was administered during conditioning, HDAC3 was removed from the *Fos* and *Nr4a2* promoters, allowing for increased H4K8 acetylation, which is correlated with increased *Fos* and *Nr4a2* transcription. These results suggest that HDAC3 is a negative regulator of cocaine-induced gene expression during the consolidation phase of CPP conditioning.

Furthermore, the molecular studies with the *Hdac3*$^{flox/flox}$ mice, which exhibit enhanced CPP acquisition compared with *Hdac3*$^{+/+}$ littermates, mirror the ChIP findings above. After the first CPP conditioning trial, during consolidation, the overall levels of H4K8Ac were greater in the NAc of *Hdac3*$^{flox/flox}$ mice harboring *Hdac3* deletions compared with *Hdac3*$^{+/+}$ mice. That increased H4K8Ac in the absence of HDAC3 is correlated with enhanced *c-Fos* mRNA expression in the NAc of *Hdac3*$^{flox/flox}$ mice relative to *Hdac3*$^{+/+}$ mice. These findings suggest that HDAC3 normally deacetylates H4K8Ac and represses *Fos* transcription during the consolidation of CPP conditioning.

Moreover, *Nr4a2* mRNA was enhanced in the NAc of *Hdac3*$^{flox/flox}$ mice harboring *Hdac3* deletions compared with *Hdac3*$^{+/+}$ mice after conditioning with cocaine, but not saline. These data indicate that HDAC3 is likely involved in cocaine-induced expression of *Nr4a2*. Nearly identical results were observed when *Grin3a* mRNA was examined. These findings suggest that repression of cocaine-activated genes in the NAc during conditioning may be one of the mechanisms by which HDAC3 suppresses CPP acquisition. This idea is supported by previous evidence showing that overexpression of HDAC4 or HDAC5 in the NAc during conditioning, but not afterward, inhibits the formation of cocaine-induced CPP (Kumar et al., 2005; Renthal et al., 2007; Taniguchi et al., 2012). Our study also suggests coordinated expression between HDAC3 and HDAC5, because NAc-specific *Hdac3* deletion results in less HDAC5 expression after CPP conditioning.

C-FOS in the NAc is an immediate early gene and transcription factor that initiates a cascade of molecular events that are integral to neuroadaptive responses to cocaine (Nestler, 2001). The *Fos* gene also represents a crossroads where drug addiction and learning and memory research converge, because C-FOS is similarly upregulated in the hippocampus immediately after a learning event and initiates a cascade of molecular events that are integral to long-term memory formation (Izquierdo and Medina, 1997; Katche et al., 2010). Therefore, HDAC3-mediated *c-Fos* mRNA transcription during CPP conditioning with cocaine may be a possible molecular mechanism by which cocaine strengthens the association of context and drug.

This is the first study to show that NR4A2 (also known as NURR1) may also be involved with HDAC3-mediated cocaine-context memory formation. NR4A2 is an orphan nuclear receptor and transcription factor (for a review of the NR4A family, see Hawk and Abel, 2011). Like C-FOS, NR4A2 is also critical for both learning and memory processes (Peña de Ortiz et al., 2000; von Hertzen and Giese, 2005; Colón-Cesario et al., 2006; McNulty et al., 2012) and reward pathway neuroplasticity (Zetterström et al., 1997; Castillo et al., 1998; Sacchetti et al., 2001). In the latter case, NR4A2 regulates dopamine neuron viability and dopamine neurotransmission in the reward pathway. In an effort to identify a mechanism by which HDAC3 loss enhances memory, McQuown et al. (2011) demonstrated that NR4A2 is absolutely critical for HDAC3-dependent modulation of memory. HDAC3$^{flox/flox}$ mice with focal deletions in the hippocampus exhibited significantly enhanced memory for a hippocampus-dependent task. This enhancement was completely blocked by siRNA against *Nr4a2* mRNA delivered to the dorsal hippocampus (McQuown et al., 2011). *Nr4a2* was also recently found to be regulated by HDAC3 in the hippocampus during extinction consolidation in mice subjected to cocaine-induced CPP (Malvaez et al., 2013). Therefore, the regulation of *Nr4a2* by HDAC3 is important in both the hippocampus (McQuown et al., 2011; Malvaez et al., 2013) and the NAc (this study), suggesting a central mechanism of action for HDAC3 in the regulation of memory.

These data provide the rationale to further investigate whether cocaine-induced histone acetylation occurs in overlapping reward and learning and memory neurocircuits at the promoters of other genes involved in both memory processes and addiction-like behaviors (such as *Grin3a*). Should that be the case, cocaine-induced histone acetylation may be one mechanism by which strong cocaine-context associative memories are formed. Those memories have been hypothesized to underlie the transformation of cocaine-seeking behaviors into stable, long-lasting behavioral abnormalities characteristic of addiction by facilitating cocaine craving (Everitt and Robbins, 2005; Hyman et al., 2006; McClung and Nestler, 2008).

This study begins to address a critical question facing epigenetic drug addiction research: which specific HDAC enzyme(s) is involved in mediating cocaine-induced behaviors? The HDAC family is comprised of class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III (the Sirtuins) and class IV (HDAC 11) enzymes (Verdin et al., 2003). As mentioned above, overexpression of class IIa HDAC4 or HDAC5 in the NAc negatively regulates cocaine reward and reinforcement, as measured by CPP and self-administration assays (Kumar et al., 2005; Renthal et al., 2007; Renthal et al., 2009; Wang et al., 2010). HDAC4 was also shown recently to be involved in learning and memory processes (Kim et al., 2012; Sando et al., 2012). Surprisingly, though, it was also shown recently that purified HDAC4 (and possibly the closely related class IIa family member HDAC5) has little to no catalytic activity on canonical HDAC substrates containing acetyl lysines (Lahm et al., 2007). HDAC4 and HDAC5 *in vivo* may require interactions with the class I HDAC3 and other corepressor proteins in multiprotein complexes to form a functionally active repressor complex, just as they do *in vitro* (Grozinger and Schreiber, 2000; Guenther et al., 2001; Fischle et al., 2002; for a review see Karagianna and Wong, 2007; Alenghat et al., 2008). In effect, HDAC3 may play a critical role in cocaine-associated neuroplasticity as the potent histone deacetylase in such proposed *in vivo* complexes.

In summarizing the current literature on HDAC function and memory, we recently proposed the "molecular brake pad" hypothesis (McQuown and Wood, 2011b). This hypothesis posits that HDACs and associated corepressor complexes may function in neurons, in part, as molecular brake pads that act as a persistent clamp that requires strong, activity-dependent signaling (such as cocaine administration) to temporarily release the transcriptional repressor complexes to activate gene expression required for long-term memory formation. Therefore, the deletion or inhibition of specific HDACs is predicted to establish a "permissive" chromatin state that can transform a subthreshold learning event that would not normally result in long-term memory into one that does, generate persistent forms of long-term memory, and facilitate transcription-dependent memory processes. These predictions have been met in the learning and memory literature (Vecsey et al., 2007; Stefanko et al., 2009; Haettig et al., 2011; McQuown et al., 2011).

The data presented in this study provide additional supporting evidence for the molecular brake pad hypothesis. The anti-HDAC3 IgG ChIP studies validate the idea that HDAC3 is present at the *Fos* and *Nr4a2* promoters when mice are exposed to the conditioning chamber but not administered cocaine. This is intriguing because without pairing the chamber with cocaine, the mouse will not form a place preference (or associative memory of context with drug). Therefore, in the absence of CPP acquisition (cocaine-context-associated memory formation), HDAC3 is loaded onto the *Fos* and *Nr4a2* promoters at the same time that H4K8Ac levels are minimal and *c-Fos* and *Nr4a2* mRNA expression is low. It takes a powerful signaling event (such as cocaine-dependent signaling) to relieve HDAC3 association with the promoters, leading to increased levels of H4K8Ac (a marker of transcriptional activation) and subsequent *c-Fos* mRNA expression. Interestingly, cocaine-induced upregulation of *Nr4a2* mRNA required HDAC3 deletion. Together, these molecular events are strongly correlated with the learned behavior of cocaine-induced CPP acquisition. Finally, as predicted by the molecular brake pad hypothesis, focal homozygous deletions of *Hdac3* in the mouse NAc transform a subthreshold learning event (low dose of cocaine paired with a CPP chamber) that does not normally evince robust CPP acquisition (cocaine-context-associated memory formation) into one that does lead to robust CPP acquisition.

The study of epigenetic mechanisms involved in cocaine action is an exciting area of investigation because there is so little known about how chromatin modification, and other associated mechanisms involving chromatin remodeling, are involved in cocaine-induced changes in behavior. In summary, this study demonstrates that HDAC3 is a critical negative regulator of cocaine-induced CPP and may be a key enzyme involved in regulating transcription for other memory processes during CPP conditioning that underlies acquisition.

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