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## Novel role and regulation of HDAC4 in cocaine-related behaviors

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### Abstract

Epigenetic mechanisms have been proposed to contribute to persistent aspects of addiction-related behaviors. One family of epigenetic molecules that may regulate maladaptive behavioral changes produced by cocaine use are the histone deacetylases (HDACs)—key regulators of chromatin and gene expression. In particular, the class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) respond to changes in neuronal activity by modulating their distribution between the nucleus and cytoplasm—a process controlled in large part by changes in phosphorylation of conserved residues. Cocaine triggers a transient nuclear accumulation of HDAC5 that functions to limit the development of cocaine reward behavior. However, the role and regulation of the close family member, HDAC4, in cocaine behaviors remain largely unknown. In this study, we report that cocaine and cAMP signaling in striatum produced differential phosphorylation and subcellular localization of HDAC4 and HDAC5. Unlike HDAC5, cocaine exposure induced a modest hyperphosphorylation and nuclear export of HDAC4. Genetic deletion of HDAC4 in the nucleus accumbens reduced acute cocaine-produced locomotion, maximum locomotor sensitization and cocaine reward-related behavior. Interestingly, overexpression of an HDAC4 cytoplasm-concentrated mutant (S266E) increased cocaine reward behavior in the cocaine conditioned place preference assay, suggesting that cocaine-induced nuclear export of HDAC4 might function to

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#### Author's Contributions

RDP, MBC, MT and CWC conceived and designed the experiments; RDP, MBC, MT, JK and SAM collected all data. The manuscript was written by RDP and CWC with input from all authors. All authors have reviewed the content and approved the final version for publication.

#### SUPPORTING INFORMATION

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facilitate the development of cocaine reward behaviors through a role in the cell cytoplasm. Together, our findings suggest that, despite high sequence homology, HDAC4 and HDAC5 are oppositely regulated by cocaine-induced signaling *in vivo* and have distinct roles in regulating cocaine behaviors.

### Keywords

addiction; cocaine; HDAC; striatum

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## INTRODUCTION

In recent years, increasing attention has been paid to the role of epigenetic mechanisms in regulating drug addiction (Hyman, Malenka & Nestler 2006; Renthal & Nestler 2009; Robison & Nestler 2011). Work in the last decade has described changes in chromatin state (e.g. histone and DNA modifications such as methylation and acetylation) produced by drug exposure in multiple brain regions including the nucleus accumbens (NAc). These modifications alter the accessibility of DNA to transcriptional complexes, providing a molecular mechanism for producing stable changes in gene expression. There is accumulating evidence that exposure to illicit drugs is able to alter the expression, localization, and function of a number of epigenetic enzymes responsible for regulating histone modifications that enhance or reduce transcription of different gene targets.

Histone deacetylases (HDACs) catalyze the removal of acetyl groups from multiple lysine residues on histone tails, which in turn promotes chromatin condensation and reduces access to transcription-promoting complexes (Kouzarides 1999; Bertos, Wang & Yang 2001; Jiang *et al.* 2008). Class IIa HDACs (HDAC4, 5, 7 and 9) are an HDAC subclass that shuttles between the nucleus and cytoplasm in response to cell signaling events (McKinsey *et al.* 2000a; Bertos *et al.* 2001; McKinsey, Zhang & Olson 2001; Chawla *et al.* 2003; Parra & Verdin 2010; Parra 2015). They do not appear to bind directly to genomic DNA, but regulate chromatin and gene expression through indirect association with DNA-bound transcription factors, such as the MEF2 family (Miska *et al.* 1999). Cell signaling pathways, such as those stimulated by intracellular calcium and cAMP levels, modulate class IIa HDAC nucleocytoplasmic shuttling by regulating conserved CaMK sensitive serine residues (HDAC4 S246/467 and HDAC5 259/498; Grozinger & Schreiber 2000; McKinsey *et al.* 2000b; Wang *et al.* 2000; Schlumm *et al.* 2013). When these residues are dephosphorylated, class IIa HDACs localize to the nucleus, where they are thought to repress gene expression. In addition to binding with MEF2, class IIa HDACs also interact with class I HDACs, and other proteins, as part of a large repressor complex (Miska *et al.* 1999; Grozinger & Schreiber 2000; Huang *et al.* 2000; Fischle *et al.* 2002). In contrast, phosphorylation of the conserved residues promotes nuclear export, disrupts binding to MEF2 transcription factors, and increases binding to the 14–3–3 family of cytoplasmic anchoring proteins, presumably resulting in de-repression of several target genes (Wang *et al.*, 2000; Grozinger & Schreiber 2000). Classically, nuclear export of class IIa HDACs is viewed as a termination of HDAC action in the nucleus, but recent evidence indicates that class IIa HDACs may also have cytoplasmic targets and functions (Cho & Cavalli 2012; Cho *et al.* 2013).

HDAC4, a member of the class IIa family, is expressed in multiple brain regions, including the forebrain and striatum (Bolger & Yao 2005; Tsankova *et al.* 2006; Renthall *et al.* 2007), and in the forebrain, it is important for spatial learning and memory (McQuown *et al.* 2011; Kim *et al.* 2012). Viral-mediated expression of HDAC4 or HDAC5 in the adult NAc of wild-type mice reduces cocaine reward behaviors (Kumar *et al.* 2005; Wang *et al.* 2010; Taniguchi *et al.* 2012), and compared with wild-type animals, HDAC5-deficient mice display increased cocaine reward sensitivity after repeated cocaine administration—a process that is rescued by NAc expression of HDAC5 during the prior cocaine administration (Renthall *et al.* 2007). In a prior study, we observed that non-contingent cocaine administration in mice triggers a transient dephosphorylation and nuclear accumulation of endogenous HDAC5 in the striatum, and expression of a mutant form of HDAC5 that cannot be phosphorylated at Ser279 (S279A) reduced the development, but not expression, of cocaine reward in the conditioned place preference assay (CPP, Taniguchi *et al.* 2012). HDAC4 has an analogous serine residue (S266) that has been shown to be a PKA-sensitive site that regulates nucleocytoplasmic localization in non-neuronal cells (Paroni *et al.* 2008; Walkinshaw *et al.* 2013), but the role of this phosphorylation site in regulating HDAC4 neuronal localization and its response to cocaine stimulation are unknown. Despite published evidence that overexpression of HDAC4 in the NAc can modulate cocaine reward, little is known about how or whether cocaine administration regulates HDAC4 levels and subcellular distribution in the NAc, and whether altering HDAC4 localization can function, like HDAC5, to influence drug reward behavior.

In the present study, we show that despite high sequence conservation, HDAC4 is regulated differently than HDAC5 in response to cocaine administration or following elevation of cAMP in striatal neurons. Given the opposite regulation, we predicted that loss of HDAC4 might oppositely influence cocaine-related behaviors compared with loss of HDAC5. We find that loss of HDAC4 in the NAc reduces cocaine reward and acute and sensitized locomotor responses to cocaine. Moreover, we find that overexpression of cytoplasmically localized HDAC4 mutant (S279E) significantly increases cocaine CPP, suggesting that the cocaine-induced nuclear export of HDAC4 might serve to regulate the development of cocaine reward-context learning. Our findings suggest that HDAC4 and HDAC5 have distinct regulation and functional roles following cocaine administration.

## METHODS

### Animals

All *Hdac4<sup>loxP/loxP</sup>* and C57Bl/6 mice (Charles River, Wilmington, MA) used in this study were adult males tested between 9 and 12 weeks old. C57Bl/6 mice were acclimated to the colony for at least 1 week following delivery. *Hdac4<sup>loxP/loxP</sup>* contained loxP sites flanking exon 5 of the *Hdac4* gene and have been previously characterized to generate an out-of-frame mutation in the presence of Cre recombinase (Potthoff *et al.* 2007). Experimental littermate animals were generated from homozygous crosses of *Hdac4<sup>loxP/loxP</sup>* animals. All animals were housed on a 12-hour light–dark cycle with access to food and water *ad libitum*. All procedures were in accordance with the McLean Hospital Institutional Care and Use (IACUC) guidelines.

## Cell culture

Primary embryonic (E18) striatal cultures were generated from Long Evans rats (Charles River, Wilmington, MA) as previously described (Pulipparacharuvi *et al.* 2008; Taniguchi *et al.* 2012). For biochemistry experiments, crude striatal cells (grossly dissected whole striatum) were plated at  $1.3 \times 10^6$  on  $5 \mu\text{g/ml}$  poly-D-lysine coated 6-well plates. For transfection and immunocytochemistry experiments, enriched striatal cultures (subdissected from coronal slice) were plated at  $1 \times 10^5$  on etched-glass coverslips coated with  $5 \mu\text{g/ml}$  poly-D-lysine/ $0.5 \mu\text{g/ml}$  laminin solution in a 24-well plate. HEK293T cells were cultured on 6-well plates at  $8 \times 10^5$ – $1 \times 10^6$  cells per well. Cells were transfected using Lipofectamine LTX (Life Technologies, Carlsbad, CA), and cell lysate was collected 48 hours after transfection.

## Immunocytochemistry

Enriched striatal cultures were transfected at 8 days *in vitro* using a calcium phosphate method (Renthal *et al.* 2007; Pulipparacharuvi *et al.* 2008). For forskolin localization experiments, at 10 days *in vitro* cells were stimulated for 3 hours with DMSO or  $10 \mu\text{M}$  forskolin prior to fixation. For nuclear export inhibition studies, at 10 days *in vitro* cells are treated for 3 hours with leptomycin B ( $3 \text{ ng/ml}$ ; Sigma-Aldrich, St. Louis, MO) or vehicle prior to fixation. Immunocytochemistry was conducted as described in Taniguchi *et al.* 2012. Briefly coverslips were incubated in anti-GFP antibody (1:10 000), anti-Flag (1:500) or anti-HDAC4 antibody (1:1000) followed by anti-mouse FITC (1:500) diluted in 1X TBS. After secondary incubation, coverslips were counterstained with Hoechst 33342 (1:50 000) to visualize the nucleus. The localization of HDAC4-GFP, HDAC4-Flag, HDAC5-GFP or HDAC4-Flag mutants was categorized as cytoplasmic mixed (not displayed), or nuclear using the GFP or Flag signal for each neuron under experimenter-blind conditions. The percentage for each category was calculated from the total number of transfected neurons counted in each condition, and the average across experiments is shown.

## Plasmids and viruses

p1005+ GFP HDAC4-Flag WT was generated by PCR amplification of hHDAC4-Flag from pBJ-hHDAC4-Flag (Dr. Eric Olson, UTSW). PCR primers were designed to add XbaI restriction sites on 5' and 3' end of full-length hHDAC4-Flag. p1005+ GFP (Dr. Rachael Neve, MIT) backbone and PCR product were digested with XbaI, and compatible cohesive ends were ligated to produce p1005+ GFP HDAC4-Flag WT. p1005+ GFP HDAC4-Flag 3SA was generated by PpuMI and PflMI digestion of pcDNA-HDAC4.3SA-Flag (Addgene, Cambridge, MA), p1005+ GFP HDAC4-Flag WT was identically digested to excise the wild-type portion of the HDAC4 coding sequence and compatible cohesive end ligations generated p1005+ GFP HDAC4-Flag 3SA. p1005+ GFP HDAC4-Flag S266E and S266A was generated by site-directed mutagenesis of pBS-hHDAC4-Flag using primers that produce a serine to glutamine substitution at S266. pBS-hHDAC4-S266E/A-Flag was digested with PpuMI and PflMI digestion, and p1005+ GFP HDAC4-Flag S266E/A was generated using the same digestion/ligation as p1005+ GFP HDAC4-Flag 3SA. Diagnostic digests and sequencing were performed to confirm proper orientation and sequence. pEGFP-

N1-HDAC5-GFP (Dr. Eric Olson, UTSW) and pcDNA3.1-GFP-HDAC4 (Addgene) were used to examine *in vitro* localization.

### Sample preparation from cocaine-injected mice

C57BL/6 mice (Charles River) were injected once per day for seven consecutive days (intraperitoneal; i.p.) with saline or 20 mg/kg cocaine (Sigma-Aldrich) and sacrificed 4 hours after the last injection. Striata were rapidly dissected from 1 mm coronal sections prepared using a mouse brain matrix. To probe the phosphorylation status of HDAC4, samples are prepared as previously described (Taniguchi *et al.* 2012). Briefly, striata are dissolved in tissue lysis buffer with inhibitors and diluted with ninefold volume of radioimmunoprecipitation assay (RIPA, without SDS) buffer prior to sequential immunoprecipitation of HDAC5 and HDAC4, using anti-HDAC5 monoclonal antibody (Abcam, #50001) and anti-HDAC5 polyclonal antibody (cell signaling, #2082) followed by anti-HDAC4 polyclonal antibody (cell signaling, #2072). Cytosolic and nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Waltham, MA) according to manufacturer's instructions. Samples were collected for protein quantification using detergent compatible Lowry colorimetric assay. Respectively, 80 and 50  $\mu$ g of nuclear and cytoplasmic amounts were used for western blot analysis. Ratios are calculated relative to the saline condition and normalized to  $\beta$ -tubulin in the cytoplasmic and lamin A/C in the nuclear fraction.

### Western blotting

Lysates from stimulated cultured neurons and cocaine-treated animals were first immunoprecipitated to isolate HDAC5. Subsequently, HDAC4 was immunoprecipitated from the HDAC5-immunodepleted lysates. Protein extracts were subjected to SDS-PAGE, transferred to PVDF membrane, blocked with 10 percent (*w/v*) non-fat dry milk or LI-COR blocking buffer diluted in 1X PBS and incubated with antibody solutions. Primary antibodies were diluted in 1X TBS with 0.05 percent Tween-20 containing 3 percent (*w/v*) bovine serum albumin or LI-COR blocking buffer with 0.01 percent Tween-20 as follows: anti-HDAC4 (1:500), anti-P-S266 (1:500, lab made), anti-Flag (1:500), anti- $\beta$ -tubulin (1:10 000) or anti-lamin A/C (1:500). Secondary antibodies were diluted in 1X TBS with 0.05 percent Tween-20 containing 10 percent (*w/v*) non-fat dry milk as follows or LI-COR blocking buffer with 0.01 percent Tween-20 and 0.1 percent SDS: anti-mouse HRP conjugated IgG (1:10 000), anti-rabbit HRP conjugated IgG (1:10 000), IRDye 680CW anti-mouse IgG (1:20 000) and IRDye 680RD anti-rabbit IgG (1:20 000). Blots were developed by enhanced chemiluminescence (ECL-Prime, Amersham Pharmacia Biotech, Little Chalfont, UK) or imaged on LI-COR Odyssey Clx (LI-COR Biosciences, Lincoln, NE). Densitometry analysis was performed with NIH ImageJ (<https://imagej.nih.gov/ij/>) or LI-COR Image Studio (LI-COR Biosciences).

### Viral-mediated gene transfer

Herpes simplex viruses (HSV) expression plasmids for the HDAC4 S266E mutant were packaged into high-titer viral particles as described previously (MIT viral core, Neve & Lim 2013). High-titer HSV-GFP was purchased from the MIT viral vector core. High titer AAV2/2-GFP and AAV2/2-Cre-GFP were purchased from Vector Biolabs (Malvern, PA).

Stereotaxic surgery was performed on anesthetized mice as previously described (Penrod *et al.* 2015). Virus was delivered bilaterally to the NAc (+1.6 mm anterior, +1.5 mm lateral and -4.4 mm ventral from bregma) at a 10° angle. Virus was delivered using Hamilton syringes at a rate of 0.1 µl/minute for a total of 0.5–0.7 µl of virus. Behavioral testing began after a recovery period (72 hours for HSV experiments for peak expression and 3 weeks for AAV experiments for peak expression and recombination). Viral placements were confirmed using GFP signal, which was co-expressed by all viruses.

### HDAC4 recombination validation

*Hdac4*<sup>loxP/loxP</sup> received AAV2/2-GFP or AAV2/2-Cre-GFP as described previously. Three weeks after viral infusion, animals were rapidly decapitated; brains were extracted, frozen on dry ice and stored at -80° C until ready for dissection. Brains were transferred from -80° C and placed into a cryostat at -25° C. Brains were mounted onto the cryostat chuck with Tissuetek and fast frozen with cryospray (Freeze-It, Thermo Fisher Scientific). Forty-micrometer sections were taken until the NAc was revealed. Bilateral punches were taken using a 1-mm stainless steel punch tool and placed in cold microcentrifuge tubes for later RNA extraction. Brains were sectioned through the depth of the punch to confirm that punches were taken from NAc. NAc punches were lysed in QIAzol lysis reagent (Qiagen Germantown, MD), and RNA was isolated using miRNAeasy collection kit (Qiagen). mRNA was converted to cDNA using SuperScriptIII-RT (Invitrogen Carlsbad, CA) and random hexamer primers. HDAC4 expression was quantified using RT-qPCR with probes *Hdac4* 5'-CGAGCACAGAGGTGAAGATG-3' (forward) and 5'-GGTGGAGAGCTCTGGTCAA-3' (reverse) and *GAPDH* 5'-AGGTCGGTGTGAACGGATTTG-3' (forward) and 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse). Fold changes relative to *GAPDH* were determined using the  $\Delta\Delta C_t$  method, in which mean fold change ( $2^{-\Delta C_t^{AVE}}$ ) and SEM ( $(\text{abs}((2^{-\Delta C_t^{AVE}} \times 2^{-\Delta C_t^{SEM}}) - (2^{-\Delta C_t^{AVE}/2^{-\Delta C_t^{SEM}}))/2))$ ) were determined and expressed relative to GFP control condition.

### Locomotor sensitization

Animals were sensitized to repeated cocaine injections, as previously described (Smith *et al.* 2016). Briefly, following a 1-hour habituation session in the test chamber, the mice receive saline or cocaine (i.p.) injections and are returned to the chamber for an additional hour. All mice received four daily saline injection sessions followed by daily cocaine injections (15 mg/kg) for the next 7 days. *Hdac4* cKO<sup>NAc</sup> cocaine locomotor sensitization was assessed 2–3 weeks after low dose (5 mg/kg) CPP. *Hdac4* cKO<sup>NAc</sup> acute cocaine locomotor responding in drug-naïve mice was assessed at 3 weeks following initial viral delivery. For each locomotor session, the sum of the first 30 or 60 minutes of locomotor activity is displayed.

### Conditioned place preference

Mice were conditioned to cocaine using a balanced, accelerated paradigm to accommodate the timing of transient HSV expression (Pliakas *et al.* 2001; Smith *et al.* 2016). Briefly, after recovery from viral-mediated gene transfer (i.e. 72 hours for HSV studies), the mice freely explore a three-chambered apparatus, and time in each chamber is recorded. On the next 2 days, animals received two daily pairings with either side of the chamber: i.p. saline in the

morning and i.p. cocaine (5 or 10 mg/kg) in the afternoon. Twenty-four hours after the last conditioning sessions, animals were returned to the chamber and allowed to freely explore both sides. Time spent in each chamber was recorded, and time spent in paired or unpaired (middle and unpaired) sides on pre-test and post-test is displayed. Only animals bilaterally expressing virus (as determined by GFP expression) in the NAc were included in final analysis.

### Luciferase assay

3X MRE-luciferase and TK-Renilla luciferase plasmids have been previously described (Flavell *et al.* 2006). Primary striatal cultures were transfected using calcium phosphate method at 8 days *in vitro*, and 48 hours later, the cells were stimulated with or without depolarization buffer (isotonic, 60 mM KCl final; Pulipparacharuvi *et al.* 2008) for 6 hours, and dual luciferase activity levels were measured as recommended by the manufacturer (Promega, Madison, WI).

### Immunohistochemistry

To confirm placement and viral-mediated gene transfer, mouse brains were rapidly extracted and processed for immunohistochemistry as previously described (Penrod *et al.* 2015). Briefly, brains were dissected, fixed, and cryoprotected. Fifty-micrometer sections from the olfactory bulbs to the dorsal hippocampus were generated using a freezing microtome. Free-floating sections were blocked in 3 percent (*w/v*) bovine serum albumin and incubated in chicken anti-GFP (1:1000, Aves) for 3–16 hours followed by anti-chicken 488 conjugated IgY (1:200). Sections were then counterstained with the nuclear dye, Hoechst 33342 (1:50,000 Thermo Fisher Scientific, Waltham, MA), dehydrated and mounted for fluorescent microscopy visualization.

### Data analysis

Student's *t*-tests and one-way, two-way or repeated measures ANOVAs with Sidak's multiple comparison *post hoc* tests were used, as appropriate. Grubb's outlier analysis was used on final datasets. Statistical analysis and graphical depictions were prepared using GRAPHPAD PRISM software (GraphPad, La Jolla, CA).

## RESULTS

### HDAC4 and HDAC5 phosphorylation are differentially regulated by cAMP and cocaine

Previous work has demonstrated that elevation of cAMP levels stimulates dephosphorylation at multiple HDAC5 residues, which results in a robust accumulation of HDAC5 protein in the cell nucleus (McKinsey *et al.* 2000b; McKinsey *et al.* 2001; Chawla *et al.* 2003; Vega *et al.* 2004; Taniguchi *et al.* 2012; Walkinshaw *et al.* 2013). The HDAC5 S279 phosphorylation site, which resides within the nuclear localization sequence, regulates the nuclear export rate, and dephosphorylation of this site is induced by cocaine or elevated cAMP signaling in striatal neurons (Taniguchi *et al.* 2012). Interestingly, HDAC4 and HDAC5 show full sequence conservation of the residues surrounding this site (Fig. 1a), and as predicted, we found that the HDAC5 P-S279 antibody (Taniguchi *et al.* 2012) also site-specifically recognizes HDAC4 P-S266 (Fig. 1b). To test whether HDAC4 P-S266 has a similar effect on

subcellular localization as HDAC5 P-S279, we generated a phospho-mimetic mutant (S266E) of HDAC4 and tested its distribution in striatal neurons. Similar to the HDAC5 S279E mutant (Taniguchi *et al.* 2012), the HDAC4 S266E protein concentrated in the cytoplasm of striatal neurons (Fig. 1c) and reduced nuclear accumulation under conditions of nuclear export inhibition (leptomycin B treatment, Fig. 1d;  $F_{\text{Interaction}}(3, 16) = 4.895$ ,  $P = 0.0134$ ; Sidak's *post hoc*, cytoplasmic versus nuclear  $P < 0.01$  WT, S266A, 3SA, n.s. S266E), suggesting that P-S266/S279 has a similar effect on subcellular distribution of these closely related proteins.

Because elevation of cAMP signaling (10  $\mu\text{M}$  forskolin) in striatal neurons triggers the rapid and robust (~75 percent) dephosphorylation of HDAC5 P-S279 (Taniguchi *et al.* 2012), we asked whether HDAC4 P-S266 is similarly regulated. To examine HDAC4 phosphorylation specifically, a sequential immunoprecipitation strategy was employed. HDAC5 was immunodepleted from the tissue lysate using a HDAC5-specific antibody, and subsequently, HDAC4 was immunoprecipitated using a HDAC4-specific antibody (or vice versa, Supporting Information Fig S1a). Surprisingly, we observed that forskolin treatment did not significantly decrease P-S266 levels (Fig. 2a), suggesting distinct regulation of these two closely related HDACs. Consistent with this observation, elevation of cAMP levels promoted accumulation of GFP-tagged HDAC5 into the cell nucleus (Fig. 2b, left;  $F_{\text{Interaction}}(2, 12) = 6.385$ ,  $P = 0.0129$ ; Sidak's *post hoc*, nuclear compartment  $P < 0.05$ ), whereas cAMP elevation did not alter GFP-tagged HDAC4's cytoplasmic distribution (Fig. 2b, right;  $F_{\text{Compartment}}(2, 12) = 0.3519$ ,  $P = 0.0057$ ) in cultured primary striatal neurons. Like forskolin treatment, cocaine administration (i.p.) in mice produces a transient dephosphorylation of HDAC5 S279 and enrichment of HDAC5 in the nuclear fraction from isolated striatal tissues (Taniguchi *et al.* 2012). To test whether chronic cocaine administration regulates HDAC4 in a similar fashion, we repeatedly injected wild-type mice with cocaine (20 mg/kg; i.p.) or saline and then analyzed P-S266 and the nuclear/cytoplasmic ratio of HDAC4. At 4 hours following the last cocaine injection, a time point where HDAC5 S279 is significantly dephosphorylated (~35 percent; Taniguchi *et al.* 2012) and enriched in the nucleus, we observed an approximately fourfold increase in P-S266 HDAC4 levels (Fig. 2c, right;  $P = 0.0525$ ). Consistent with the role of this residue in regulating HDAC4 and HDAC5 subcellular localization, we detected a significant decrease in HDAC4 nuclear levels (Fig. 2d, right;  $t = 2.623$ ,  $df = 9$ ,  $P = 0.0277$ ). These data suggest that HDAC4 and HDAC5, despite high sequence homology, are distinctly regulated by cAMP signaling and cocaine in striatum.

### HDAC4 in the NAc is required for multiple cocaine behavioral responses

Because cocaine administration regulates the subcellular localization of HDAC4, we tested the importance of this gene in two cocaine-induced behaviors—cocaine reward, which measures an animal's preference to explore a chamber previously associated with drug experience, and locomotor sensitization, which measures a naïve and adaptive motor response that develops with repeated cocaine administration. Because total *Hdac4* KO mice do not survive past weaning (Vega *et al.* 2004), we investigated the role of HDAC4 in cocaine behaviors using a conditional gene deletion approach. Young adult *Hdac4<sup>f/f</sup>* mice (Potthoff & Olson 2007) received bilateral infusions of AAV2-Cre-GFP into the NAc to



generate conditional *Hdac4* KO mice (*Hdac4* cKO<sup>NAc</sup>) or littermate controls (AAV2-GFP infusions). Three weeks after virus infusion to allow for recombination (Fig. 3a;  $t = 2.236$ ,  $df = 10$ ,  $P = 0.0494$ ), all mice were tested sequentially in CPP (5 or 10 mg/kg cocaine; Pliakis *et al.*, 2001; Smith *et al.* 2016) and locomotor sensitization tests. *Hdac4* cKO<sup>NAc</sup> mice failed to condition to a low dose of cocaine (5 mg/kg;  $F_{\text{Conditioning}}(1, 29) = 10.1$ ,  $P = 0.0035$ ; Sidak's *post hoc* pre versus post, GFP  $P < 0.01$ , *Hdac4* cKO<sup>NAc</sup> n.s.), but conditioned like wild-type controls to high-dose cocaine in the CPP assay (10 mg/kg, Fig. 3b;  $F_{\text{Conditioning}}(1, 37) = 35.49$ ,  $P < 0.0001$ ; Sidak's *post hoc* pre versus post, GFP and *Hdac4* cKO<sup>NAc</sup>  $P < 0.001$ ), suggesting that HDAC4 in the NAc enhances an animal's sensitivity to the rewarding effects of cocaine, particularly at low doses. The mice were then left undisturbed in their home cages for 2–3 weeks before locomotor sensitization testing. All mice were subjected to four daily injections of saline and then seven consecutive daily injections of cocaine (15 mg/kg; i.p.), and locomotion was monitored for 1 hour (Smith *et al.* 2016). Compared with control mice, *Hdac4* cKO<sup>NAc</sup> mice displayed normal locomotion following the saline injections (Fig. 3c). There was a small, but non-significant, reduction in locomotion after the first cocaine injection (Fig. 3c, Coc1), but a significant reduction in sensitized locomotion developed after repeated cocaine administration (Fig. 3c;  $F_{\text{Session}}(3, 87) = 115$ ,  $P < 0.001$ ;  $F_{\text{Interaction}}(3, 87) = 3.402$ ,  $P = 0.0212$ ; Sidak's *post hoc*, Coc7  $P < 0.01$ ). Because all of the mice had received prior, low-dose cocaine in the preceding CPP test, we next tested drug-naïve *Hdac4* cKO<sup>NAc</sup> and control mice to examine possible differences in naïve cocaine locomotor responses to a range of cocaine doses. We observed a significant reduction in drug-induced locomotion in the drug-naïve *Hdac4* cKO<sup>NAc</sup> mice at both 15 and 30 mg/kg cocaine (Fig 3c;  $F_{\text{Genotype}}(3, 98) = 11.45$ ,  $P = 0.0010$ ;  $F_{\text{Dose}}(3, 98) = 11.24$ ,  $P < 0.001$ ;  $F_{\text{Interaction}}(3, 98) = 3.78$ ,  $P = 0.0129$ ; Sidak's *post hoc*, 15 mg/kg  $P < 0.05$ , 30 mg/kg  $P < 0.01$ ). Together, these findings indicate that HDAC4 in the NAc is required for normal cocaine reward behavior and both naïve and sensitized locomotor responses to cocaine.

### Cytoplasmic HDAC4 enhances cocaine reward

Chronic cocaine administration increases the phosphorylation and cytoplasmic localization of HDAC4 (Fig. 2c and d), and loss of HDAC4 reduces cocaine reward behavior (Fig. 3a), particularly at a threshold dose of drug (5 mg/kg). We therefore speculated that the cocaine-induced HDAC4 nuclear export might function to facilitate cocaine reward behavior. To test this idea, we generated recombinant HSV that overexpress a cytoplasm-localized HDAC4 mutant (S266E) or GFP alone (Fig. 1c and d). Animals bilaterally overexpressing HDAC4 S266E, but not GFP, in the adult NAc displayed significant cocaine conditioned place preference at a threshold dose (Fig. 4a;  $F_{\text{Conditioning}}(1, 26) = 10.3$ ,  $P = 0.0035$ ; Sidak's *post hoc*, pre versus post, SE  $P < 0.05$ ). Unlike overexpression of the nuclear mutant, HDAC4–3SA, which dramatically reduced depolarization-induced MEF2-dependent transcription, overexpression of the HDAC4 S266E mutant had no significant effect on basal or depolarization-induced MEF2-dependent transcription activity in striatal cultures (Fig. 4b;  $F_{\text{Construct}}(1, 48) = 27.84$ ,  $P < 0.0001$ ,  $F_{\text{Interaction}}(2, 48) = 7.222$ ,  $P = 0.0018$ ; Sidak's *post hoc*, basal versus KCl, GFP and S266E  $P < 0.001$ , 3SA ns), suggesting that overexpression of HDAC4 S266E does not function as a dominant negative protein in striatal neurons. Together, these findings suggest that phosphorylated HDAC4 can enhance cocaine reward-like behavior, possibly through a role in the cytoplasm.

## DISCUSSION

In this study, we report that, unlike HDAC5 (Belfield *et al.* 2006; Ha 2010; Mihaylova *et al.* 2011; Taniguchi *et al.* 2012) and HDAC4 in other cell types (Du *et al.* 2008; Kozhemyakina *et al.* 2009; Mihaylova *et al.* 2011; Liu & Schneider 2013; Walkinshaw *et al.* 2013), elevation of cAMP signaling in striatal neurons fails to trigger robust dephosphorylation and accumulation of HDAC4 in the cell nucleus, suggesting distinct and cell type-specific regulatory mechanisms between these closely related class IIa HDACs. Moreover, we find that cocaine administration produces an increase in HDAC4 phosphorylation (statistical trend) and a significant reduction in HDAC4 nuclear levels in striatal tissues, which is opposite of the effects of cocaine on HDAC5 (Fig. 2, Taniguchi *et al.* 2012). While HDAC4 in the adult NAc appears to be dispensable for cocaine reward-like behavior in the conditioned place preference test at higher doses (10 mg/kg), it is required for the development of preference to low doses (5 mg/kg), perhaps indicating that novel mechanisms recruited by higher doses of cocaine are able to compensate for the loss of HDAC4. We also find that HDAC4 is required in the adult NAc for acute locomotor responses to cocaine—particularly at moderate to high doses—and for cocaine locomotor sensitization (Fig. 3), suggesting a NAc-specific role for HDAC4 in naïve and adaptive motor responses to cocaine. We show that a mutant form of HDAC4 with a phosphomimetic mutation at a key regulatory serine residue (S266E) promotes cytoplasmic accumulation of HDAC4 and cocaine reward, the opposite localization and behavioral phenotype observed for HDAC5 (Taniguchi *et al.* 2012). Taken together with previous work, our findings add to the growing evidence that despite high homology and often overlapping expression patterns, there are cell type-specific and/or brain region-specific roles and regulation for the different class IIa HDAC family members.

Our current observations raise a number of interesting questions for future study. Our analysis of the phospho-site mutants on HDAC4 and HDAC5 suggests strongly that phosphorylation at the conserved residues serves a similar role in regulating subcellular localization. However, while cAMP signaling triggers a robust dephosphorylation and nuclear accumulation of HDAC5 (Fig. 2; Belfield *et al.* 2006; Ha 2010; Mihaylova *et al.* 2011; Taniguchi *et al.* 2012), HDAC4 appears to be largely insensitive to cAMP signaling in cultured striatal neurons. Consistent with these observations, HDAC4 and HDAC5 phosphorylation and subcellular localization were distinctly regulated by *in vivo* cocaine exposure. Although somewhat surprising given the high homology between HDAC4 and HDAC5, multiple studies in non-neuronal cells report differential sensitivity to signaling events that regulate phosphorylation/nuclear export as well as interdependence between closely related HDACs to promote coordinated export events (Chang *et al.* 2005; Backs *et al.* 2006; Zhang *et al.* 2007; Backs *et al.* 2008; Métrich *et al.* 2010; Liu *et al.* 2012). Similar to our current findings that cocaine triggers nuclear export of HDAC4 (Fig. 2d), a previous study (Renthal *et al.* 2007) reported that chronic cocaine administration increased the levels of P-S259—another highly conserved phosphorylation site that promotes cytoplasmic localization of the class IIa HDACs (McKinsey *et al.* 2000a, b). This suggests that chronic cocaine administration might trigger hyperphosphorylation at multiple HDAC4 residues that regulate subcellular localization.

Because *Hdac5 KO* mice display enhanced cocaine reward behavior (Renthal *et al.* 2007), particularly after prior cocaine experience, and overexpression of HDAC4 in the NAc reduces cocaine reward behavior and drug self-administration (Kumar *et al.* 2005; Wang *et al.* 2010), we predicted that genetic disruption of HDAC4 in the adult NAc might enhance behavioral responses to cocaine. However, we noted that *Hdac4 cKO<sup>NAc</sup>* mice failed to condition to a near-threshold dose of cocaine (5 mg/kg), which suggests that endogenous HDAC4 increases the sensitivity to cocaine reward, albeit to a modest extent. Moreover, we detected reductions in both naïve and sensitized cocaine locomotor responses in *Hdac4 cKO<sup>NAc</sup>* mice, suggesting that HDAC4 enhances the sensitivity to cocaine in multiple behavioral readouts.

Because repeated cocaine exposure promoted HDAC4 hyperphosphorylation and increased cytoplasmic localization, we examined the behavioral consequence of overexpression of a cytoplasmically accumulated HDAC4 mutant. We reasoned that cytoplasmic localization on HDAC4 might reflect the activation of gene expression and removal of its gene repressor functions in the cell nucleus. In this case, like expression of HDAC5 S279E (Taniguchi *et al.* 2012), we would predict that overexpression of HDAC4 S266E would have no effect on cocaine CPP because it is not retained in the nucleus and unable to repress transcription. However, if cocaine-induced relocalization of HDAC4 serves a cytoplasmic role, then overexpression of the HDAC4 S266E mutant might alter cocaine CPP. Interestingly, we observed that overexpression of HDAC4 in the cytoplasm actually enhanced cocaine reward behavior at a near-threshold conditioning experience. In the two 5 mg/kg conditioning experiments (Figs 3b & 4a), the behavior of the control groups is variable. We find that a number of variables contributed to the conditioning effect, particularly at a near-threshold dose, including experimenter sex, background strain, and surgical or other prior experience. In these experiment, animals differed in strain (*Hdac4<sup>Fl/Fl</sup>* versus C57Bl/6 J), housing experience (breed in colony versus ordered from vendor) and surgical experience (AAV infusion with 2–3 weeks of recovery versus HSV infusion with 2–3 days of recovery). Despite the difficulty in comparing the experiments directly, the concordance between the two experiments (loss of function reduces while overexpression enhances conditioning) supports the interpretation that cocaine-induced cytoplasmic localization of HDAC4 promotes cocaine responding.

The finding of a cytoplasmic role for HDAC4 is surprising, but not unprecedented. For example, previous work has found that cytoplasmically localized HDAC5 can enhance tubulin deacetylation (Cho & Cavalli 2012; Cho *et al.* 2013). In the future, it will be interesting to test whether cytoplasmic HDAC4 promotes cocaine CPP independent of its ability to regulate nuclear gene expression and/or whether the cytoplasmic mutant of HDAC4 functions as a dominant negative by associating with nuclear factors and titrating them away from the nucleus. Regarding the latter possibility, HDAC4 can interact with multiple nuclear proteins, including other HDACs, repressor complex members and transcription factors (Miska *et al.* 1999; Huang *et al.* 2000; Fischle *et al.* 2002; Backs *et al.* 2008) whose removal from the nucleus could promote cocaine reward. However, arguing against this possibility, we did not observe a significant repression or enhancement of MRE-luciferase activity of HDAC4 S266E on basal or depolarization-induced MEF2-dependent transcription (Fig. 4b).

Overall, our current findings indicate that the same cocaine regimen that promotes HDAC5 dephosphorylation and nuclear accumulation in striatum enhances HDAC4 phosphorylation and cytoplasmic localization. Our previous work suggested that cocaine and D1 dopamine receptor stimulation of cAMP signaling activates a PP2A phosphatase-dependent dephosphorylation of HDAC5, which promotes nuclear accumulation (Taniguchi *et al.* 2012). Because neither elevation of cAMP in striatal neurons nor cocaine administration produces HDAC4 dephosphorylation and nuclear accumulation, we speculate that HDAC4 is a poor substrate for PP2A or that there are competing kinase reactions, such as PKA, that enhance HDAC4 phosphorylation at S266. This differential regulation of HDAC4 and HDAC5 is possibly mediated by differential affinity for the numerous phosphatase and kinase cascades downstream of the coordinated glutamatergic and dopaminergic activity produced by cocaine exposure, and future research will be required to determine the underlying signaling mechanisms that differentially regulate these closely related HDACs in the striatum. Our behavioral findings also suggest distinct, and possibly opposite, roles for HDAC4 and HDAC5 in multiple cocaine behaviors. In the future, understanding the relevant signaling cascades and downstream target genes or cell processes that ultimately mediate HDAC4's and HDAC5's effects on cocaine behavior will be essential for understanding their roles in drug addiction-related behaviors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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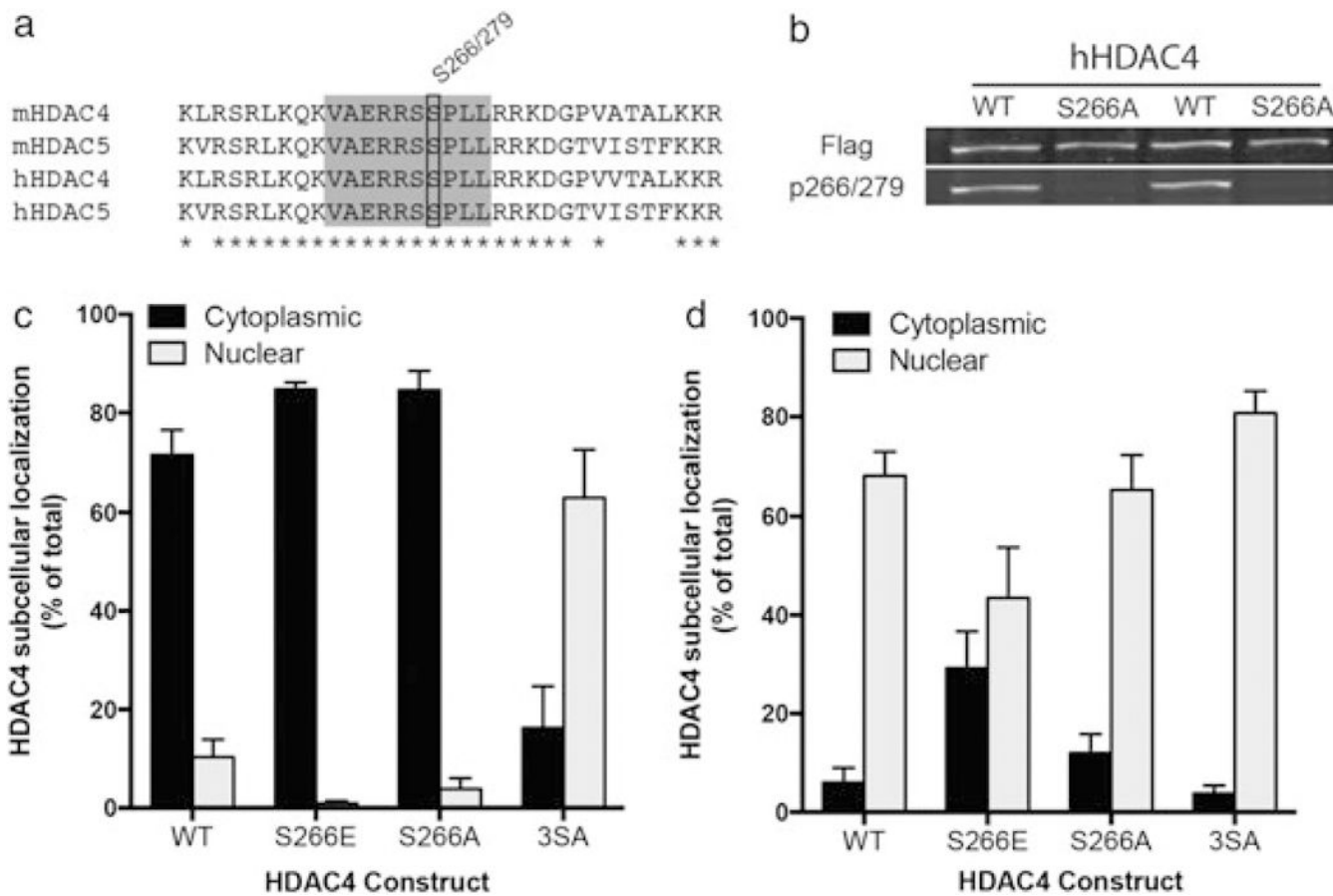
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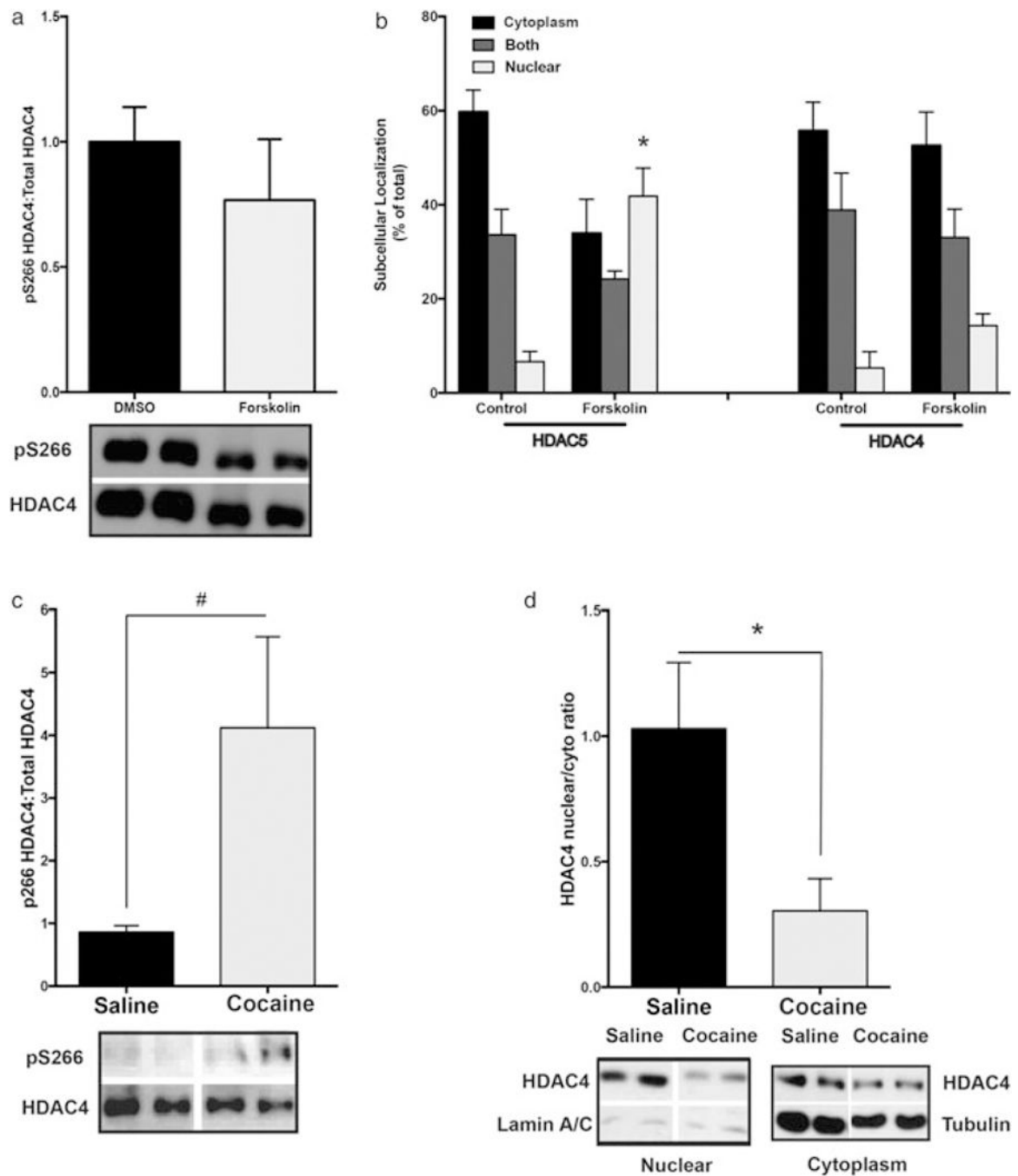
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**Figure 1.** HDAC4 S266 drives cytoplasmic localization when phosphorylated. (a) Residue conservation comparison between mouse and human HDAC4 and HDAC5 near the S266/S279 residue (black box). Gray box encompasses the epitope used for antibody production; asterisks indicate 100 percent identity sites. (b) HDAC4 WT or HDAC S266A overexpressed in HEK293 cells show equivalent total HDAC expression (top panel) but the absence of p266 signal (bottom panel) in the alanine mutant condition. (c) Phosphomimetic mutation of S266 (S266E) promotes HDAC4 cytoplasmic accumulation. Mutation of critical serine residues to alanine (S246A/S467A/S632A) shifts HDAC4 subcellular localization to the nucleus. (d) Under conditions where nuclear export is blocked (3 hour of 10 ng/ml leptomycin B treatment), phosphomimetic mutation of S266 (S266E) causes enhanced cytoplasmic localization, indicating reduced nuclear import. In (c) and (d), transfected neurons were identified by GFP expression (driven by a second promoter), and subcellular localization was determined by Flag signal (C-terminal tag on all HDAC4 constructs)





**Figure 2.** HDAC4 phosphorylation is differentially regulated by cAMP and cocaine. (a) Forskolin treatment (10  $\mu$ M, 3 hours) does not alter HDAC4 S266 phosphorylation (from HDAC5 immunodepleted, HDAC4 immunoprecipitated lysates) compared with DMSO-treated cultures (mean  $\pm$  SEM,  $n = 6$ /condition, three independent experiments). (b) Forskolin treatment (10  $\mu$ M, 3 hours) promotes nuclear accumulation of HDAC5-GFP but does not alter HDAC4-GFP subcellular distribution (mean  $\pm$  SEM,  $n = 3$  wells/condition, ~80 cells per well, two to three independent experiments). (c) Chronic cocaine (20 mg/kg; i.p.; 7 days)

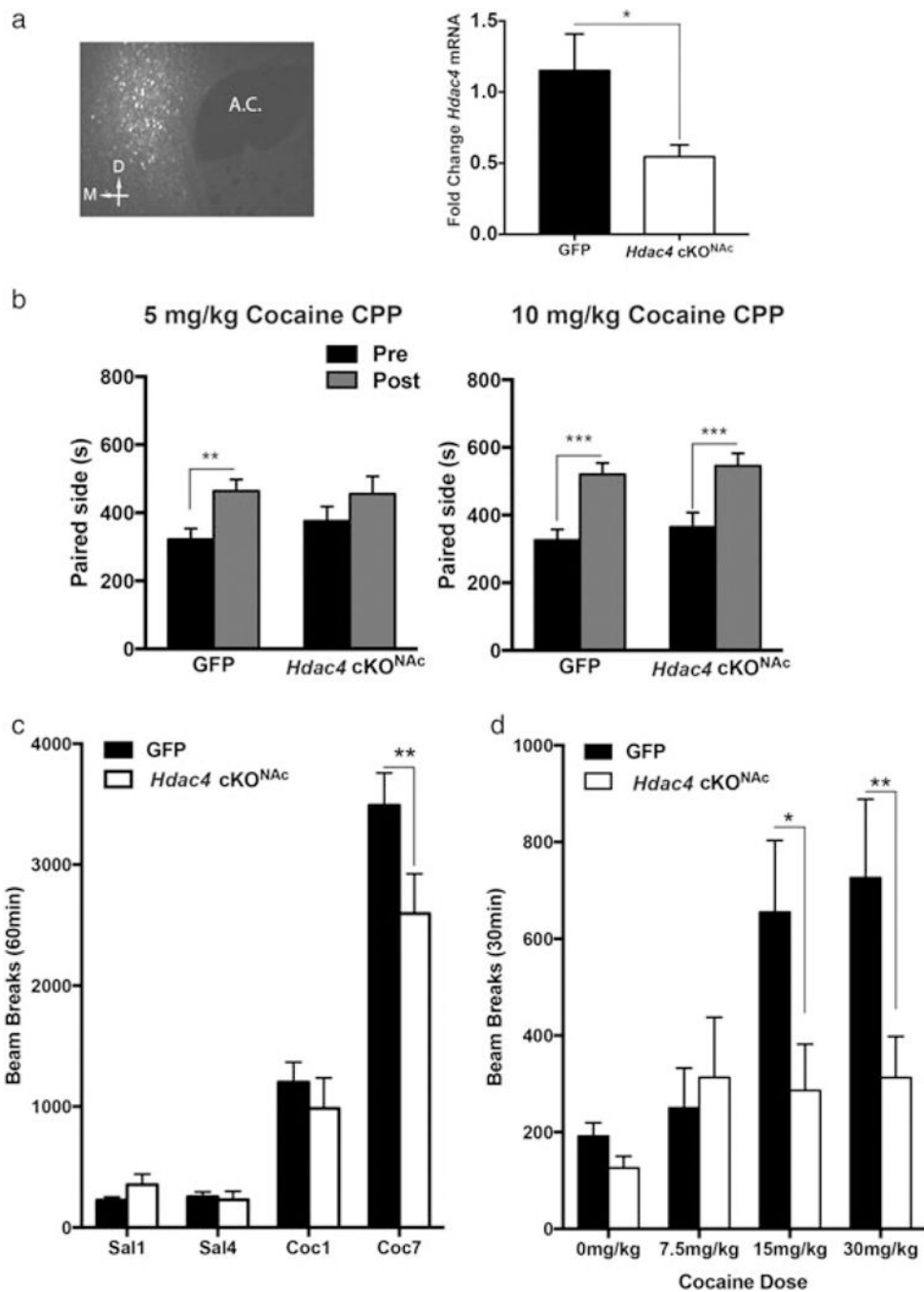
weakly increases S266 phosphorylation of HDAC4 (from HDAC5-immunodepleted, HDAC4 immunoprecipitated lysates) compared with animals treated with repeated saline injections (mean  $\pm$  SEM,  $n = 9$  mice/condition). (d) Cocaine promotes cytoplasmic accumulation of HDAC4. Nuclear/cytoplasmic ratios of HDAC4, relative to saline, normalized to lamin A/C in nuclear fraction and to  $\beta$ -tubulin in cytoplasmic fraction (mean  $\pm$  SEM,  $n = 5-6$  mice/condition). Asterisks indicate significant difference from control group in pairwise comparison; \* $P < 0.05$ , # near significant result  $P = .0525$

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**Figure 3.** Loss of HDAC4 limits locomotor responses to acute and sensitizing regimens of cocaine. (a) Representative targeted expression in the NAc >21 days post-infection. Viral-mediated Cre expression significantly reduces HDAC4 mRNA. NAc punches from AAV-GFP or AAV-Cre-GFP *Hdac4*<sup>fl/fl</sup> mice 3 weeks post-infection. HDAC4 mRNA is normalized to GAPDH and expressed as fold change compared with GFP control group (mean ± SEM, *n* = 6–7 NAc punches/condition). (b) *Hdac4* NAc cKO disrupts low-dose (5 mg/kg) cocaine-conditioned place preference (left) but does not alter high-dose (10 mg/kg) cocaine CPP (right, mean ±

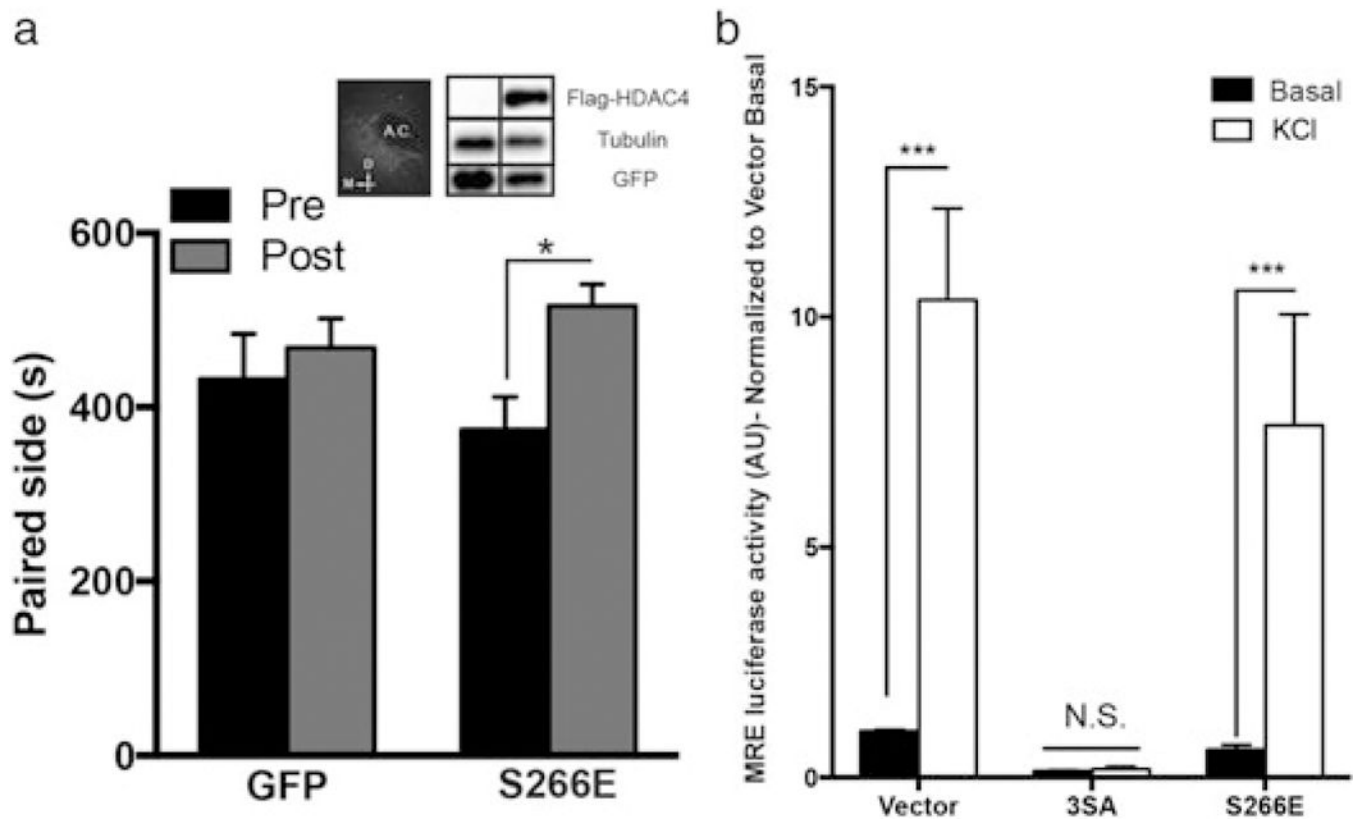
SEM,  $n = 12-19$  mice/condition). (c) *Hdac4*NAc cKO decreases locomotor sensitization magnitude (mean  $\pm$  SEM,  $n = 12-19$  mice/condition). (d) *Hdac4*NAc cKO decreases acute locomotor responses to cocaine (mean  $\pm$  SEM,  $n = 8-10$  mice/condition). Asterisks indicate significant difference from pre-test (b) or control group (a, c and d) in pairwise comparison, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . AC, anterior commissure; D, dorsal; M, medial

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**Figure 4.** Cytoplasmic accumulation of HDAC4 promotes cocaine reward. (a) Overexpression (inset shows representative targeted expression in the NAc ~7 days post-infection and viral-mediated expression in cultured striatal neurons) of a cytoplasmically localized HDAC4 mutant enhances a subthreshold conditioning experience (mean  $\pm$  SEM,  $n = 7-12$  mice/condition). (b) Overexpression of a nuclear localized, but not cytoplasmically localized, HDAC4 mutant represses MRE-luciferase activity by KCl-induced depolarization. Asterisks indicate significant difference from pre-test or control, \* $P < 0.05$ , \*\*\* $P < 0.001$ . AC, anterior commissure