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Histone Deacetylase 5 Limits Cocaine Reward through cAMP-Induced Nuclear Import

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SUMMARY

Chromatin remodeling by histone deacetylases (HDACs) is a key mechanism regulating behavioral adaptations to cocaine use. We report here that cocaine and cyclic adenosine monophosphate (cAMP) signaling induce the transient nuclear accumulation of HDAC5 in rodent striatum. We show that cAMP-stimulated nuclear import of HDAC5 requires a signaling mechanism that involves transient, protein phosphatase 2A (PP2A)-dependent dephosphorylation of a Cdk5 site (S279) found within the HDAC5 nuclear localization sequence. Dephosphorylation of HDAC5 increases its nuclear accumulation, by accelerating its nuclear import rate and reducing its nuclear export rate. Importantly, we show that dephosphorylation of HDAC5 S279 in the nucleus accumbens suppresses the development, but not expression, of cocaine reward behavior *in vivo*. Together, our findings reveal a molecular mechanism by which cocaine regulates HDAC5 function to antagonize the rewarding impact of cocaine, likely by putting a brake on drug-stimulated gene expression that supports drug-induced behavioral changes.

INTRODUCTION

Histone deacetylation by chromatin-modifying enzymes plays a critical role in shaping transcriptional responses to experience. Drug addiction is thought to represent a long-lasting, maladaptive change in the function of the brain reward circuitry, and drug-induced transcriptional responses contribute to behavioral adaptations relevant to addiction (Hyman et al., 2006; Kalivas, 2004; McClung and Nestler, 2003). Several recent studies have reported an important role for histone deacetylase (HDAC) activity in the regulation of cocaine-induced behaviors in rodent models of addiction (Hui et al., 2010; Kumar et al., 2005; Renthal et al., 2007, 2009; Sanchis-Segura et al., 2009; Wang et al., 2010). However, how cocaine regulates HDAC function in brain reward circuitry, and whether regulation is important for its ability

to modulate addiction-related behavioral responses, is poorly understood.

The class IIa HDACs emerged recently as important modulators of cocaine-induced behavioral responses *in vivo* (Kumar et al., 2005; Renthal et al., 2007; Wang et al., 2010). The class IIa HDACs (HDAC4, 5, 7, and 9) are unique among the HDAC family proteins in that they shuttle between the nucleus and the cytoplasm in cells (Belfield et al., 2006; Bertos et al., 2001; McKinsey et al., 2000a, 2001). Nucleocytoplasmic shuttling is governed by a basic residue-rich nuclear localization sequence (NLS) located within the N-terminal half of the proteins and a nuclear export sequence (NES) located within the C-terminal region (McKinsey et al., 2000a, 2001). Numerous studies have reported that CaMK superfamily proteins, in response to an intracellular calcium rise, increase phosphorylation at two conserved sites, S259 and S498, which serve to (1) increase binding of HDAC5 to the 14-3-3 cytoplasmic-anchoring proteins, (2) disrupt binding between HDAC5 and myocyte enhancer factor 2 (MEF2) transcription factors in the nucleus, and (3) promote cytoplasmic localization of HDAC5 (Chawla et al., 2003; McKinsey et al., 2000a, 2000b, 2001; Sucharov et al., 2006; Vega et al., 2004).

HDAC5 in the nucleus accumbens (NAc) was shown recently to reduce the rewarding impact of cocaine and inhibit cocaine experience-dependent reward sensitivity (Renthal et al., 2007), suggesting that it plays an active role in the nucleus to repress gene expression that promotes cocaine reward behavior. One of the only known HDAC5-interacting proteins in the nucleus is the MEF2 family of transcription factors, and HDAC5 is known to antagonize MEF2-dependent transcription (Lu et al., 2000). Consistently, expression of active MEF2 in the NAc enhances cocaine reward behavior (Pulipparacharuvil et al., 2008), which is opposite to the effects of HDAC5 expression in the NAc (Renthal et al., 2007). Activation of D1 class dopamine receptors (D1-DARs), or elevation of cyclic adenosine monophosphate (cAMP) levels, reduces basal and calcium-stimulated MEF2 activity in striatal or hippocampal neurons (Belfield et al., 2006; Pulipparacharuvil et al., 2008), which motivated us to explore the possibility that cocaine and cAMP signaling might regulate HDAC5's nuclear localization and/or function in the striatum *in vivo*.

In the present study, we uncover a signaling mechanism by which cocaine and cAMP signaling promote transient nuclear

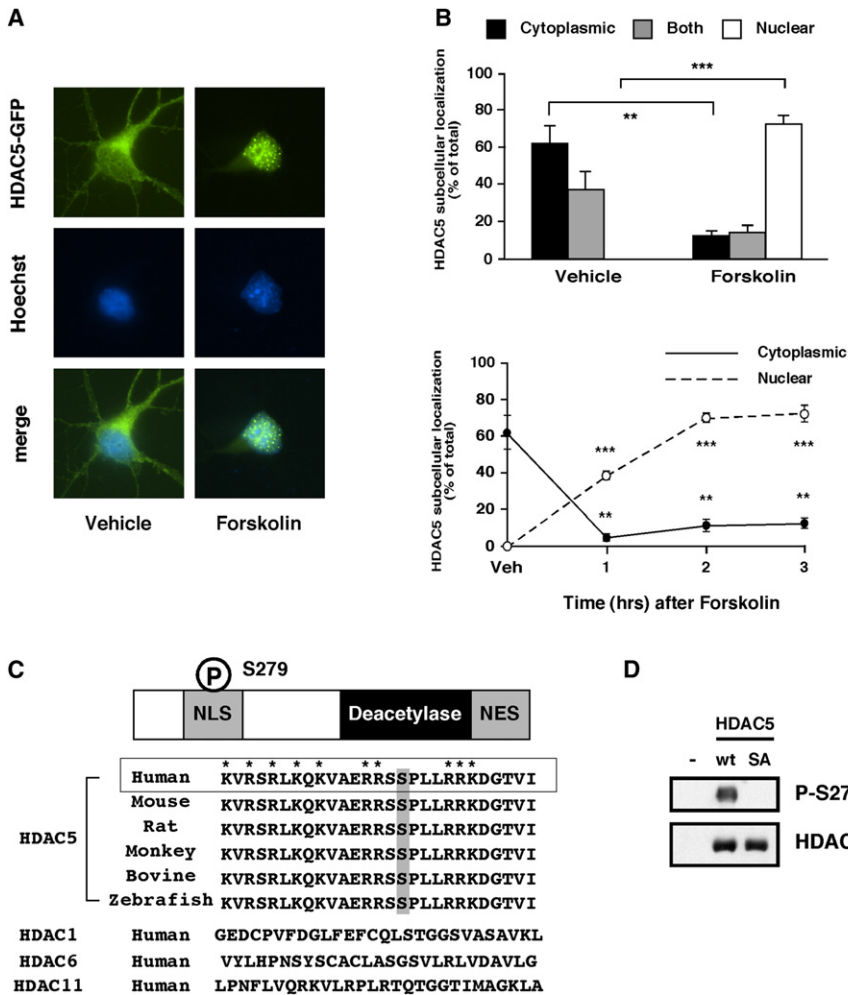


Figure 1. Elevation of cAMP Induces Nuclear Import of HDAC5 in Striatal Neurons

(A and B) Striatal neurons transfected with hHDAC5-EGFP were treated with either vehicle or forskolin (10 μ M). (A) Representative image showing nuclear import of HDAC5-EGFP after forskolin treatment for 3 hr. Sections were counterstained with Hoechst. (B) The top panel shows that the localization of HDAC5 was categorized as cytoplasmic, nuclear, or both for individual cells by an experimenter blind to treatment. 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 (mean \pm SEM; ** p < 0.01 and *** p < 0.001, Student's t test; n = 3 wells/condition, \sim 96 transfected cells were counted in each well). The bottom panel illustrates kinetics of nuclear import of HDAC5 after forskolin treatment (mean \pm SEM; ** p < 0.01 and *** p < 0.001 compared to vehicle condition, Student's t test; n = 3 wells/condition, \sim 108 transfected cells were counted in each well). (C) The molecular structure of HDAC5. HDAC5 is depicted (upper portion), and is composed of an NLS, a HDAC domain, and an NES. The Cdk5 phosphorylation site (S279) is shown within the NLS region. The lower portion depicts the amino acid sequence surrounding the S279 site for HDAC5 compared to other species and nonclass IIa HDACs. The asterisk (*) indicates positively charged amino acids.

(D) Characterization of P-S279 HDAC5 antibodies. HEK293T cells were transfected with GFP-tagged WT and S279A (SA) HDAC5 expression plasmids. Phosphorylation site-specific immunoreactivity was detected only in the lysate containing WT HDAC5 and not in lysate containing the S279A phosphorylation mutant. Total expression levels of transfected hHDAC5-EGFP were confirmed using anti-GFP antibody.

accumulation of HDAC5 through dephosphorylation-dependent regulation of NLS function in striatal neurons *in vitro* and *in vivo*, and demonstrate that this regulatory process is essential for the ability of HDAC5 to limit cocaine reward in the NAc *in vivo*. Taken together with previous work, our findings reveal that transient and dynamic regulation of this epigenetic factor plays an important role in limiting the rewarding impact of cocaine after repeated drug exposure.

RESULTS

cAMP Signaling Promotes Nuclear Import of HDAC5 in Striatal Neurons

To test whether cAMP signaling regulates striatal HDAC5, we transiently transfected a plasmid expressing HDAC5-EGFP fusion protein into cultured primary striatal neurons, and then analyzed the basal and cAMP-stimulated steady-state subcellular distribution. Under basal culture conditions, we observed that a majority of HDAC5 is localized in the cytoplasm or is evenly distributed between the nucleus and cytoplasm (Figures 1A and 1B). However, elevation of cAMP levels with the adenylyl cyclase activator, forskolin (10 μ M), induced the rapid nuclear import

of HDAC5 (Figures 1A and 1B) where it accumulated in a predominantly punctate pattern (Figure 1A). The cAMP-induced steady-state nuclear accumulation of HDAC5 occurred over a time course of \sim 1–2 hr in striatal neurons (Figure 1B, bottom). Interestingly, the nuclear HDAC5 puncta colocalized with endogenous MEF2 proteins (see Figure S1A available online), suggesting that the nuclear HDAC5 is associated with transcriptional complexes on genomic DNA and that previously noted cAMP-dependent suppression of MEF2 activity is likely mediated by HDAC5 (Belfield et al., 2006; Pulipparacharuvil et al., 2008).

Identification of a Conserved HDAC5 Phosphorylation Site Within the NLS

We speculated that cAMP signaling might regulate nuclear accumulation by regulating HDAC5 phosphorylation. By *in silico* analysis of the HDAC5 primary amino acid sequence, we identified a highly conserved serine (S279) that was a candidate substrate for protein kinase A (PKA) or cyclin-dependent kinase 5 (Cdk5), both of which are implicated in drug addiction-related behavioral adaptations (Benavides et al., 2007; Bibb et al., 2001; Pulipparacharuvil et al., 2008; Self et al., 1998). Because S279 resides

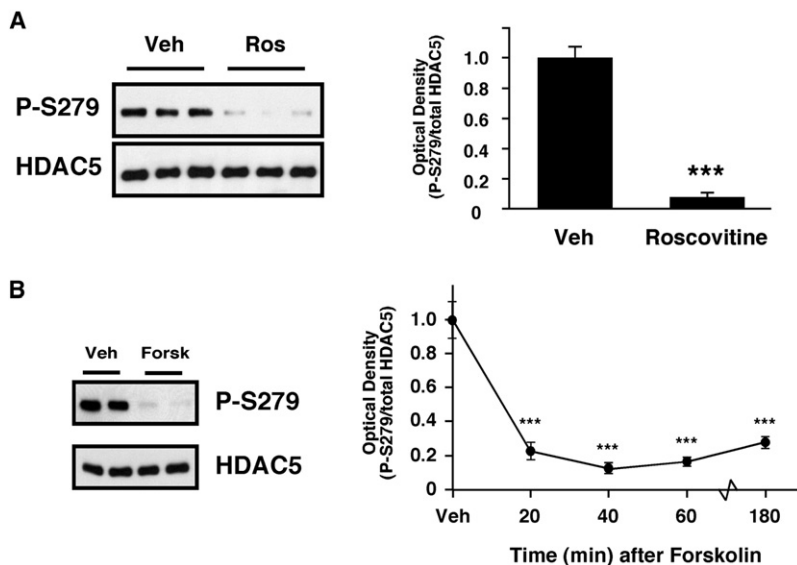


Figure 2. cAMP Signaling Promotes Dephosphorylation of S279, a Cdk5 Phosphorylation Site of HDAC5

(A) Cultured striatal neurons were treated with vehicle (Veh) or the Cdk5 inhibitor roscovitine (Ros) (50 μ M) for 3 hr. Immunoprecipitated HDAC5 from cultured striatal neurons using anti-HDAC5 antibody was immunoblotted for P-S279 and HDAC5 (mean \pm SEM; ***p < 0.001, Student's t test; n = 3/condition).

(B) Cultured striatal neurons were treated with forskolin (Forsk) (10 μ M) and lysed 20, 40, 60, or 180 min afterward. Immunoprecipitated HDAC5 was analyzed by immunoblotting with anti-P-S279 and anti-HDAC5 antibodies. Representative blots show treatment for 180 min (mean \pm SEM; ***p < 0.001 compared to vehicle, Student's t test; n = 4/condition, two independent experiments).

within the HDAC5 NLS, which is characterized by a high density of basic residues (Figure 1C, noted by asterisks), we speculated that phosphorylation at this site may modulate nucleocytoplasmic localization of HDAC5. The HDAC5 S279 site (and surrounding residues) was highly conserved from fish to humans (Figure 1C) and in both HDAC4 and HDAC9. Tandem mass spectrometry analysis of flag-epitope tagged HDAC5 in cultured cells revealed a singly phosphorylated peptide (SSPLLK: 278–283 amino acids) (Figure S1B). Therefore, we generated a phosphorylation site-specific antibody against HDAC5 S279 to study its regulation by cAMP signaling. The P-S279 peptide antibody recognizes wild-type (WT) HDAC5, but not a mutant form that cannot be phosphorylated at this site (HDAC5 S279A) (Figure 1D). It also recognizes endogenous P-HDAC5 after immunoprecipitation (IP) of total HDAC5 from cultured striatal neurons or adult striatal tissues, but not from anti-HDAC5 IPs using HDAC5 knockout (KO) mouse lysates (Figure S1C) (Chang et al., 2004), indicating that endogenous HDAC5 is basally phosphorylated at S279 in striatum in vitro and in vivo.

To determine whether Cdk5 or PKA can phosphorylate HDAC5 S279, we incubated full-length, dephosphorylated HDAC5 with recombinant Cdk5/p25 or PKA in vitro and found that either kinase can phosphorylate S279 in vitro (Figures S2A and S2B). However, when we incubated striatal neurons with specific kinase inhibitors for either Cdk5, PKA or p38 MapK (all potential kinases predicted for S279), we observed dramatically reduced P-S279 levels in the presence of Cdk5 inhibitors (Figures 2A and S2C) but observed no change in P-S279 in the presence of PKA or p38 MapK inhibitors (Figure S2C). Together, these findings indicate that whereas PKA is able to phosphorylate HDAC5 in vitro, it is not required for endogenous HDAC5 P-S279 in striatal neurons.

cAMP Signaling Dephosphorylates S279 through a PP2A-Dependent Mechanism

To test whether cAMP signaling regulates HDAC5 P-S279 levels, we cultured primary striatal neurons and elevated cAMP levels

with forskolin (10 μ M) or a nonselective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 200 μ M), for various periods of time. We observed a rapid and robust dephosphorylation of HDAC5 S279 within 20 min of forskolin or IBMX treatment (Figures 2B and S2D), an effect that was stable for at least 3 hr. In addition, forskolin induced robust dephosphorylation of endogenous HDAC5 S279 in cultured primary cortical neurons, COS7 cells (Figures S2E and S2F), as well as with overexpressed HDAC5-EGFP in HEK293T cells (data not shown). These findings suggest that cAMP-stimulated dephosphorylation of HDAC5 S279 is a conserved mechanism across multiple cell types, including nonneuronal cells.

We next sought to identify the molecular mechanisms by which cAMP signaling stimulates HDAC5 dephosphorylation of P-S279. Elevation of cAMP levels increases the activity of the protein phosphatase 2A (PP2A) in striatal neurons (Ahn et al., 2007; Ceglia et al., 2010). Consistent with this pathway, we found that okadaic acid, a potent inhibitor for PP2A and partial inhibitor of PP1, blocked cAMP-induced dephosphorylation of P-S279 in striatal neurons (Figure 3A), whereas the PP1-specific inhibitor, tautomycin, had no effect (Figure S3). In addition we observed that purified PP2A was sufficient to dephosphorylate endogenous HDAC5 P-S279 in vitro (Figure 3B). Together, these data reveal that PP2A activity is necessary and sufficient for cAMP-stimulated dephosphorylation of HDAC5 S279 in striatal neurons.

Dephosphorylation of S279 Is Required for Nuclear Import of HDAC5

To test the role of PP2A activity on nucleocytoplasmic localization of HDAC5, striatal neurons were treated with okadaic acid or tautomycin in the presence or absence of forskolin treatment. Okadaic acid treatment increased basal HDAC5 localization in the cytoplasm, and it blocked the cAMP-induced nuclear import of WT HDAC5-EGFP (Figure 4A). In contrast, tautomycin altered neither basal nor cAMP-induced localization of WT HDAC5-EGFP (Figure S4A), indicating that PP2A activity is required for cAMP-induced nuclear accumulation.

To test whether the PP2A-dependent dephosphorylation of HDAC5 S279, specifically, was required for the cAMP-induced

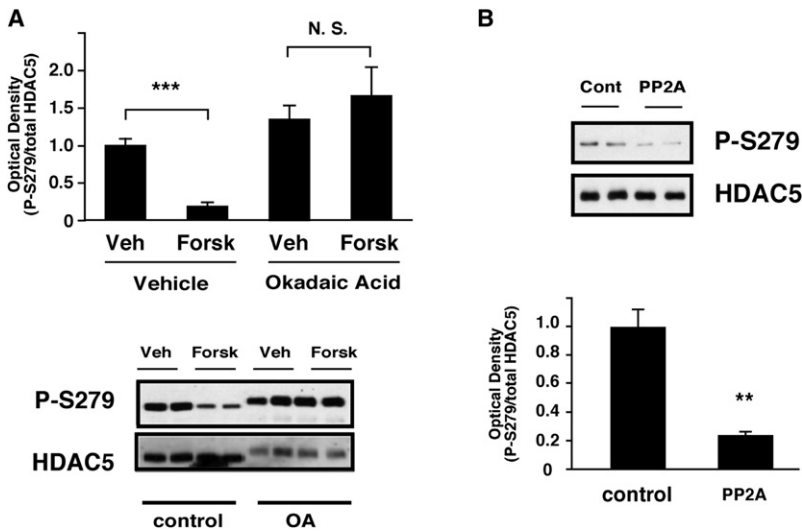


Figure 3. PP2A Activity Is Required for cAMP-Induced Dephosphorylation of S279 HDAC5

(A) Okadaic acid blocks cAMP-induced dephosphorylation of HDAC5. Cultured striatal neurons were treated with forskolin (Forsk) (10 μ M) in the presence or absence of okadaic acid (50 nM). Immunoprecipitated HDAC5 was analyzed by immunoblotting with P-S279 and anti-HDAC5 antibodies (mean \pm SEM; *** p < 0.001 compared to vehicle [Veh] condition, Student's t test; n = 6/condition from three independent experiments). N.S., not significant.

(B) S279 was dephosphorylated by PP2A in vitro. Immunoprecipitated HDAC5 protein from striatal cultures was incubated in the absence or presence of PP2A (mean \pm SEM; ** p < 0.01, Student's t test; n = 3/condition from two independent experiments). Cont, control.

nuclear import of HDAC5, we generated a phosphomimetic mutant at this site by changing S279 to a negatively charged residue, glutamic acid (E), and then analyzed the subcellular localization pattern of the HDAC5 before and after elevation of cAMP in cultured striatal neurons. Compared to WT HDAC5, we observed that most of the HDAC5 S279E protein localized in the cytoplasm under unstimulated conditions (Figure 4B). However, unlike WT HDAC5, the HDAC5 S279E mutant failed to relocalize to the nucleus by 3 hr after forskolin treatment (Figure 4B, middle). The S279E mutant did not simply disrupt the NLS function because treatment with the Crm1-mediated nuclear export inhibitor, leptomycin B (LMB) (Harrison et al., 2004; Vega et al., 2004), led to near-complete nuclear accumulation of both WT and S279E HDAC5 at similar rates (Figures S5 and 5A). These findings indicate that dephosphorylation of HDAC5 S279 is necessary for cAMP-induced nuclear accumulation.

To test whether dephosphorylation of S279 is sufficient to promote nuclear localization, we expressed in striatal neurons the nonphosphorylatable HDAC5 S279A mutant. Under basal conditions localization of the HDAC5 S279A mutant was similar to WT HDAC5 (Figure 4B, right), indicating that dephosphorylation of S279 alone is not sufficient to confer nuclear localization of HDAC5. Similar to WT HDAC5, forskolin stimulated nuclear accumulation of HDAC5 S279A, which indicates that dephosphorylation of S279 is necessary, but not sufficient, for cAMP-induced nuclear accumulation of HDAC5. Similar basal subcellular distribution and responses to cAMP were observed with HDAC5 proteins lacking EGFP fusion protein (Figure S4B).

CaMK or PKD-dependent phosphorylation of HDAC5 P-S259 and P-S498 confers cytoplasmic localization of HDAC5 in non-neuronal cells (McKinsey et al., 2000a), mediates binding to 14-3-3 cytoplasmic-anchoring proteins, and disrupts association with MEF2 transcription factors (Harrison et al., 2004; McKinsey et al., 2000b; Vega et al., 2004). Interestingly, forskolin treatment stimulated dephosphorylation of both S259 and S498 to a similar extent as S279 (Figure 4C), indicating that all three sites are negatively regulated by cAMP signaling. Consistent

with previous studies (McKinsey et al., 2000a; Vega et al., 2004), we found that HDAC5 S259A or S259A/S498A mutants were distributed evenly between the cytoplasm and nucleus or were concentrated in the nucleus (Figure 4D, left, and Figure S4C), confirming a critical role for these phosphorylation sites in striatal neurons. However, we found that the HDAC5 S259A and S259A/S498A mutants had significantly reduced (~60%) P-S279 levels (Figure S4D), confounding a straightforward interpretation of the S259A and the S259A/S498A effects on nuclear/cytoplasmic localization and suggesting that P-S279 is sensitive to the phosphorylation status of S259. Interestingly, forskolin treatment of striatal neurons stimulated strong nuclear accumulation of HDAC5 S259A or S259A/S498A (Figures 4D and S4C), indicating that dephosphorylation of S259 and S498 alone cannot account for cAMP-induced nuclear import. To test the specific importance of P-S279 in this context, we generated compound HDAC5 mutants, S259A/S279E and S259A/S498A/S279E, and observed that the S279E mutation shifted the basal subcellular localization away from the nucleus in a pattern similar to WT HDAC5 (Figures 4D and S4C). Consistent with the single mutant (S279E, Figure 4B), forskolin-induced nuclear accumulation of HDAC5 was defective in either of the compound mutants, confirming an essential and independent function for dephosphorylation of HDAC5 S279 in cAMP-induced nuclear import.

Dephosphorylation of S279 Increases the Nuclear Import Rate

We next sought to understand how dephosphorylation of HDAC5 S279 promotes nuclear accumulation in striatal neurons. As mentioned earlier, blocking Crm1-dependent nuclear export with LMB results in strong nuclear accumulation of both WT and S279E HDAC5 proteins (Figure S5), indicating that both of these proteins shuttle between the nucleus and cytoplasm under basal conditions. The steady-state, nucleocytoplasmic distribution of HDAC5 is determined by the balance of nuclear import and nuclear export kinetics. Therefore, the cAMP-induced accumulation of HDAC5 in the nucleus likely represents a change in the nuclear import rate, the nuclear export rate, or both. To evaluate these parameters, we used conditions where HDAC5 nuclear export was blocked (LMB) with or without simultaneous

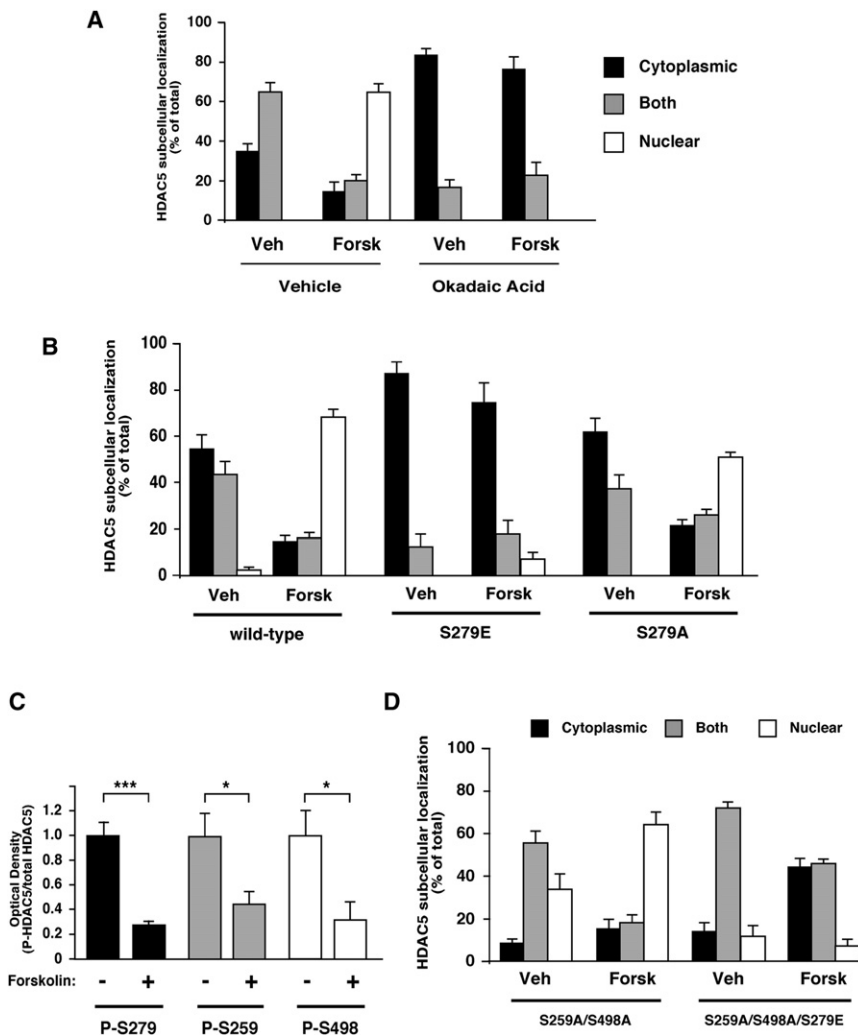


Figure 4. Dephosphorylation of S279 is Necessary for cAMP-Induced Nuclear Import of HDAC5

(A) Okadaic acid prevents cAMP-induced nuclear import of HDAC5. Transfected neurons were treated with vehicle (Veh) or forskolin (Forsk) (10 μ M) in the absence or presence of okadaic acid (50 nM) for 3 hr (mean \pm SEM, n = 3 wells in each condition, \sim 47 transfected neurons were counted in each well).

(B) The phosphorylation mimic mutant S279E prevents cAMP-induced nuclear import of HDAC5. Transfected neuron with WT, phosphorylation mimetic mutant S279E, and nonphosphorylation mutant S279A HDAC5-EGFP were treated with vehicle or forskolin (10 μ M) for 3 hr (mean \pm SEM, n = WT, S279E, and S279A, 9, 6, and 6 wells in each condition, \sim 83 transfected neurons were counted in each well).

(C) cAMP signaling induces dephosphorylation of S279, S259, and S498. Cultured striatal neurons were treated with forskolin (10 μ M) for 3 hr. Immunoprecipitated HDAC5 was analyzed by immunoblotting with anti-P-S259 and P-S498 antibodies (mean \pm SEM; *p < 0.05 and ***p < 0.001, Student's t test; n = 6–8 in each condition from two independent experiments).

(D) Dephosphorylation of S279 is required for cAMP-induced nuclear import of S259A/S498A mutant HDAC5. Transfected neurons with S259A/S498A mutant and S259A/S498A/S279E mutant HDAC5-EGFP were treated with vehicle or forskolin (10 μ M) for 3 hr (mean \pm SEM, n = 6 in each condition, \sim 81 transfected neurons were counted in each well).

elevation of cAMP. Compared to the LMB-only condition, we observed a dramatic increase in the nuclear import rate of WT HDAC5 after forskolin treatment, resulting in near-complete disappearance from the cytoplasm by 20 min (Figure 5A); this condition showed similar kinetics to forskolin-induced dephosphorylation of S279 (Figure 2B). In contrast, the import rate of HDAC5 S279E after forskolin plus LMB treatment is nearly indistinguishable from the rate of nuclear import of WT HDAC5 treated with vehicle plus LMB (Figure 5A), which indicates that dephosphorylation of S279 accelerates the nuclear import rate. We next tested potential effects of P-S279 on HDAC5 nuclear export by first incubating striatal neurons with LMB to force accumulation of WT or S279E HDAC5 into the nucleus (Figure 5B). Following washout of LMB we monitored the initial rate of nuclear export and observed that the HDAC5 S279E mutant disappeared from the nucleus more rapidly than WT HDAC5 (Figure 5B). Therefore, our findings suggest that cAMP increases the HDAC5 nuclear import rate and decreases the nuclear export rate by stimulating dephosphorylation of HDAC5 S279. In addition we observed that the HDAC5 S279E mutant coprecipitates with a cytoplasmic chaperone

protein, 14-3-3, to a significantly greater extent than WT HDAC5 in cultured cells (Figure 5C), suggesting that P-S279 enhances the affinity of 14-3-3 and HDAC5, potentially enhancing cytoplasmic retention and nuclear export of HDAC5. However, the HDAC5 S259A/S498A/S279E mutant, despite its enhanced cytoplasmic localization, fails to coimmunoprecipitate with 14-3-3 (data not shown), indicating that the primary cytoplasmic localizing function of P-S279 is not likely due to its enhancement of 14-3-3 binding.

Cocaine Stimulates Dephosphorylation of HDAC5 S279 and Nuclear Import

Cocaine and dopamine signaling regulate cAMP levels in striatum. To test whether dopamine signaling regulates HDAC5 phosphorylation in striatum in vivo, we injected adult mice with a dopamine D1 class receptor agonist, SKF81297 (5 mg/kg), or a dopamine D2 class receptor agonist, quinpirole (5 mg/kg), and analyzed striatal HDAC5 P-S279 levels in vivo. We found that SKF81297 administration stimulates significant dephosphorylation of HDAC5 S279 at 30 min and 3 hr after injections, whereas exposure to quinpirole stimulated a trend toward increased HDAC5 P-S279 levels (Figures S6A and S6B). We next injected adult C57BL/6 mice with cocaine (20 mg/kg) and analyzed both HDAC5 P-S279 levels and nuclear/cytoplasmic

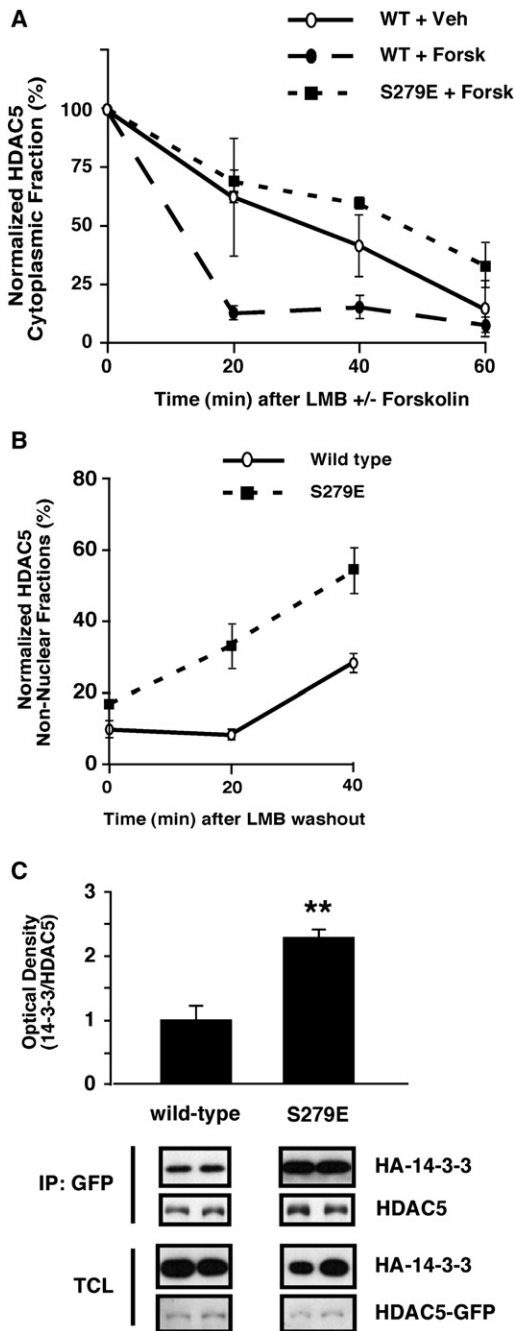


Figure 5. Phosphorylation of S279 Controls Nuclear Import and Export Rate of HDAC5 and Binding Affinity to 14-3-3

(A) Phosphorylation of S279 decreases the nuclear import rate of HDAC5. Transfected neurons were treated with or without forskolin (Forsk) (10 μ M) in the presence of LMB (10 ng/ml). The percentage of cytoplasmic HDAC5 protein in each time point was normalized to the percentage of cytoplasmic HDAC5 in the basal condition (mean \pm SEM, ANOVA, $F_{2, 24} = 8.85$, $p < 0.01$; Tukey's post hoc analysis, WT/vehicle [Veh] versus WT/Forsk, $p < 0.05$, WT/Forsk versus S279E/Forsk, $p < 0.01$; $n = 3$ wells/condition and each time point, ~ 45 transfected neurons were counted in each well).

(B) Phosphorylation of S279 accelerates nuclear export of HDAC5. Transfected neurons were treated with LMB (10 ng/ml) for 8 hr. An average of the percentage of nuclear exported HDAC5 (localized in cytoplasm or evenly

distributed between nucleus and cytoplasm) to the total transfected neurons at two time points following washout of LMB is shown. There were significant effects of both HDAC5 mutant (mean \pm SEM, ANOVA, $F_{1, 12} = 35.214$, $p < 0.001$) and time after washout (ANOVA, $F_{2, 12} = 27.0$, $p < 0.001$); $n = 3$ wells for each condition, average 50 transfected neurons were counted in each well. (C) S279E mutant has greater binding affinity to 14-3-3 than WT HDAC5. HEK293T cells were cotransfected with GFP-tagged HDAC5 and HA-tagged 14-3-3 epsilon. Two days after transfection, HDAC5-EGFP protein was immunoprecipitated using anti-GFP antibodies. IPs were analyzed by western blotting using HDAC5 and HA-tag antibodies (mean \pm SEM; ** $p < 0.01$, Student's *t* test; $n = 3$ in each condition).

localization of endogenous HDAC5 in the striatum. We compared mice injected 7 days with saline (vehicle control), 7 days with cocaine (cocaine-experienced), or 6 days with saline and one cocaine injection on the seventh day (cocaine-naive), and analyzed HDAC5 P-S279 levels at 1, 4, and 24 hr after the last injection (Figure 6A). By first immunoprecipitating total HDAC5, we were able to measure HDAC5-specific P-S279 levels as confirmed in HDAC5 KO mice (Figure S1C). We observed a significant dephosphorylation of HDAC5 at 1 and 4 hr following the last injection in both the cocaine-naive and cocaine-experienced mice, but phosphorylation at S279 had returned to baseline levels by 24 hr after the last cocaine injection. We next analyzed the levels of P-S259 and P-S498 HDAC5 after cocaine, and similar to P-S279 regulation, we observed a significant reduction of all three sites (Figure 6B). Taken together, these findings reveal that cocaine stimulates the coordinated dephosphorylation of P-S259, P-S279, and P-S498 on HDAC5.

We next analyzed the effects of cocaine on nuclear/cytoplasmic distribution of endogenous striatal HDAC5 using a biochemical fractionation approach. Similar to the subcellular distribution of HDAC5 in primary striatal neurons in culture (e.g., Figure 1B), a majority of the striatal HDAC5 cofractionated with cytoplasmic proteins (Figure 6C). Following the same dosing paradigm detailed above, administration of cocaine to naive or cocaine-experienced mice resulted in a significant accumulation of HDAC5 in the nucleus at 4 hr after the last injection, and like the regulation of P-S279, nuclear accumulation was transient and returned to saline control levels by 24 hr after the last injection (Figure 6D). Taken together, these results reveal that cocaine administration stimulates the rapid and transient dephosphorylation of HDAC5 and subsequent nuclear accumulation of endogenous HDAC5 in vivo.

Dephosphorylation of HDAC5 S279 Antagonizes Cocaine Reward

To test the importance of cocaine-induced dephosphorylation of HDAC5 S279 for the development of cocaine reward behavior, we utilized viral-mediated gene transfer to express full-length, HDAC5 WT or mutants (S279A or S279E) bilaterally in the NAc of WT, adult male mice (Figure 7A) prior to a cocaine-conditioned place preference (CPP) assay. This assay involved pairing one of two distinct chambers with either cocaine or saline injections for 2 consecutive days. Subsequently, the mice were given equal access to both chambers, and time spent in either the cocaine-paired or saline-paired chamber was measured. As

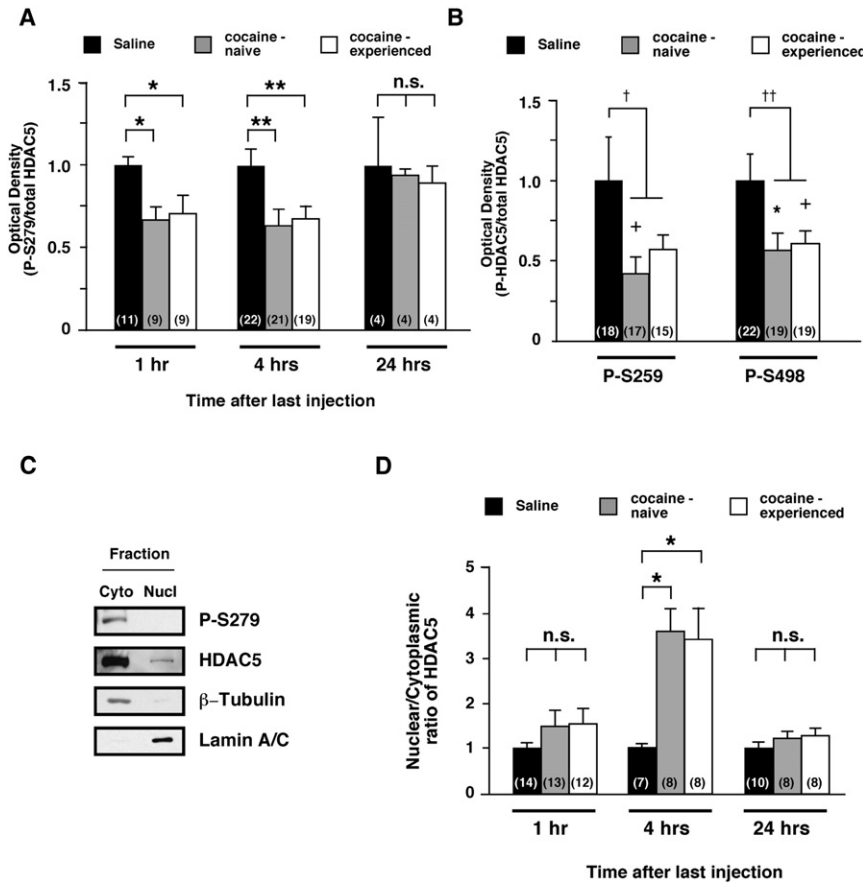


Figure 6. Cocaine Exposure Transiently Decreases Phosphorylation of HDAC5

Adult C57Bl/6 mice were injected with saline for 7 days (1 ml/kg, i.p., 1×/day; saline), with saline for 6 days followed by 1 day of cocaine (20 mg/kg; i.p.; cocaine naive), or with cocaine for 7 days (20 mg/kg; i.p.; cocaine experienced).

(A) Striatal tissue was dissected out 1, 4, or 24 hr after the last injection, and immunoprecipitated HDAC5 was subjected to western blotting using P-S279 and HDAC5 antibodies (mean ± SEM, separate univariate ANOVAs were performed for each time point: 1 hr, $F_{2, 26} = 5.481$, $p = 0.01$, 4 hr, $F_{2, 59} = 7.006$, $p < 0.01$, and 24 hr, $F_{2, 9} = 0.091$, $p > 0.05$; significant Tukey's post hoc results compared to saline are indicated by * $p < 0.05$ and ** $p < 0.01$, respectively; n = saline, cocaine naive, or cocaine experienced: 1 hr [11, 9, and 9]; 4 hr [22, 21, and 19]; and 24 hr [4, 4, and 4]). n.s., not significant.

(B) Immunoprecipitated HDAC5 from striatal tissue at 4 hr after the last injection was analyzed by western blotting using P-S259, P-S498, and HDAC5 antibodies (mean ± SEM, separate univariate ANOVAs were performed for P-S259 and P-S498; P-S259, $F_{2, 47} = 2.894$, $p = 0.065$, P-S498, $F_{2, 57} = 4.297$, $p < 0.05$, Tukey's post hoc results compared to saline are indicated by * $p < 0.05$ and + $p = 0.06$; n = saline, cocaine naive, and cocaine experienced, P-S259 [18, 17, and 15], P-S498 [22, 19, and 19]). Combined cocaine-treated groups were significantly different from saline control groups for both P-S259 and P-S498. Student's t tests, † $p < 0.05$ and †† $p < 0.01$.

(C) Striatal tissue was fractionated into cytoplasmic (Cyto) and nuclear (Nucl) fractions, and confirmed by blotting with β -tubulin and Lamin A/C, respectively.

(D) Subcellular fractionation after cocaine exposure demonstrated transient nuclear accumulation of HDAC5 in striatum. Nuclear/cytoplasmic ratios of HDAC5, relative to saline, were calculated by comparing arbitrary units of nuclear and cytoplasmic HDAC5 (normalized to Lamin A/C in nuclear fraction and to β -tubulin in cytoplasmic fraction; mean ± SEM, separate univariate ANOVAs, 1 hr, $F_{2, 36} = 1.283$, $p > 0.05$, 4 hr, $F_{2, 20} = 7.446$, $p < 0.01$, 24 hr, $F_{2, 23} = 2.005$, $p > 0.05$; for Tukey's post hoc analysis * $p < 0.05$, n = saline, cocaine naive, and cocaine experienced: 1 hr [14, 13, and 12], 4 hr [7, 8, and 8], and 24 hr [10, 8, and 8]). n.s., not significant.

expected, the control virus (GFP)-injected mice spent significantly more time in the cocaine-paired chamber (Figure 7C), indicating a clear positive preference for the context in which cocaine was experienced. Similar to a previous report (Renthal et al., 2007), the overexpression of WT HDAC5 reduced cocaine-associated place preference, but it did not reach significance. Mice expressing the HDAC5 S279E mutant protein had a cocaine place preference similar to the GFP-only control virus-injected mice, whereas mice expressing HDAC5 S279A dephosphorylation mutant showed significantly reduced cocaine place preference (Figure 7C; S279A, 81 s, versus S279E, 246 s). We observed similar expression levels of the HDAC5 WT, S279A, and S279E mutants in striatal neurons (Figure 7B), indicating that the results are not likely due to differences in HDAC5 protein expression levels. As expected, we observed that mice injected with the lower dose of cocaine used in the CPP assay (5 mg/kg) showed a significant transient reduction of HDAC5 P-S279 levels (Figure 7D), although the magnitude and duration were somewhat attenuated when compared to the higher doses of cocaine

(Figure 7D; data not shown). The absence of an effect by the HDAC5 S279E mutant is consistent with its localization in the cytoplasm in striatal neurons. These findings indicate that dephosphorylation of HDAC5 S279 in the NAc is required for HDAC5 to limit the rewarding impact of cocaine in vivo.

Because HDAC5 dephosphorylation was required for its ability to reduced cocaine reward behavior, we next asked whether HDAC5 dephosphorylation suppresses the development of cocaine CPP, which is the period where regulation of P-HDAC5 is observed (Figures 6 and 7D), or whether HDAC5 might be influencing the expression of CPP behavior during the test. To test this idea, we first performed cocaine versus saline context pairing prior to bilateral expression of HDAC5 S279A or GFP-only vector in the NAc and then tested for the expression of cocaine CPP. Unlike expression of HDAC5 S279A during the cocaine/context pairings (development of CPP), we observed no significant differences between vector and HDAC5 S279A treatments during the expression of cocaine CPP behavior on the test day (Figure 7E), indicating that dephosphorylation of

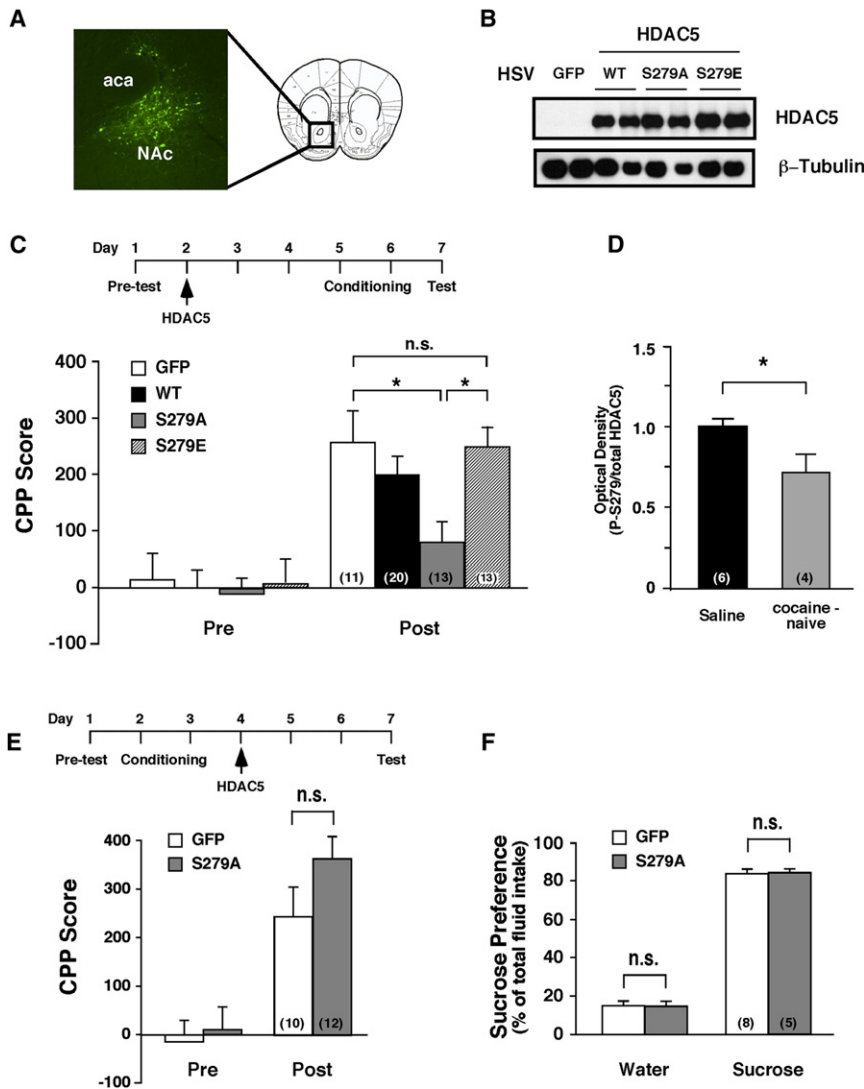


Figure 7. Dephosphorylation of HDAC5 S279 in the NAc Limits the Development of Cocaine Reward Behavior

(A) Representative image of HSV-mediated gene expression in the NAc. GFP expression was used to confirm virus injection placement.

(B) Western blotting showing similar expression levels of WT HDAC5, S279A, and S279E mutants in virus-infected cultured dissociated striatal neurons.

(C) The top view illustrates the time course of CPP paradigm; the bottom view shows that the CPP to cocaine (5 mg/kg) was attenuated by overexpression of the S279A mutant, but not by the S279E mutant (mean ± SEM, ANOVA, $F_{3, 53} = 3.797$, $p < 0.05$; * $p < 0.05$, Tukey's post hoc analysis; $n = 11, 20, 13$, and 13 in GFP, WT, S279A, and S279E, respectively). CPP score was defined as the time (s) spent in the cocaine side minus the saline side for both pre- (Pre) and post-conditioning (Post) trials. n.s., not significant.

(D) Immunoprecipitated HDAC5 from striatal tissue at 2 hr after last cocaine (5 mg/kg) injection was analyzed by western blotting using P-S279 and HDAC5 antibodies (mean ± SEM, * $p < 0.05$, Student's t test; $n = 6$ and 4 in each condition).

(E) The top view illustrates the time course of CPP paradigm; the bottom view shows that the CPP to cocaine (5 mg/kg) was not attenuated when the HDAC5 S279A mutant was overexpressed specifically during the expression of reward behavior. The stereotactic surgery to introduce HDAC5 S279 mutant in the NAc was performed at day 4 following conditioning (mean ± SEM, n.s. indicates $p > 0.05$, Student's t test; $n = 10$ and 12 in each condition). CPP score was defined as the time (s) spent in the cocaine side minus the saline side for both pre- (Pre) and post-conditioning (Post) trials.

(F) Sucrose preference was not limited by overexpression of the HDAC5 S279A mutant. Mice injected with GFP or HDAC5 S279A mutant in the NAc showed indistinguishable preferences

for either water or 1% sucrose (mean ± SEM, n.s. indicates $p > 0.05$, Student's t test; $n = 8$ and 5 in each condition). Preference was defined as the percentage of water or 1% sucrose intake relative to total liquid consumption in each 24 hr period for 4 days.

HDAC5 S279 resists the development of cocaine reward behavior but does not reduce its expression.

Because HDAC5 dephosphorylation limits the development of cocaine reward, we next asked whether this mechanism might also regulate natural reward behavior, or whether the effect of HDAC5 is more specific for cocaine reward. To this end, we performed bilateral NAc injections of GFP-only control virus or the HDAC5 S279A virus and then measured a natural reward behavior, sucrose preference. When sucrose preference was measured daily for 4 consecutive days, we observed no differences in 1% sucrose preference between mice expressing HDAC5 S279A mutant or GFP-only vector control (Figures 7F and S7), suggesting that HDAC5 does not regulate natural reward behavior and may have a more specific role for substance abuse.

DISCUSSION

Taken together, our findings reveal a molecular mechanism by which cocaine and cAMP signaling regulate HDAC5 nuclear accumulation to limit adaptations that increase the rewarding impact of cocaine (Figure 8). Our findings support the noted role for HDAC5 in limiting cocaine reward behavior (Renthal et al., 2007); however, our observations that cocaine induces transient, delayed dephosphorylation and nuclear import of HDAC5 to suppress cocaine reward are a significant departure from previous ideas of how cocaine regulates HDAC5 function in vivo (Renthal et al., 2007). We observed a significant regulation of HDAC5 phosphorylation and nuclear levels that strongly suggests that dynamic regulation of this epigenetic factor plays a crucial role in limiting the impact of cocaine reward in vivo.

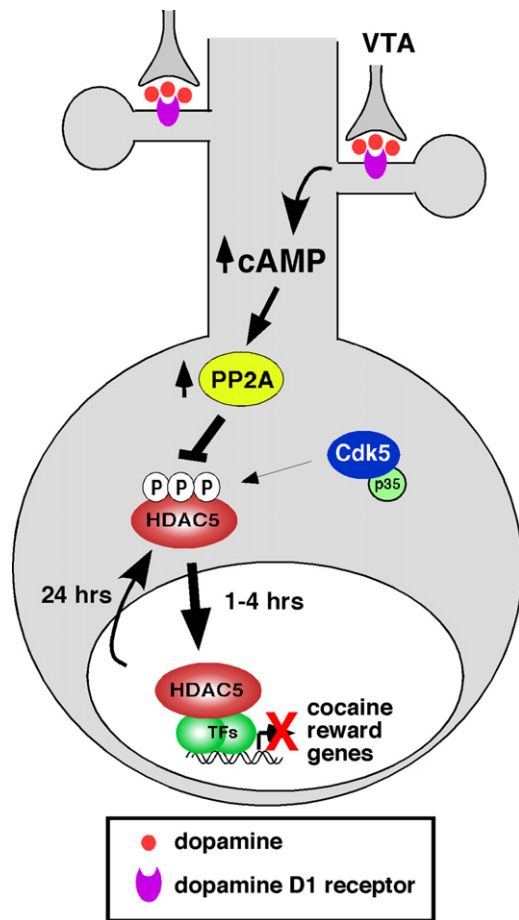


Figure 8. Working Model for How Cocaine and cAMP Signaling Regulate HDAC5 Nuclear Import and Limit Cocaine Reward Behavior

Several studies have reported that cocaine exposure increases P-S259 HDAC5 levels by western blotting or immunohistochemistry (Dietrich et al., 2012; Host et al., 2011; Renthall et al., 2007), but with the near-perfect conservation of amino acids spanning the P-S259 site in HDAC4, HDAC5, HDAC7, and HDAC9, it is important to note that the P-S259 antibody recognizes multiple class IIa HDAC proteins, not only HDAC5. In contrast to these reports, our study revealed a robust decrease in P-S259 and P-S498 levels on HDAC5 (Figure 6B). Our analysis of the P-S279 HDAC5 site, which is also highly conserved in HDAC4 and HDAC9, revealed that total P-S279 immunoreactivity was not specific to HDAC5 (i.e., HDAC5 KO mouse tissues had significant residual P-S279 immunoreactivity). To achieve HDAC5-specific analysis of these conserved sites, we had to immunoprecipitate total HDAC5 protein prior to western blotting with the phosphorylation site-specific antibodies (e.g., Figure S1C). In the future it will be important to determine whether the reported increases in P-S259 signal after cocaine exposure reflect specific regulation of HDAC5 or might instead represent regulation of other class IIa HDAC(s).

The binding of HDAC5 to 14-3-3 proteins is mediated by phosphorylation of S259 and S498 sites, and this association is

thought to be important for HDAC5 cytoplasmic localization (Chawla et al., 2003; McKinsey et al., 2000a, 2000b, 2001; Sucharov et al., 2006; Vega et al., 2004). Similar to previous work, we observe that the HDAC5 S259A/S498A mutant protein is largely localized within the nucleus or evenly distributed between nucleus and cytoplasm. However, this mutant has significantly reduced P-S279 levels (Figure S4D), which suggests that the increase in nuclear localization of this mutant may be due, at least in part, to reduced P-S279 levels. This conclusion is strengthened by the observation that combining the S279E phosphomimetic mutation with the S259A/S498A mutations results in increased cytoplasmic distribution of HDAC5 and resistance to cAMP-induced nuclear import. The S259A/S498A/S279E HDAC5 mutant does not bind to 14-3-3 (data not shown), which strongly suggests that P-S279 exerts its effect on HDAC5 nuclear import through a 14-3-3-independent mechanism. Several studies have reported that phosphorylation close to, or within, an NLS can mask a protein's interaction with other proteins or inactivate its NLS function (Jans et al., 1991; Moll et al., 1991). Due to the strong concentration of positively charged residues within the HDAC5 NLS, we speculate that the introduction of three negative charges by organic phosphate at S279 might neutralize the NLS charge or induce a conformational change that reduces association with nuclear import proteins.

During review of our manuscript, a study reported regulation of P-S279 HDAC5 by PKA in COS7 cells (Ha et al., 2010), and provided evidence that P-S279 promoted nuclear retention in these cells. Similar to this study, we had also found that purified PKA phosphorylates HDAC5 S279 in vitro (Figure S2A); however, we found that basal phosphorylation at this site, at least in striatal neurons, did not require PKA activity (Figure S2C). In addition our direct measurements of endogenous HDAC5 P-S279 levels revealed that forskolin treatment of COS7 cells, striatal neurons, cortical neurons, or acute, adult striatal slices actually decreased P-S279 HDAC5 levels (Figures 2B and S2; data not shown), which seems incompatible with the proposed role for P-S279 in the COS7 cells. We speculate that the expression of constitutively active PKA in COS7 cells may regulate additional HDAC5 sites that influence nuclear localization and require P-S279 or that overexpressed HDAC5-EGFP is regulated differently than endogenous HDAC5 in COS7 cells. Additional experiments will be required to help resolve the different conclusions drawn by these two studies, but in striatal neurons it seems clear that HDAC5 P-S279 does not promote nuclear accumulation, but quite the opposite.

Our observations about the role and regulation of HDAC5 P-S279 in cocaine-induced behavioral plasticity raise a number of interesting questions for future study. For example what is the nuclear function of HDAC5 that limits cocaine reward? Nestler and colleagues (Renthall et al., 2007) reported that the enzymatic HDAC domain of HDAC5 is required for reducing cocaine reward, suggesting that the ultimate substrate is histone deacetylation and indirect suppression of HDAC5 target genes. Indeed, many hundreds of genes were aberrantly increased or decreased by cocaine in the HDAC5 KO mice at 24 hr after repeated cocaine injections. Because these were total HDAC5 KO mice, lacking HDAC5 expression throughout the lifetime of

the animal, it is difficult to know whether these are direct effects of HDAC5 on the identified genes. Moreover, the time point analyzed (i.e., 24 hr) is during a phase when HDAC5 phosphorylation and nucleocytoplasmic localization are similar to saline control conditions. In the future it will be interesting to determine the target genes that are bound and regulated by HDAC5 after cocaine, particularly at those time points when enhanced HDAC5 nuclear function is observed following cocaine exposure. It is possible, and perhaps likely, that regulation of multiple HDAC5 gene targets contributes to the reduction of cocaine reward behavior, and dissecting out the relative contributions of each gene target will represent a major challenge going forward.

It is interesting to note that the HDAC5 S279A mutant suppressed cocaine reward to a greater extent than WT HDAC5 (Figure 7C). There are several possible explanations for this difference, including the following.

- (1) The HDAC5 S279A mutant *in vivo* resides constitutively in the nucleus, whereas the WT HDAC5 is only transiently localized in nucleus upon cocaine exposure. In this case the levels of the P-S259/P-S498 would presumably be low such that P-S279 plays the dominant major role in subcellular localization (unlike the striatal cultures).
- (2) The HDAC5 S279A mutant has reduced nuclear export kinetics compared to WT HDAC5, and as a result, resides in the nucleus for a longer time after cocaine exposure. We found that P-S279 HDAC5 increases nuclear export kinetics (Figure 5B). If HDAC5 S279A cannot be rephosphorylated following nuclear import, then HDAC5 S279A may remain in the nucleus and exert longer-lasting effects following cocaine exposure.
- (3) HDAC5 P-S279 may regulate not only its nuclear/cytoplasmic localization, as documented in our study, but might also regulate its function as a transcriptional corepressor in the nucleus. As such, the HDAC5 S279A mutant may be a more effective corepressor via unknown mechanisms. Due to technical limitations, we were unable to visualize the subcellular distribution of the HDAC5 mutants *in vivo*. Nevertheless, our findings in this study reveal an important role for dephosphorylation of P-S279 HDAC5 in the regulation of cocaine reward behavior.

Our findings in striatal cultured neurons revealed a high degree of colocalization of HDAC5-EGFP with endogenous MEF2A and MEF2D, two of the well-studied transcription factor proteins that interact with HDAC5, suggesting MEF2 as a possible mediator of HDAC5 function in reducing cocaine reward sensitivity after repeated cocaine experience. Consistent with this idea, we reported recently that expression of constitutively active MEF2 in the NAc enhances cocaine reward behavior (Pulipparacharuvil et al., 2008), which is opposite of the effect of HDAC5 expression in this region. In the future, it will be important to determine whether HDAC5 exerts its effects on cocaine reward through binding to MEF2 proteins, or whether the critical nuclear target of HDAC5 in the mediation of cocaine reward may be one or more previously undescribed transcription factors. The identifi-

cation of HDAC5 target genes after cocaine exposure may help determine whether MEF2 and HDAC5 bidirectionally regulate cocaine reward through a common pathway or whether these proteins regulate cocaine behavior through distinct transcriptional mechanisms *in vivo*.

Similar to our observed regulation of HDAC5 P-S279, previous studies in striatal neurons have reported that cAMP signaling increases PP2A activity (Ahn et al., 2007), which then dephosphorylates the Cdk5 substrates, Wave1 (Ceglia et al., 2010) and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) (Bibb et al., 1999; Nishi et al., 2000). Acute cocaine does not alter the levels or activity of Cdk5 or levels of p35 in striatum (Kim et al., 2006; Takahashi et al., 2005), suggesting that the decrease in P-S279 is due to increased phosphatase activity rather than decreased Cdk5 activity. Interestingly, cocaine and cAMP signaling have been shown to induce transient DARPP-32 nuclear accumulation via dephosphorylation in striatal neurons (Stipanovich et al., 2008). Similar to our findings with HDAC5, nuclear accumulation of DARPP-32 attenuates cocaine reward behavior, which is proposed to involve epigenetic gene regulation (Stipanovich et al., 2008). Together, these findings indicate that whereas cocaine induces rewarding effects, the striatum stimulates negative feedback processes, such as enhanced HDAC5 and DARPP-32 nuclear levels, to attenuate the reward impact of future cocaine exposures. As such, these proteins may represent critical intrinsic mechanisms for counteracting the maladaptive changes in reward circuit function, and understanding these negative feedback processes may reveal new avenues for the treatment of drug addiction.

Taken together, our findings reveal that cocaine regulates the transient nuclear accumulation of HDAC5, and this likely occurs through a molecular mechanism involving PP2A phosphatase-dependent dephosphorylation of HDAC5 at three critical phosphoserines: S259, S279, and S498. The removal of phosphate from these sites likely increases the NLS function and decreases binding to 14-3-3 proteins, and promotes the repression of HDAC5 target genes in the nucleus. Importantly, our findings reveal that dephosphorylation of S279 HDAC5 is critical for its ability to limit the development of cocaine reward-related behavioral adaptations, but not natural reward behavior. Because cocaine-experienced HDAC5 KO mice have enhanced place preference to cocaine, and this effect is rescued by NAc expression of WT HDAC5 (Renthal et al., 2007), our combined findings suggest that HDAC5 provides a delayed braking mechanism on gene expression programs that support the development, but not expression, of cocaine reward behaviors. As such, deficits in this process may contribute to the development of maladaptive behaviors associated with addiction following repeated drug use in humans.

EXPERIMENTAL PROCEDURES

Plasmid

Herpes simplex virus (HSV)-flag-*human* HDAC5 plasmid was provided by Dr. Eric Nestler, and using PCR, we generated a nontagged version of HDAC5 for subcloning. The PCR fragment was subcloned into Bluescript vector, and the insert region was confirmed by DNA sequencing. The nontagged HDAC5 was then digested with XbaI and subcloned into HSV vector. Serine to alanine (S259A, S279A, S498A) and serine to glutamate (S279E)

mutants of HDAC5 were generated by QuickChange Site-Directed Mutagenesis Kit.

Mice

All C57BL/6 mice (Charles River) used in this study were adult males tested between 10 and 12 weeks old. They were housed on a 12 hr light-dark cycle with access to food and water ad libitum. All procedures were in accordance with the Institutional Animal Care and Use (IACUC) guidelines.

Generation of P-S279 HDAC5 Antibodies

Rabbits were injected with a synthesized P-HDAC5 encompassing HDAC5 amino acids 274–285, where position S279 was phosphorylated (Covance). The injected peptide was conjugated to Keyhole Limpet Hemocyanin via an N-terminal cysteine residue. Anti-P-S279 antibodies were affinity purified with peptide-conjugated Sepharose beads (SulfoLink; Pierce).

Dissociated Striatal Cultures

Embryonic striatal neurons (E18/19) were cultured from Long-Evans rats (Charles River) as described previously (Cowan et al., 2005; Pulipparacharuvil et al., 2008). Details can be found in the Supplemental Experimental Procedures section.

Immunocytochemistry

Striatal neurons (E18 rat) were plated at 100,000 cells/well (24-well plate; Corning), grown on PDL/Laminin-coated glass coverslips for 8 days, and then transfected using calcium phosphate method (Pulipparacharuvil et al., 2008). Two days later, cells were stimulated with indicated agents. Neurons were fixed in 4% paraformaldehyde/2% sucrose in 1X PBS for 20 min at room temperature, permeabilized, and stained with indicated primary and secondary antibodies (see Supplemental Experimental Procedures). The localization of HDAC5 was categorized as cytoplasmic, nuclear, or both (evenly distributed across nucleus and cytoplasm) for each neuron under experimenter-blind conditions.

Sample Preparation from Cocaine-Injected Mice

C57BL/6 mice (Charles River) were injected once per day (intraperitoneally [i.p.]) with saline or cocaine (5 or 20 mg/kg) before rapid isolation of brain tissues at indicated times after injection. HDAC5 was immunoprecipitated from diluted total striatal lysates and analyzed by standard western blot analysis with indicated antibodies (see Supplemental Experimental Procedures for dilutions and sources). Cytosolic and nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology) according to the manufacturer's instructions.

Mass Spectrometry Analysis

HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) FBS, penicillin-streptomycin (1X; Sigma-Aldrich), and L-glutamine (4 mM; Sigma-Aldrich). HEK293T cells were transfected with HSV-flag-hHDAC5 using calcium phosphate and harvested 2 days after transfection. Flag-HDAC5 was prepared from HEK293T cell extracts in RIPA buffer (50 mM Tris [pH 7.4], 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholates, 10 mM NaF, 10 nM okadaic acid, and complete protease inhibitor cocktail tablet [1X; Roche]) by IP with anti-flag antibody (M2)-conjugated beads. The protein was separated by SDS-PAGE and stained with Coomassie brilliant blue. The HDAC5 band was excised from the gel, washed, and then digested with trypsin. The tryptic digests were analyzed with an EC-MS/MS system.

In Vitro PKA and Cdk5 Kinase Assay

Flag-HDAC5 was prepared from transfected HEK293T cell extracts by IP with anti-flag antibody (M2)-conjugated beads in RIPA buffer. For the PKA phosphorylation, immunoprecipitated beads were washed and suspended in PKA phosphorylation buffer (50 mM PIPES [pH 7.3], 10 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, and protease inhibitor) and incubated with or without recombinant PKA catalytic subunit (Sigma-Aldrich) or alkaline phosphatase (Roche) in the presence of 1 mM ATP at 30°C. For the Cdk5 phosphorylation assay, the immunoprecipitated flag-HDAC5 on the beads was washed and

resuspended in alkaline phosphatase buffer (Roche) and incubated with alkaline phosphatase at 37°C for 2 hr. Dephosphorylated beads were washed with RIPA buffer three times and Cdk5 kinase assay buffer (10 mM MOPS [pH 7.2], 10 mM MgCl₂, 1 mM EDTA) three times, and the immunoprecipitated HDAC5 was incubated with or without Cdk5-p25 (Sigma-Aldrich) in the presence of 1 mM ATP at 30°C. After boiling SDS sample buffer to elute from the beads, the incubated IP samples were subjected to western blotting analysis.

In Vitro PP2A Dephosphorylation Assay

Immunoprecipitated HDAC5 from striatal neurons in RIPA buffer was washed with dephosphorylation buffer (50 mM Tris-HCl [pH 8.5], 20 mM MgCl₂, 1 mM DTT, protease inhibitor cocktail [1X; Roche]) five times and incubated with or without 2.5 U of purified PP2A (Promega) at 30°C for 60 min. Proteins were subjected to western blotting analysis.

Viral-Mediated Gene Transfer

Expression plasmids for HDAC5 WT, S279A, and S279E mutants in HSV vector were packaged into high-titer viral particles as described previously (Barrot et al., 2002). Stereotactic surgery was performed on mice under general anesthesia with a ketamine/xylazine cocktail (10 mg/kg:1 mg/kg). Coordinates to target the NAc (shell and core) were +1.6 mm anterior, +1.5 mm lateral, and -4.4 mm ventral from bregma (relative to dura) at a 10° angle. Virus was delivered bilaterally using Hamilton syringes at a rate of 0.1 μl/min for a total of 0.5 μl. Viral placements were confirmed by GFP signal, which was coexpressed in each virus.

CPP

Mice were conditioned to cocaine using an unbiased accelerated paradigm to accommodate the timing of transient HSV expression (Barrot et al., 2002; Renthal et al., 2007). Additional details can be found in the Supplemental Experimental Procedures.

Sucrose Preference

Singly housed mice were provided tap water in two identical double-ball-bearing sipper-style bottles for 2 days followed by 2 days of 1% (w/v) sucrose solution to allow for acclimation and to avoid undesired effects of neophobia (Green et al., 2006). The next day mice underwent stereotactic injections of control or HDAC5 virus into the NAc (bilaterally, as described above for CPP assays). Forty-eight hours after viral injection, mice were again given two bottles: one containing water, and the other containing 1% sucrose solution. The consumption of water versus sucrose was measured after 24, 48, 72, and 96 hr of access to the bottles to determine preference for sucrose (Renthal et al., 2007). Bottle positions of water and sucrose were swapped each day of testing to avoid potential drinking side bias.

Statistics

One-way, two-way, or repeated-measures ANOVAs with Tukey's multiple comparison post hoc tests were used to analyze the following: western blotting, phosphorylation level of S279, nuclear/cytoplasmic ratio of HDAC5 with cocaine exposure, CPP, sucrose preference, and rates of nuclear export and import of HDAC5. Student's t tests were used to analyze HDAC5-EGFP localization, western blotting for phosphorylation level of S279 for samples treated with roscovitine, forskolin, okadaic acid, tautomycin, and for the in vitro dephosphorylation assay with PP2A, cocaine-treated samples compared to saline controls, and averaged sucrose preference data.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.10.032.

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