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Histone Deacetylase 5 Epigenetically Controls Behavioral Adaptations to Chronic Emotional Stimuli

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SUMMARY

Previous work has identified alterations in histone acetylation in animal models of drug addiction and depression. However, the mechanisms which integrate drugs and stress with changes in chromatin structure remain unclear. Here, we identify the activity-dependent class II histone deacetylase, HDAC5, as a central integrator of these stimuli with changes in chromatin structure and gene expression. Chronic, but not acute, exposure to cocaine or stress decreases HDAC5 function in the nucleus accumbens (NAc), a major brain reward region, which allows for increased histone acetylation and transcription of HDAC5 target genes. This regulation is behaviorally important, as loss of HDAC5 causes hypersensitive responses to chronic, not acute, cocaine or stress. These findings suggest that proper balance of histone acetylation is a crucial factor in the saliency of a given stimulus and that disruption of this balance is involved in the transition from an acute adaptive response to a chronic psychiatric illness.

INTRODUCTION

Some individuals are more vulnerable to chronic psychiatric illnesses, such as drug addiction or depression, but the neural and molecular mechanisms responsible for this are poorly understood. Although addiction and depression are known to have a strong genetic component and involve aberrant changes in gene expression (Hyman et al., 2006; Nestler et al., 2002), recent evidence has suggested that epigenetic mechanisms, such as histone acetylation, may also contribute (Tsankova et al., 2007). In animal models of addiction, chronic cocaine was shown to induce long-lasting changes in histone acetylation, which serve to relax the chromatin structure and permit greater access to transcriptional activators (Kumar et al., 2005; Levine et al., 2005). In a model of depression, stable changes in chromatin structure were observed nearly a month after stress treatment, indicating epigenetic mechanisms may play a significant role in the pathogenesis and maintenance of chronic psychiatric illness (Tsankova et al., 2006).

One of the most appealing features linking epigenetic mechanisms to psychiatric illness is their ability to respond to, integrate, and translate diverse environmental stimuli into structural changes, which govern the expression of specific genes (Kouzarides, 2007). Although the term epigenetics is used in several ways, we use the broad definition of epigenetics, as "the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states" (Bird, 2007). This elaborate epigenetic process is carried out by many classes of chromatin-modifying enzymes, which serve as mediators between the environment and the genome. Although recent data have begun to describe chromatin modifications in animal models of psychiatric illness, limited focus has been paid to the specific enzymes that mediate these processes. Therefore, we focus here on histone deacetylases (HDACs), a family of enzymes capable of repressing gene expression by removing acetyl groups from histones to produce a less accessible chromatin structure.

There are three distinct classes of HDACs, classes I, II, and III, based on their homology to the yeast genes *rpd3*, *hda1*, and *sir2*, respectively (Kurdistani and Grunstein, 2003). We focus here on the class II HDAC, HDAC5, due to its activity-dependent regulation in neurons (Chawla et al., 2003) and, as we show, its relative enrichment in the nucleus accumbens (NAc), a key brain reward region

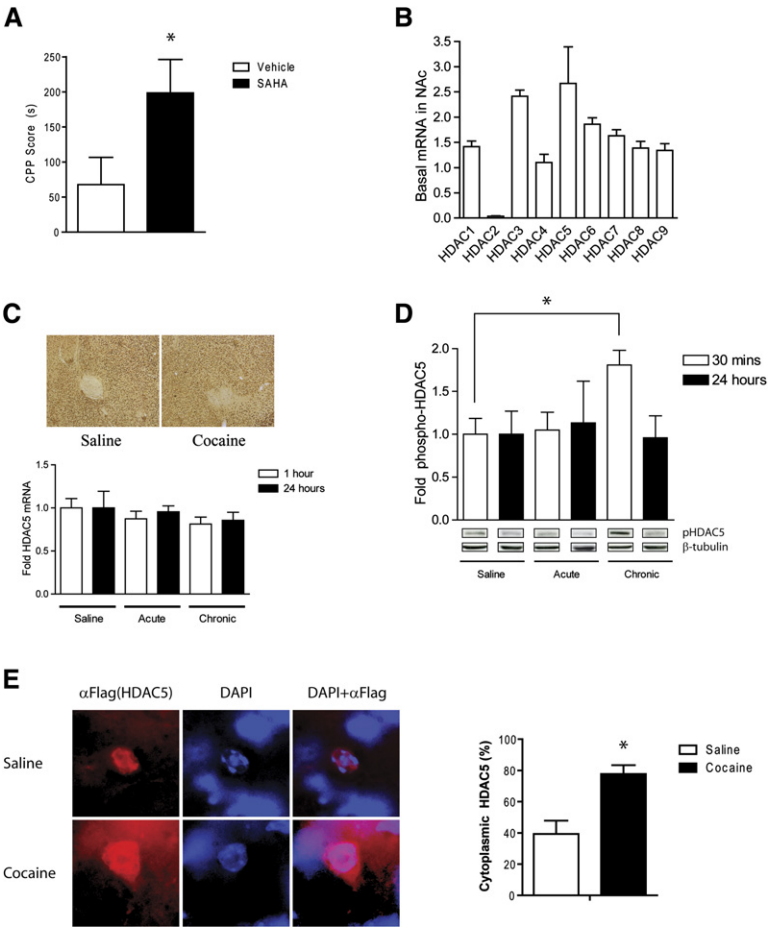


Figure 1. Cocaine Induces HDAC5 Phosphorylation and Nuclear Export in the Nucleus Accumbens

(A) Intra-NAc inhibition of HDACs with 100 μM SAHA significantly enhances cocaine reward. $p < 0.05$, n = 17–20, Student's t test.

(B) Basal expression levels of HDAC mRNA within the NAc expressed as fold difference from the average level of all HDACs 1–9 from eight mice (72 values). *HDAC3* and *HDAC5* show the highest expression within the NAc.

(C) Neither acute nor chronic cocaine regulates *HDAC5* mRNA in the nucleus accumbens 30 min or 24 hr later as measured by both qPCR in C57BL/6 mice (bottom, n = 4) and LacZ expression in HDAC5+/− mice where LacZ was knocked into the *HDAC5* locus (top, n = 3).

(D) Chronic cocaine administration causes a significant induction of phosphorylated HDAC5 (Ser259) 30 min later [analysis of variance, ANOVA, F (2,25) = 5.80, *$p < 0.05$, n = 8–9] that is not observed after an acute dose and returns to baseline by 24 hr [F (2,19) = 0.27, $p > 0.05$, n = 4].

(E) Representative image of chronic cocaine shuttling HDAC5 out of the nucleus 30 min after the last cocaine dose, blocking its actions within the nucleus (left). DAPI was used for a nuclear stain. Quantification of the percent of cytoplasmic cells to total cells counted (right, $p < 0.05$, n = 12–20 infected cells/mouse, three mice per group, Student's t test).

Data are presented as mean ± SEM.

implicated in cocaine and stress responses. We found that chronic, but not acute, exposure to either cocaine or stress regulates HDAC5 in this brain region, via distinct mechanisms, to attenuate its repressive influence on transcription. We further show that mice lacking HDAC5 exhibit dysregulation of histone acetylation, gene expression, and ultimately behavioral hyperadaptation to chronic, but not acute, cocaine or stress. Together, these data establish HDAC5 in the NAc as a central regulator of an animal's adaptive response to these chronic stimuli and suggest a role for HDAC5 in the molecular machinery gating the transition to a pathological psychiatric state.

RESULTS

Cocaine Regulates HDAC5 in the NAc

Recent studies from our laboratory have shown that histone acetylation is induced in the NAc in response to acute and chronic cocaine administration (Kumar et al., 2005). We have also shown that systemic administration of nonspecific HDAC inhibitors augmented behavioral responses to cocaine, consistent with a scheme whereby cocaine-induced hyperacetylation of histones contributes to the drug's behavioral effects. To determine whether the effects of systemic HDAC inhibitors are acting at the level of the NAc, we delivered suberoylanilide hydroxamic acid (SAHA), a highly specific inhibitor of class I and class II HDACs, directly into this brain region. The rewarding effects of cocaine were then analyzed in a conditioned place preference paradigm, where an animal's preference to a cocaine-paired environment is measured. We found that mice receiving intra-NAc delivery of SAHA during their place conditioning display significantly higher rewarding responses to cocaine than vehicle-treated controls (Figure 1A). These findings localize the role of HDAC function in cocaine reward specifically to the NAc.

In order to identify the specific HDAC(s) in the NAc important for cocaine reward, we screened the expression levels of class I and class II HDACs (HDACs 1–9), since these are the enzymes inhibited by SAHA and are thus implicated in cocaine reward. We found that *HDAC3* and *HDAC5* had the highest expression in the NAc, while *HDAC2* had the lowest. The relative levels of the other HDACs in the NAc were similar (Figure 1B). However, the mRNA levels of these HDACs, including *HDAC5*, were unaffected by cocaine administration (Figure 1C and data not shown).

Since class II HDACs (4, 5, 7, and 9) are phosphorylated and exported out of the cell nucleus through an activity-dependent mechanism, we tested whether such

phosphorylation might be regulated by cocaine. Among the class II HDACs, we focused on HDAC5 because of its relative enrichment in NAc and because its subcellular localization was the most tightly regulated by depolarization in cultured neurons (Chawla et al., 2003). We found that 30 min after an injection of cocaine, HDAC5 phosphorylation is strongly induced in mice previously exposed to a course of chronic cocaine injections, an effect not seen after the first cocaine dose (Figure 1D). No change in HDAC5 phosphorylation was observed 24 hr after an acute or chronic cocaine injection. The phosphospecific HDAC5 band observed via western blotting was only present, as expected, in cytoplasmic fractions (see Figure S1A in the Supplemental Data available with this article online), suggesting that cocaine is increasing nuclear export of HDAC5 via phosphorylation. Since antibodies to total HDAC5 are inadequate, we directly visualized the subcellular localization of HDAC5 under saline and chronic cocaine-treated conditions by infecting the NAc with a herpes simplex virus (HSV) vector expressing Flag-tagged HDAC5. We first confirmed that overexpressed Flag-tagged HDAC5 could be efficiently phosphorylated in vivo by cocaine treatment as well as in vitro in cell culture (Figure S1B). By staining brain sections through the NAc with anti-Flag antiserum, we next demonstrated a statistically significant increase in nuclear export in the NAc in vivo after chronic cocaine administration (Figure 1E). We hypothesized that HDAC5 phosphorylation and nuclear export in response to chronic cocaine is at least partially mediated by CaMKII, based on the role this enzyme plays in HDAC5 trafficking in cultured neurons (Chawla et al., 2003) and the rapid activation of CaMKII that occurs in the NAc after chronic cocaine (Mattson et al., 2005). Indeed, we found that the CaMK inhibitor, KN-93, significantly attenuates KCl-induced HDAC5 phosphorylation in NAc punches ex vivo (Figure S2). Such translocation of HDAC5 out of the nucleus is thought to block HDAC5 function, since only its nuclear form can deacetylate histones (Czubryt et al., 2003; Zhang et al., 2002). The rapid phosphorylation and nuclear export of HDAC5 only after chronic cocaine exposure suggests a mechanism by which genes induced specifically by chronic cocaine can be regulated epigenetically. Importantly, the subsequent return of HDAC5 into the nucleus would allow for only a transient increase in cocaine-induced histone acetylation and gene activation, with a return to baseline function between cocaine exposures.

Overexpression of HDAC5 in the NAc Regulates Cocaine Reward

To address the behavioral significance of HDAC5 regulation by cocaine in the NAc, we generated HSV vectors to overexpress HDAC5 in vivo. As previously reported, HSV-mediated transgene expression is maximal 1–4 days after injection and is largely dissipated by 7 days (e.g., Barrot et al., 2002; Carlezon et al., 1998; Green et al., 2006), a time course of expression confirmed for HDAC5 in the present study (data not shown). This transient expression makes HSV vectors excellent tools for overexpressing HDAC5 selectively during the training phase of place conditioning as opposed to the expression of a place preference (Figure 2A). Using an unbiased conditioned place preference paradigm, we observed that bilateral overexpression of HDAC5 in the NAc attenuated the rewarding effects of cocaine compared to animals that received HSV-GFP injections (Figure 2C). To provide insight into the mechanism by which HDAC5 reduces the rewarding responses to cocaine, we generated viral vectors expressing mutant forms of HDAC5: one mutant lacked the catalytic histone deacetylase domain; another mutant lacked the myocyte enhancing factor-2 (MEF2)-interacting domain, which has been shown to be important for class II HDAC function in nonneural cells (Lu et al., 2000). As shown in Figure 2C, the histone deacetylase domain was necessary for HDAC5 to attenuate cocaine reward, whereas loss of the MEF2-interacting domain had no effect. Equivalent expression of each of these transgenes was confirmed in vitro by western blotting and in the NAc in vivo by qPCR (Figure 2B). Moreover, treating mice systemically with the HDAC inhibitor, trichostatin A, which acts at the catalytic domain of HDACs (Finnin et al., 1999), completely blocked the repressive actions of HDAC5 overexpression on cocaine reward (Figure 2C). These data further substantiate a deacetylation-dependent mechanism for HDAC5 regulation of cocaine reward. Interestingly, the repressive action of HDAC5 on cocaine reward is not a property of all class II HDACs. The overexpression of a related class II HDAC, HDAC9, in the NAc had no effect (Figure 2C), although overexpression of HDAC4 exerted a similar effect (Kumar et al., 2005). Together, these findings indicate that the cocaine-induced regulation of endogenous HDAC5 in the NAc regulates an animal's behavioral responses to cocaine.

Loss of HDAC5 Hypersensitizes Mice to the Chronic but Not Acute Effects of Cocaine

The pronounced effect of HDAC5, but not HDAC9, overexpression on cocaine reward prompted us to investigate cocaine behaviors in HDAC5 and HDAC9 knockout mice. Unfortunately, HDAC4 knockout mice do not survive into adulthood and hence could not be included in this study (Vega et al., 2004). Surprisingly, both HDAC5 and HDAC9 mutant mice displayed normal cocaine reward at several doses of cocaine (Figure S3). However, since HDAC5 phosphorylation and nuclear export were only induced after repeated cocaine administration, we hypothesized that HDAC5 might play a more significant role in responses to chronic cocaine. To address this question, we treated mice for 1 week with higher, sensitizing doses of cocaine prior to conditioned place preference training and subsequently assessed their sensitivity to cocaine reward (Figure 3A). We found that prior cocaine exposure sensitizes HDAC5 knockout mice to the rewarding effects of cocaine significantly more than wild-type littermate control mice (Figure 3B). This increased response to chronic cocaine was not evident in HDAC9 knockout animals (Figure 3C).

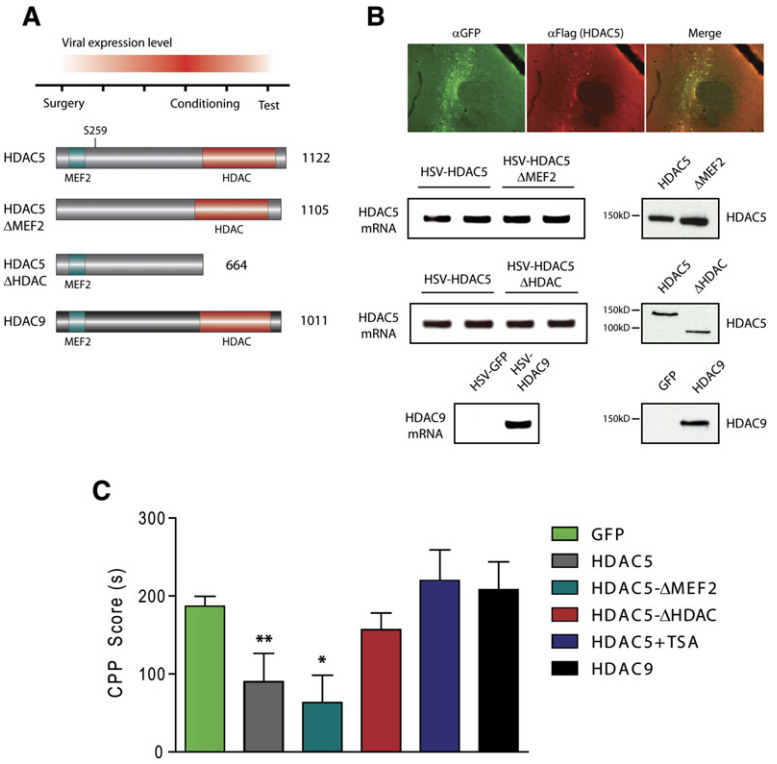


Figure 2. Viral-Mediated Expression of HDAC5 Regulates Cocaine Reward

(A) Time course of HSV expression during the conditioned place preference paradigm (top). HDAC5 domain structure, depicting the MEF2-interacting domain, the Ser259 site important for nuclear export, and the catalytic HDAC domain. Also shown are the HDAC5 mutants and HDAC9 used for the behavioral experiments with their total amino acid number listed (bottom).

(B) Representative staining of HSV-HDAC5 in the NAc. GFP is coexpressed in all neurons infected with HDAC5 (top). Expression levels of human *HDAC5* mRNA from wild-type and mutant HDAC5 viruses infected in NAc tissue in vivo, as measured by RT-PCR. The expression of human HDAC9 is also shown to confirm viral expression in vivo. Western blots for HDAC5 and HDAC9 of wild-type and mutant virus-infected PC12 cells are shown to the right of its respective in vivo mRNA data. In vivo mRNA levels and in vitro protein levels for the HDAC5 mutants were comparable to that of wild-type (bottom).

(C) Viral overexpression of HDAC5 significantly reduces conditioned place preference to cocaine [ANOVA, F (5,123) = 4.21, $^{**}p < 0.01$, n = 63 or 19]. Deletion of the catalytic HDAC domain blocks this effect ($p > 0.05$, n = 63 or 8), while deletion of the MEF2-binding domain maintains its activity against cocaine reward ($^{*}p < 0.05$, n = 63 or 17). Treating HDAC5-overexpressing mice with the HDAC inhibitor trichostatin A blocks the inhibitory effect of HDAC5 on cocaine reward ($p > 0.05$, n = 63 or 7). A related class II HDAC, HDAC9, demonstrated no effect on cocaine reward ($p > 0.05$, n = 63 or 11).

Data are presented as mean ± SEM.

Since the NAc is the site of cocaine-induced HDAC5 phosphorylation, as well as the site of HDAC inhibitor action, we examined whether restoration of HDAC5 expression, specifically within the NAc of HDAC5 knockout mice, could rescue the hypersensitivity phenotype. Mice received bilateral intra-NAc injections of HSV-HDAC5 or HSV-GFP during the chronic course of cocaine administration prior to conditioned place preference training, as this is the period responsible for both elevations of HDAC5 phosphorylation in wild-type mice and for reward hypersensitivity in HDAC5 knockout mice. We found that restoration of HDAC5 expression completely normalized the reward hypersensitivity seen in the knockouts, which argues that the HDAC5 knockout phenotype is both localized within the NAc and is not developmental in nature (Figure 3D). Notably, expressing HDAC5 in the NAc of wild-type mice during pre-exposure to chronic cocaine did not affect cocaine reward as it did when expressed during the conditioning phase (i.e., Figure 2C). This could reflect the fact that the inhibitory effect of HDAC5 overexpression on cocaine reward requires expression during training or that the higher dose of cocaine used in the pretreatment period was capable of overcoming any inhibitory effect of HDAC5. Together, these data indicate that HDAC5 within the NAc epigenetically regulates the adaptive responses that occur during repeated exposure to cocaine and suggest a possible mechanism by which loss of HDAC5 results in increased histone acetylation, aberrant gene expression, and ultimately increased responses to cocaine reward.

Altered Patterns of Gene Expression and Histone Acetylation in HDAC5 Knockout Mice which Mirror the Behavioral Phenotype

To better understand the mechanism by which HDAC5 knockout mice hyperadapt to chronic cocaine exposure, we investigated HDAC5 target genes that may contribute to their reward hypersensitization. Because no HDAC5 target genes have been previously identified in brain, we performed genome-wide expression microarrays in the NAc of HDAC5 mutant mice and their wild-type littermate controls after repeated cocaine or saline administration. To achieve the highest quality data, we performed these experiments on six biological replicates, pooling NAc from four mice for each replicate.

We first compared the set of genes differentially expressed between HDAC5 knockouts treated with chronic cocaine and wild-types treated with chronic cocaine, since this is the condition in which behavioral differences in cocaine reward were observed. This analysis identified 1616 significantly regulated genes in the NAc (>1.2-fold, $p < 0.05$). One interesting observation from this

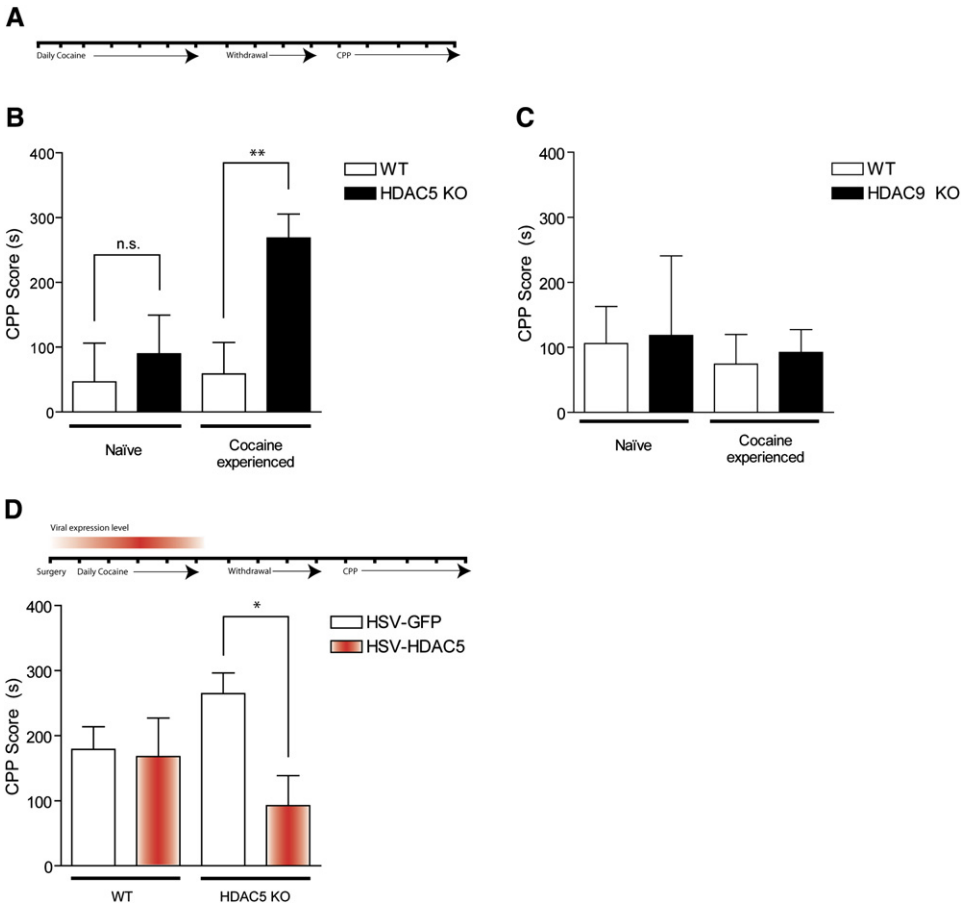


Figure 3. Mice Lacking HDAC5 Differentially Adapt to Chronic Cocaine Exposure

(A) Behavioral paradigm to assess the effect of prior cocaine exposure on subsequent cocaine reward in HDAC5 knockout (KO) and wild-type (WT) mice. Each tick mark represents 1 day.

(B) HDAC5 KO mice and littermate controls were given daily cocaine injections for 7 days and then tested for cocaine CPP after 4 days of withdrawal. While cocaine-naive HDAC5 KO mice do not display a greater preference for cocaine than their littermate controls, after prior exposure to cocaine, HDAC5 KO mice develop a significant hypersensitization to cocaine reward [ANOVA, significant effect of cocaine, $F(1,62) = 6.81$, $p < 0.05$, significant effect of genotype, $F(1,62) = 4.01$, $p < 0.05$, Bonferroni post hoc test: naive WT versus naive HDAC5 knockout, $p > 0.05$, $n = 13$, cocaine-experienced WT versus cocaine-experienced HDAC5 knockout, $^{**}p < 0.01$, $n = 18$].

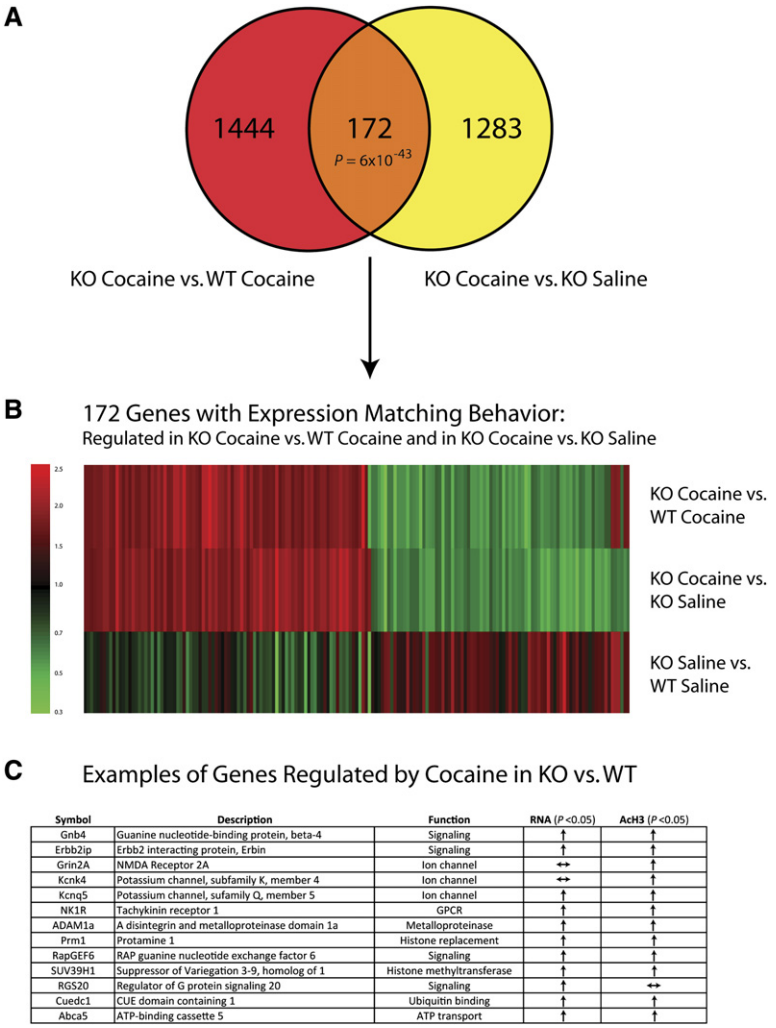
(C) Neither cocaine-naive nor cocaine-experienced HDAC9 KO mice show a significant difference to cocaine reward from their littermate controls as measured by conditioned place preference (ANOVA, no effect of drug or genotype, $n = 5–20$).

(D) Modified paradigm from (B), where viral introduction of HDAC5 specifically into the NAc occurs prior to the first cocaine treatment (top). Prior to the first cocaine dose, viral expression of HDAC5 in the NAc of naive HDAC5 KO mice and their littermate controls rescues the hypersensitization of reward observed in the KO mice after cocaine exposure [ANOVA: significant effect of virus, $F(1,32) = 4.48$, $p < 0.05$, Bonferroni post hoc test, HSV-GFP versus HSV-HDAC5 in HDAC5 KO mice $^{*}p < 0.05$, $n = 7–9$].

Data are presented as mean ± SEM.

comparison is that a vast majority of these 1616 genes are not significantly different between cocaine-naive knockout and wild-type mice (Figure S4A). This is consistent with the behavioral observations that HDAC5 mutant mice display hypersensitivity to cocaine only after prior chronic cocaine exposure. Due to the large number of regulated genes, we used Ingenuity pathway analysis to identify statistically enriched signaling pathways that may contribute to the HDAC5 knockout phenotype. Examples of highly regulated pathways are depicted in Figure S4B and covered in the Discussion.

To identify the most biologically relevant genes from this large gene set, we filtered the 1616 genes for the ones which were also significantly regulated by cocaine in the knockout (knockout cocaine versus knockout saline, >1.2-fold, $p < 0.05$). Since the reward phenotype in HDAC5 knockout mice requires prior exposure to chronic cocaine, we hypothesized that this filter would help identify the genes that most closely match the behavioral phenotype. We identified a set of 172 genes that satisfied these criteria (Figure 4A; see Table S1 for complete gene list). The enrichment of this set of 172 genes is highly



Symbol	Description	Function	RNA (P <0.05)	AcH3 (P <0.05)
Gnb4	Guanine nucleotide-binding protein, beta-4	Signaling	↑	↑
Erbb2ip	Erbb2 interacting protein, Erbin	Signaling	↑	↑
Grin2A	NMDA Receptor 2A	Ion channel	↔	↑
Kcnk4	Potassium channel, subfamily K, member 4	Ion channel	↔	↑
Kcnq5	Potassium channel, sufamily Q, member 5	Ion channel	↑	↑
NK1R	Tachykinin receptor 1	GPCR	↑	↑
ADAM1a	A disintegrin and metalloproteinase domain 1a	Metalloproteinase	↑	↑
Prm1	Protamine 1	Histone replacement	↑	↑
RapGEF6	RAP guanine nucleotide exchange factor 6	Signaling	↑	↑
SUV39H1	Suppressor of Variegation 3-9, homolog of 1	Histone methyltransferase	↑	↑
RGS20	Regulator of G protein signaling 20	Signaling	↑	↔
Cuedc1	CUE domain containing 1	Ubiquitin binding	↑	↑
Abca5	ATP-binding cassette 5	ATP transport	↑	↑

Figure 4. Microarray Analysis Identifies Expression Patterns that Mirror the Phenotype Observed in HDAC5 Knockout Mice

(A) Gene expression microarrays were performed on NAc from HDAC5 knockout (KO) and wild-type (WT) littermate controls that received either chronic cocaine or saline and were sacrificed 24 hr later. Venn diagrams display the number of genes significantly regulated (1.2-fold and $p < 0.05$) between pair-wise comparisons of cocaine-treated HDAC5 KO versus cocaine-treated WT mice (left) and cocaine-treated HDAC5 KO versus saline-treated HDAC5 KO (right). There were 172 genes that are significantly enriched in both comparisons ($p < 6 \times 10^{-43}$).

(B) Heatmap analysis displays the 172 genes significantly enriched from (A) and shows how they are expressed in the following comparisons: cocaine-treated HDAC5 KO versus cocaine-treated WT (row 1), cocaine-treated HDAC5 KO versus saline-treated HDAC5 KO (row 2), and saline-treated HDAC5 KO versus saline-treated WT (row 3). It can be seen that nearly all of these genes are similarly regulated in rows 1 and 2, while few are significantly regulated in row 3. This expression pattern mirrors the enhanced cocaine reward seen in cocaine-treated (rows 1 and 2) but not naive (row 3) HDAC5 KO mice.

(C) List of representative upregulated genes in cocaine-treated HDAC5 KO mice, whose significant regulation was confirmed by qPCR and/or ChIP on independent tissue samples. The right two columns indicate whether qPCR confirmed significantly increased mRNA levels in the NAc of KO mice and whether ChIP identified increased histone H3 acetylation on its promoter region ($p < 0.05$). These genes were selected for validation based on their potential biological relevance.

statistically significant, with $p < 6 \times 10^{-43}$. Moreover, after adjusting for multiple comparisons, the false discovery rate for this set of genes is 0.145, which is comparable to other studies of heterogeneous cell populations in vivo. Indeed, we confirmed the significant regulation of representative genes on this list by qPCR analysis of independent tissue samples (Figure 4C).

Since these 172 candidate genes were identified without regard to whether they were up- or downregulated, we generated a heatmap of these genes to illustrate their direction of regulation. Nearly all of the 172 candidate genes were regulated in the same direction in cocaine-treated HDAC5 knockout versus wild-type mice (row 1) as they were by cocaine- versus saline-treated knockout mice (row 2, Figure 4B). This pattern indicates that the gene expression differences observed between cocaine-exposed knockouts and wild-types were induced by cocaine and not pre-existing in the naive state. Indeed, levels of these 172 genes were not affected, or were even oppositely affected, in cocaine-naive knockout mice versus cocaine-naive wild-type controls (row 3, Figure 4B). Since HDAC5 is itself a transcriptional repressor, we were surprised to see a substantial subset of genes downregulated in the NAc of HDAC5 knockout mice. However, several of the behaviorally relevant genes that were upregulated can act as transcriptional repressors, such as the histone methyltransferase, Suv39H1 (Bannister et al., 2001; Lachner et al., 2001), and may account for this observation.

This work identifies a set of genes whose expression mirrors the behavioral observation that chronic cocaine induces distinct adaptations in the NAc of knockout versus wild-type mice, while expression levels of these genes are normal in naive knockout mice. Notably, many of these 172 candidate genes have been implicated in dopamine transmission in the NAc, in NAc excitability, or in cocaine responses (Figure 4C). In HDAC5 knockout mice, cocaine induced *RapGEF6*, a guanine nucleotide exchange factor that activates Rap1 and can promote ERK signaling (Kuiperij et al., 2003; Lee et al., 2002), which is a crucial pathway for cocaine reward (Lu et al., 2006). Also regulated by

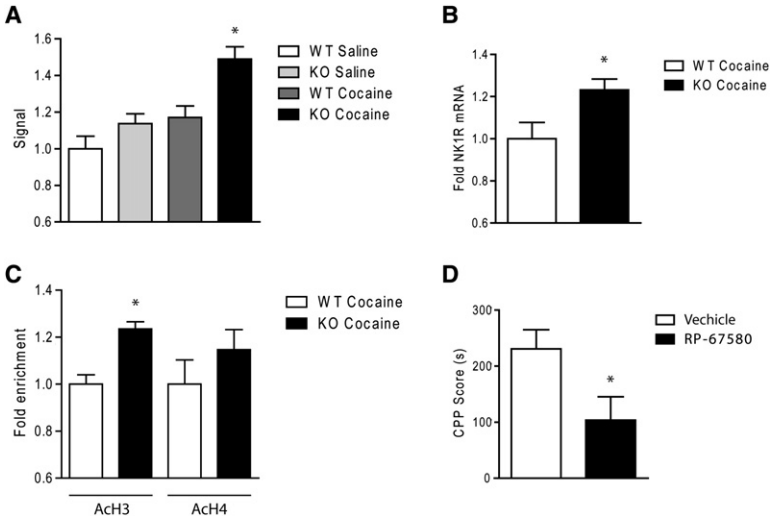


Figure 5. Microarray Target, NK1 Receptor (NK1R), Regulates Cocaine Reward

(A) Microarray expression data of *NK1R* normalized to wild-type (WT) saline. *NK1R* microarray expression is significantly increased in the HDAC5 knockout (KO) cocaine group only ($p < 0.05$, Student's t test).

(B) Quantitative PCR verifies a significant increase in *NK1R* mRNA levels in HDAC5 KO mice. qPCR was performed on an independent cohort of HDAC5 WT and KO mice treated with chronic cocaine ($p < 0.05$, $n = 6$).

(C) Quantitative ChIP shows a significant increase in histone H3 acetylation, but only a trend in H4 acetylation, at the *NK1R* gene promoter in HDAC5 KO mice treated with chronic cocaine ($p < 0.05$, $n = 6$).

(D) Reduced conditioned place preference to cocaine in mice treated with the NK1R antagonist RP-67580 (1.5 mg/kg) versus mice treated with vehicle ($p < 0.05$, $n = 8$).

Data are presented as mean ± SEM.

cocaine in the NAc of HDAC5 knockout mice is *Wnt-5a*, another key activator of ERK signaling (Almeida et al., 2005). Upregulation of *Gnb4*, a G protein that activates GIRK currents (Ruiz-Velasco et al., 2002), would be expected to reduce NAc excitability and lead to heightened cocaine responses (Dong et al., 2006). Ingenuity pathway analysis also revealed the enhancement of two pathways in cocaine-treated HDAC5 knockout mice that are known to regulate cocaine reward (Figure S4B), dopamine D1 receptor signaling (Nazarian et al., 2004), and cytoskeletal remodeling (Toda et al., 2006). The dysregulation of these genes, and perhaps others that we have identified, may therefore contribute to the reward hypersensitization exhibited by HDAC5 knockout mice.

To explore the mechanism by which these behaviorally relevant genes are upregulated by cocaine in HDAC5 knockout mice, we investigated whether their promoters had elevated levels of histone acetylation, an epigenetic mark of gene activation. Indeed, most of the gene targets identified by microarray also had significantly elevated histone H3 acetylation at their promoters, including *RapGEF6*, *Gnb4*, *Suv39H1*, and the NK1 receptor (NK1R), among many others (Figure 4C and Figure S4C). Since earlier studies have implicated substance P, an endogenous ligand for NK1R, in dopamine transmission and cocaine responses (Kalivas and Miller, 1984; Kombian et al., 2003; Placenza et al., 2005), and pharmacological antagonists are available, we chose NK1R as a prototypical example of a gene regulated in our microarray analysis (Figure 5A). We confirmed that its mRNA was elevated in cocaine-treated knockout mice (Figure 5B), that its promoter was hyperacetylated under these conditions (Figure 5C), and that blockage of this receptor, via systemic administration of the NK1R antagonist RP-67580, attenuated cocaine reward (Figure 5D). Similar effects on cocaine reward were seen with intra-NAc delivery of RP-67580 (data not shown). Thus, the increased levels of *NK1R* in the NAc of cocaine-treated HDAC5 knockout mice appear to contribute to the enhanced cocaine reward phenotype. This effect of RP-67580 is unexpected, since NK1R knockout mice display normal responses to cocaine reward (Murtra et al., 2000), perhaps due to developmental compensations. These observations therefore validate the utility of our microarray analysis in identifying molecular substrates of cocaine reward and HDAC5 action in the NAc.

Together, this work provides mechanistic insight into how the loss of HDAC5 tilts the epigenetic balance toward increased histone acetylation, which over repeated cocaine doses leads to elevations in steady-state mRNA levels of specific genes and ultimately to enhanced reward behavior. These data also identify the genome-wide profile of HDAC5-regulated genes within the brain.

Loss of HDAC5 Hypersensitizes Mice to Chronic but Not Acute Stress

The regulation of HDAC5 in the NAc by chronic cocaine prompted us to consider whether HDAC5 plays an analogous role in the adaptation to chronic stress, as the NAc is also an important substrate for stress and depression-like behavior (Nestler and Carlezon, 2006). Moreover, the neuroadaptive responses to cocaine and stress appear to be highly intertwined (Ahmed and Koob, 1997; Erb et al., 1996; Koob and Kreek, 2007). We utilized social defeat stress, a chronic stress paradigm that mimics many of the behavioral aspects of human depression (Berton et al., 2006). Importantly, the social avoidance induced by chronic defeat stress is reversible by chronic, but not acute, antidepressant treatment (Berton et al., 2006; Tsankova et al., 2006). We observed that chronic social defeat stress significantly downregulated *Hdac5* mRNA levels in the NAc (Figure 6A) without altering the phosphorylation state or subcellular distribution of HDAC5 (Figure S5). Thus, chronic stress, like chronic cocaine, reduces HDAC5

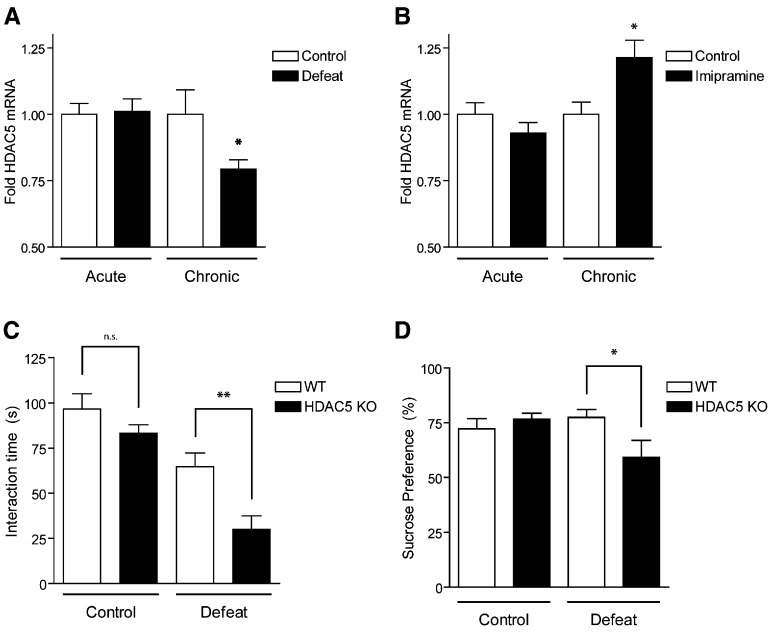


Figure 6. HDAC5 Regulates Adaptation to Chronic Stress

(A) Mice underwent acute (1 day) or chronic (10 days) of social defeat stress and were sacrificed 24 hr after the last defeat. Chronic but not acute defeat stress significantly reduced *Hdac5* mRNA in the NAc as measured by qPCR [$t(7) = 2.67$, $^*p < 0.05$, n = 3–6, with each n representing four mice pooled].

(B) Mice were treated acutely (1 injection) or chronically (28 daily injections) with the antidepressant imipramine (20 mg/kg). *Hdac5* mRNA was upregulated in their NAc by chronic but not acute imipramine treatment [$t(26) = 2.2$, $^*p < 0.05$, n = 14].

(C) HDAC5 knockout mice and their littermate controls underwent chronic social defeat stress for 10 consecutive days. Social interaction with a novel mouse was then measured 24 hr after the last defeat. While naive, unstressed HDAC5 knockout (KO), and wild-type (WT) displayed similar social interaction, the experience of chronic defeat stress induced a significantly stronger social aversion in HDAC5 KO mice than their littermate controls [ANOVA, significant effect of social defeat $F(1,36) = 37.50$, $p < 0.0001$, significant effect of genotype $F(1,36) = 12.04$, $p < 0.01$, Bonferroni post hoc test: control WT versus control KO, $p > 0.05$, defeated WT versus defeated KO, $^{**}p < 0.01$, n = 9–11].

(D) Sucrose preference test in control or defeated WT and HDAC5 KO mice demonstrates stress-induced anhedonia selectively in the mutants [$t(14) = 2$, $^*p = 0.05$, n = 8]. Defeated wild-type mice of this background did not demonstrate stress-induced anhedonia, which is observed in C57BL/6 mice (not shown).

Data are presented as mean ± SEM.

function, but through a distinct mechanism. An acute episode of social defeat did not alter *Hdac5* levels. Conversely, chronic treatment with the antidepressant imipramine significantly increased *Hdac*5 mRNA levels in the NAc (Figure 6B), an effect not seen with acute drug exposure. No other HDAC tested, which included HDAC1, 2, 3, 4, and 9, demonstrated reciprocal regulation by stress and imipramine (data not shown).

This regulation of HDAC5 expression is behaviorally important, as HDAC5 knockout mice develop more severe social avoidance after chronic social defeat stress as compared to their wild-type littermate controls (Figure 6C). Defeated HDAC5 knockout mice also became anhedonic to the natural rewarding effects of sucrose, another hallmark of a depressive-like condition, while defeated wild-type mice of this background did not show this response (Figure 6D). In contrast, knockout and wild-type mice did not differ in their behavioral responses to an acute defeat episode, nor did they differ in several models of acute stress and acute anxiety-like behavior, such as the forced swim test, elevated plus maze, and open field test (Figure S6). These data thereby implicate HDAC5 in the NAc as an epigenetic regulator of behavioral adaptations to chronic stress and chronic antidepressant action. Furthermore, these findings are consistent with a conserved role of HDAC5 in mediating the transition between acute emotional stimuli and chronic psychopathology, including drug addiction and depression.

DISCUSSION

Chromatin remodeling has been implicated in several chronic psychiatric conditions as a potential mechanism by which environmental stimuli cause long-lasting changes in gene expression and behavior (Tsankova et al., 2007). The enzymes that catalyze these histone modifications are therefore crucial mediators between the environment and the genome. We show here that the activity-dependent class II HDAC, HDAC5, is a central regulator of the actions of chronic cocaine and chronic stress in the NAc. Through its control of histone acetylation, HDAC5 contributes to the normal behavioral adaptations to these two types of emotional stimuli. These findings suggest a novel role for HDAC5 and chromatin remodeling in the NAc in an animal's vulnerability to transition from acute emotional insults to a chronic psychiatric illness.

One of the best examples of a molecule involved in this pathological transition is ΔFosB, which accumulates in the NAc uniquely after repeated cocaine administration and sensitizes rodents to addictive-like behaviors (Kelz et al., 1999). Similarly, ΔFosB is induced in the NAc after chronic, but not acute, exposures to several types of stress (Perrotti et al., 2004). ΔFosB, therefore, illustrates the characteristics of a molecular switch involved in the transition from acute responses to chronic pathological effects of drugs or stress (McClung et al., 2004). HDAC5 also satisfies many of the key functional characteristics of

a molecular switch, since it too responds specifically to chronic, but not acute, cocaine or stress to regulate behavioral adaptations to those stimuli.

In the NAc, chronic cocaine increases HDAC5 phosphorylation at Ser259, an effect not observed after an acute dose. Studies in nonneural cells suggest that phosphorylation of Ser259 (along with a cooperatively regulated site at Ser498) provides docking sites for 14-3-3 proteins, which mediate the export of HDAC5 out of the nucleus (McKinsey et al., 2000a, 2000b). The kinetics of this phosphorylation are also notable, as HDAC5 phosphorylation was observed 30 min after repeated cocaine administration but returned to normal by 24 hr. Previous reports from cardiac myocytes as well as cultured cerebellar granule neurons (Linseman et al., 2003) suggest that CaMKII is an important HDAC5 kinase. A recent report has shown that cocaine rapidly activates CaMKII in the NAc within 10 min (Mattson et al., 2005), a time frame consistent with its role as an upstream HDAC5 kinase in the NAc. Indeed, we found that CaMKII was necessary for depolarization-induced HDAC5 phosphorylation in acutely dissected NAc tissue, further highlighting CaMKII as a putative kinase for cocaine-induced regulation of HDAC5 in the NAc. This rapid but transient phosphorylation and nuclear export of HDAC5 likely allows for pulses of increased histone acetylation, target gene activation, and over time, behavioral adaptations to repeated cocaine exposure. This model is supported by experiments in which HDAC5 was virally overexpressed within the NAc, a manipulation which elevates nuclear HDAC5 levels and attenuates cocaine reward. Experiments with HDAC5 mutant proteins and HDAC inhibitors establish that this action of HDAC5 is mediated through its catalytic histone deacetylase domain. This is a significant finding, since prior studies of class II HDACs in nonneural cells have focused on the MEF2-binding domain of these proteins as the most important moiety for their physiological activity. Rather, our data show that the catalytic domain of HDAC5 is most important for its regulation of cocaine reward. We further show that HDAC5 knockout mice are significantly more sensitive to the rewarding effects of cocaine, but only in mice previously exposed to the drug chronically. This phenotype is rescued by restoring HDAC5 expression specifically in the NAc, the same site where endogenous HDAC5 phosphorylation is enhanced by chronic cocaine. Importantly, HDAC5 knockout mice are phenotypically normal with respect to the acute effects of cocaine, conditions under which endogenous HDAC5 is not highly phosphorylated. This was surprising, since acute cocaine reward was dramatically reduced by HDAC5 overexpression. However, only chronic cocaine regulates endogenous HDAC5 to control genes important for cocaine reward, so it is possible that supraphysiological levels of HDAC5 repress these same target genes below baseline, thereby affecting acute reward behavior when overexpressed. These findings show that the regulation of HDAC5 in the NAc by cocaine is consistent with its role as a molecular switch between acute drug exposures and a chronically addicted state.

We used gene expression microarray analysis to identify genes differentially regulated in the NAc between HDAC5 knockout mice and their wild-type littermate controls after chronic cocaine or saline administration. Patterns of gene expression revealed by this analysis broadly support the hypothesis that HDAC5 controls a set of genes specifically induced by chronic cocaine. Many of the genes significantly regulated between cocaine-exposed knockout versus cocaine-exposed wild-type mice follow a pattern of expression that mirrors the behavioral responses to cocaine. That is, they are induced by cocaine in the HDAC5 knockout mice significantly more than by cocaine in wild-type mice, but are not significantly altered in the knockout in the cocaine-naive state. This is important because it provides a molecular description for how HDAC5 knockout mice more readily sensitize to cocaine and perhaps transition to an addicted state. The mechanism by which this dysregulation in gene expression occurs involves increased histone H3 acetylation on the promoters of the genes we identified through microarray analysis. The NK1 receptor provides a proof of principle: it is upregulated in the NAc by chronic cocaine in HDAC5 knockout mice only, this upregulation is associated with hyperacetylation of histone H3 at the NK1R gene promoter, and the upregulation contributes to enhanced responses to cocaine reward. Taking a more global approach to the microarray data, we used Ingenuity pathway analysis to identify signaling pathways that were uniquely regulated by cocaine in the NAc of HDAC5 knockout mice. This analysis revealed several key pathways important for neural plasticity and reward behavior (see Figure S4), including dopamine receptor signaling (Nazarian et al., 2004), ATF2/CREB signaling (Carlezon et al., 2005), NF-κB (Ang et al., 2001; S.J. Russo et al., 2004, Soc. Neurosci., abstract), NFAT (Mermelstein, 2007), cytoskeletal remodeling proteins (Toda et al., 2006), and ion channels (Pulvirenti et al., 1992; Dong et al., 2006). Together, these data directly implicate chromatin remodeling as a mechanism driving altered gene activation and behavioral responses to cocaine. We therefore conclude that, in the wild-type response to chronic cocaine, HDAC5 is phosphorylated and exported out of the nucleus to permit controlled activation of its downstream target genes (Figure 7). Within 24 hr, HDAC5 then returns to the cell nucleus to limit the expression of cocaine-regulated genes by histone deacetylation. In the knockout, however, HDAC5 is not present to limit the expression of these genes, which consequently begin to accumulate during repeated cocaine exposures, ultimately culminating in increased sensitivity to cocaine reward.

Since behavioral responses to drugs of abuse and stress are closely linked (Koob and Kreek, 2007), effects mediated in part by the NAc (Nestler and Carlezon, 2006), we determined whether HDAC5 plays an analogous role in an animal's adaptation to chronic stress. As with cocaine-elicited behaviors, naive HDAC5 knockout mice were phenotypically normal in several tests of acute stress and acute anxiety-like behavior, but exhibited increased sensitivity to the deleterious effects of chronic social defeat stress,

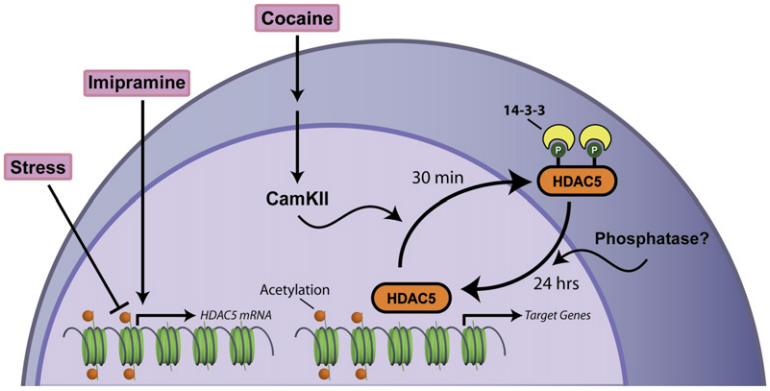


Figure 7. Model of HDAC5 Action in the NAc in Cocaine and Stress Responses

Chronic cocaine rapidly induces the phosphorylation at Ser259 and nuclear export of HDAC5, most likely via CaMKII. Ser498 is also likely phosphorylated, since the two sites are known to be cooperatively regulated. This blocks HDAC5 action within the nucleus and allows for increased histone acetylation and transcription of HDAC5 target genes. HDAC5 returns to the nucleus within 24 hr to limit the expression of cocaine-induced gene transcription. Chronic stress reduces HDAC5 function by downregulating the transcription of HDAC5 itself, resulting in increased sensitivity to stress. Chronic treatment with the antidepressant imipramine may reverse some of these stress-induced behavioral maladaptations by increasing HDAC5 expression.

including decreased social interaction and decreased sucrose preference. Moreover, as with cocaine, chronic but not acute social defeat stress decreased HDAC5 function in the NAc, albeit through a distinct mechanism. These data demonstrate a crucial role for HDAC5 in regulating behavioral adaptations to chronic stress as well as chronic cocaine and suggest that HDAC5 contributes to a molecular switch between acute stress responses and more long-lasting depression-like maladaptations (Figure 7). This dual action in cocaine and stress is reminiscent of the opioid peptide dynorphin, which also regulates behavior in animal models of depression (Newton et al., 2002; Pliakas et al., 2001) and cocaine reward (Carlezon et al., 2005; McLaughlin et al., 2006). However, dynorphin levels were unaffected in HDAC5 knockout mice in both microarray and qPCR experiments (data not shown), indicating the existence of several distinct mechanisms underlying chronic cocaine and stress responses in the NAc. Of further clinical relevance, chronic but not acute imipramine treatment, which is known to reverse many of the effects of chronic social defeat stress (Berton et al., 2006; Tsankova et al., 2006), upregulated *Hdac5* mRNA expression in the NAc, suggesting that HDAC5 function in the NAc may be an important target for antidepressant action (Figure 7). This reciprocal regulation by social defeat and imipramine in the NAc was specific for HDAC5, as no other HDAC tested was regulated in this way. It is interesting to note, however, that the function of HDAC5 in other brain regions may be very different, as we have previously demonstrated that chronic imipramine administration in socially defeated mice downregulates HDAC5 expression in the hippocampus and that this downregulation may mediate an antidepressant-like response in this brain region. Thus, HDAC5 is antidepressant when expressed in the NAc and prodepressant when expressed in the hippocampus, making it another example of a growing number of important signaling proteins (e.g., CREB, BDNF), which exhibit opposite regulation and behavioral effects in these two brain structures (Berton et al., 2006; Carlezon et al., 2005; Eisch et al., 2003; Shirayama et al., 2002).

Together, our data draw fascinating parallels to observations of HDAC5 function in cardiac tissue. The hearts of naive HDAC5 knockout mice, for example, are phenotypically normal. However, when knockout mice are exposed to chronic cardiac stress via thoracic aortic banding, their hearts hypertrophy to significantly greater sizes than wild-type control mice (Chang et al., 2004). Our findings in brain, along with those in heart, suggest that HDAC5 may serve as a central mediator between chronic environmental stimuli and the downstream adaptive responses in chromatin remodeling and gene expression that protect several tissues from diverse types of repeated challenges.

The functions of HDAC5 described here provide new insight into the pathogenesis of drug addiction, depression, and other stress-related syndromes. We propose that HDAC5 contributes to the behavioral transition between short-term physiological and long-term pathological responses to emotional stimuli, since its regulation of chromatin structure is attenuated only by repeated stimulation, and its complete loss in mutant mice results in significant hypersensitivity in animal models of chronic but not acute drug addiction and stress. These findings implicate epigenetic mechanisms in an individual's vulnerability to repeated adverse stimulation. This fundamentally new insight into the molecular underpinnings of chronic maladaptation in brain could lead to the development of improved treatments for addiction, depression, and other chronic psychiatric disorders.

EXPERIMENTAL PROCEDURES

Drugs

Cocaine-HCl, imipramine, and trichostatin A were purchased from Sigma. RP-67580 was purchased from Tocris. Suberoylanilide hydroxamic acid (SAHA) was synthesized as described previously in WO 93/07148 PTC/US92/08454 (Breslow et al., 1993).

Behavior

Conditioned Place Preference

We used an unbiased paradigm similar to that described previously (Kelz et al., 1999). Briefly, mice were conditioned using a standard

CPP paradigm except for the studies in Figure 2, which required an accelerated paradigm to accommodate the timing of HSV expression. The standard protocol involved once daily conditioning sessions over 4 days, which lasted 30 min each. The mice were injected with cocaine (either 1.25, 2.5, or 5 mg/kg) or saline on opposites sides of a place preference box (gray side or striped side) on alternating days and were then tested for their preference on the day following the last conditioning. The CPP score was defined as the time spent (s) on the cocaine-paired side minus the time spent on the saline paired side (s). See Supplemental Methods for more detailed CPP and Sensitization-CPP methodology.

Social Defeat Stress

Social defeat was performed as described previously (Berton et al., 2006). Briefly, 10 min defeats were carried out by placing an experimental mouse in the home cage of a different aggressive CD1 mouse each day for 10 days. After each defeat, the mice were separated by a plastic barrier with holes to allow nonphysical aggressive interaction to continue for 24 hr. Social interaction with a novel mouse was measured 24 hr after the last defeat. Acute defeat consisted of a single 10 min defeat session with social interaction measured 24 hr later. See Supplemental Methods for details of anxiety and sucrose preference tests.

Viral-Mediated Gene Transfer

Expression plasmids for HDAC5, HDAC5ΔHDAC (Δ670-1122), HDAC5ΔMEF2 (Δ175-192), and HDAC9 were subcloned into herpes simplex virus and 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. Coordinates to target both the nucleus accumbens shell and core were, +1.6 mm A/P, +1.5 mm lateral, and −4.4 mm D/V from bregma (relative to dura). 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 Crestle Violet staining to visualize needle tracks.

RNA Isolation

Bilateral NAc were dissected from mice treated with the indicated regimen of cocaine, social defeat, or imipramine and frozen on dry ice. Frozen brain tissue was then thawed in TriZol (Invitrogen) and processed according to the manufacturer's protocol. RNA was purified with RNAesy Micro columns (QIAGEN) and processed as indicated by the manufacturer. Spectroscopy confirmed that the RNA had 260/280 and 260/230 ratios >1.8. Agilent bioanalyzer confirmed RNA quality (RIN) was excellent (>9.3). With the imipramine samples, RNA was subsequently amplified one round using RNAamp (Arcturus), in order to generate enough RNA for studying multiple genes.

Western Blotting

Standard western blotting techniques were performed. Briefly, 50 μg of NAc lysate was added to an 8% SDS-PAGE gel and transferred to a PVDF membrane. To detect phospho-HDAC5, we used a phospho-Ser259-specific HDAC5 antibody as described previously (Harrison et al., 2006). β-tubulin (Sigma) was used to normalize the phospho-HDAC5 band since a reliable total HDAC5 antibody could not be identified for endogenous HDAC5 in NAc lysates. To visualize HSV-overexpressed HDAC5, however, an antibody to total HDAC5 (Upstate) worked well. The HDAC9 antibody was from Biovision. Phospho-Thr286-CaMKII and Lamin antibodies were obtained from Cell Signaling Technology.

Immunohistochemistry

HDAC5 heterozygous mice were treated with 20 mg/kg cocaine or saline for 7 days. They were sacrificed 24 hr later. Immunohistochemistry for β-galactosidase was performed as described previously (Barrot et al., 2002; Russo et al., 2007). C57BL/6 mice received HSV-HDAC5 bilaterally injected in their NAc. Four days post-op, 30 min after their seventh dose of cocaine (twice daily), mice were sedated with a lethal dose of chloral hydrate and perfused with 4% paraformaldyhyde. NAc sections were stained with anti-Flag antiserum (Sigma) and DAPI to visualize the nucleus. Socially defeated mice were processed similarly, but since the treatment is for 10 days, the virus was not delivered until day 7 of defeat. Cells were counted as nuclear if Flag staining was confined to the area also stained with DAPI, and cytoplasmic if Flag staining extended beyond the nucleus, even if some staining remained in the nucleus.

Chromatin Immunoprecipitation

ChIP for acetylated H3 (AcH3) and H4 were performed as described in Kumar et al. (2005) with minor modifications. Briefly, chromatin was sonicated to an average of about 500 bp. Sonicated chromatin was then immunoprecipitated using anti-AcH3 (06-599) and anti-AcH4 (06-598) antibodies from Upstate (now Millipore) and a rabbit IgG control. The IP was collected using Protein A beads from Upstate (06-157), which were washed repeatedly to remove nonspecific DNA binding. The chromatin was eluted from the beads and cross-links were removed overnight at 65°C in the presence of proteinase K and EDTA. DNA was then purified and quantified using real-time PCR.

Reverse Transcription and Quantitative PCR

Reverse transcription of total RNA was carried out using Superscript III (Invitrogen) as indicated by the manufacturer using random hexamers. qPCR was then run using approximately 25 ng of cDNA per reaction, primers, and SYBR Green (ABI). Each reaction was run in triplicate and quantified using the ΔΔCt method as previously described (Tsankova et al., 2006). A complete list of primers used in this study is in Table S6.

Microarrays and Analysis

Total NAc RNA was pooled from four mice per biological replicate and amplified by the UT Southwestern Microarray facility with the TotalPrep RNA Amplification kit (Illumina/Ambion). The cRNA pools from six biological replicates were labeled and hybridized to Illumina Mouse V6-1.1 full genome expression arrays. We used a cutoff of 1.2-fold and a $p < 0.05$ for each pair-wise comparison. The false discovery rate of the genes used to make the heatmap in Figure 4B was 0.145 (calculated by permutation). See Supplemental Methods for more detailed microarray methodology. All microarray data have been deposited in the GEO database under accession GSE9134.

Statistical Analysis

One- or two-way ANOVAs were performed to determine significance for conditioned place preference, social defeat, western blotting, microarrays, and real-time PCR data with greater than two groups. Student's t tests were used for other comparisons, including PCR and chromatin immunoprecipitation experiments. All values included in the figure legends represent mean ± SEM (*$p \leq 0.05$; **$p < 0.01$).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/56/3/517/DC1/.

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Accession Numbers

All microarray data have been deposited in the GEO database under accession GSE9134.