EPIGENETIC MECHANISMS IN DRUG ADDICTION

APPROVED BY SUPERVISORY COMMITTEE

Eric J. Nestler, M.D., Ph.D.

Eric N. Olson, Ph.D.

Ege Kavalali, Ph.D.

Q. Richard Lu, Ph.D.

DEDICATION

I first and foremost would like to thank my parents for their continued love and support. Their guidence has given me both the confidence to approach tough challenges and the skills to solve them creatively. I also thank Drs. Eva Lee and Song Zhao who took the time to teach me basic biology and inspire my interest in biomedical research. I thank my thesis advisor, Dr. Eric Nestler for his mentorship in every aspect of a scientific career, from the research itself to teaching the community; he somehow does it all and has been an amazing role model. I also thank Dr. Mike Brown, whose unbounded curiousity has kept me fascinated by even the most esoteric biology. And finally, my wife Nora and daughter Amber who make each day so exciting that the completion of this thesis is truly just icing, albeit very tasty icing, on the cake.

EPIGENETIC MECHANISMS IN DRUG ADDICTION

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WILLIAM RUSSELL RENTHAL

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EPIGENETIC MECHANISMS IN DRUG ADDICTION

WILLIAM RUSSELL RENTHAL, Ph.D.

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ERIC J. NESTLER, M.D., Ph.D.

Changes in gene expression in brain reward regions are thought to contribute to the pathogenesis and persistence of drug addiction. Recent studies have begun to focus on the molecular mechanisms by which drugs of abuse, and related environmental stimuli, such as drug-associated associated cues or stress, converge on the genome to alter specific gene programs. Increasing evidence suggests that these stable gene expression changes in neurons are mediated in part by epigenetic mechanisms that alter chromatin structure on specific gene promoters. Indeed, genome-wide analysis using chromatin immunoprecipitation coupled with promoter microarrays *in vivo*, identified on which genes chronic cocaine exposure alters histone acetylation and methylation in the nucleus accumbens, a key brain reward region. In addition to providing novel insight into basic transcriptional mechanisms co-opted by cocaine, these data revealed a new class of cocaine-regulated genes, the sirtuins, which potently regulate reward behavior. In order to further understand the mechanisms by which cocaine regulates chromatin structure, I investigated enzymes which control levels of histone acetylation, histone deacetylases (HDACs).

Chronic, but not acute, exposure to cocaine decreased the function of a class II HDAC, HDAC5, in the NAc, 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, but not acute, cocaine. I have also identified a key role of the class I HDAC, HDAC1, which interacts with the drug-induced transcription factor, ΔFosB, to repress *c-fos* gene induction in striatum after chronic psychostimulant exposure. Taken together, these findings suggest that proper balance of histone acetylation in the NAc is a crucial factor in the saliency of cocaine action, and that disruption of this balance may be involved in the transition from acute adaptive responses to chronic psychiatric illness.

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* = these authors contributed equally

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ΔcJun	truncated form of cJun, dominant negative of ΔFosB
ΔFosB	Truncated form of FosB that is induced by chronic drug use
4OHT	4-hydroxytamoxifen
AAV	Adeno-associated virus
acH3,acH4	Acetylated histone H3 or H4
ADCY3	Adenylyl cyclase 3
ADORA1	Adenosine A1 receptor
AGS3	Activator of G-protein signaling 3
AKT	AKT/PKB protein kinase
AMPA	Glutamate receptor (AMPA)
ARC	Activity-regulated cytoskeleton-associated protein
ATF1	Activating transcription factor 1
ATF2	Activating transcription factor 2
BDNF	Brain derived neurotrophic factor
bp	Base pair
Ca2+/CaM	Calcium/calmodulin
CamKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CaN	Calcineurin
CaNΔCT	Constitutively active calcineurin
CART	Cocaine and amphetamine regulated transcript
CB1	Cannabinoid receptor 1
CBP	CREB-binding protein
CCKaR	Cholecystokinin A receptor
CDK5	Cyclin-dependent kinase 5
c-Fos	FBJ murine osteosarcoma viral oncogene
ChIP	Chromatin immunoprecipitation
ChIP-chip	ChIP combined with microarrays
ChIP-Seq	ChIP combined with massively parallel sequencing
CLOCK	Clock transcription factor
CREB	cAMP response element binding protein
D1R	Dopamine D1 receptor
DAPI	Nuclear stain (4',6'-diamidino-2-phenylindole)
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DRD3	Dopamine D3 receptor
EDTA	Ethylene diamine tetraacetic acid

EGR	Early growth response protein
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
GABRB1	Gamma-aminobutyric acid receptor beta 1
GBP2	Guanine nucleotide binding protein 2
Gcn5	Histone acetyltransferase Gcn5
GFP	Green fluorescent protein
Gnb4	G protein beta-4 subunit
GR	Glucocorticoid receptor
H2A,H2B,H3,H4	Histone H2A, H2B, H3, H4
H3K27	Histone H3 methylated at lysine 27 (repressive)
H3K4	Histone H3 methylated at lysine 4 (activating)
H3K9	Histone H3 methylated at lysine 9 (repressive)
H3S10	Histone H3 phosphorylated at serine 10 (activating)
HAT	Histone acetyltransferase
hda1	Yeast histone deacetylase (similar to Class II HDACs)
HDAC	Histone deacetylase
HDM	Histone demethylase
HEK293	Human embryonic kidney cells
HMT	Histone methyltransferase
HOMER	Homer protein
HP1	Heterochromatin protein 1
HSV	Herpes simplex virus
K3K36	Histone H3 methylated at lysine 36 (active, elongating)
KCl	Potassium chloride
KCNE3	Potassium voltage-gated channel subfamily E member 3
KDM3A/JHDM2A	Lysine demethylase 3A/JHDM2A
KDM4D/JMJD2D	Lysine demethylase 4D/JMJD2D
KMT1A/SUV39H1	Lysine methyltransferase 1A/SUV39H1
LCM	Laser capture microdissection
LT-VSCC	L-type voltage sensitive calcium channel
MAPK	Mitogen activated protein kinase
MBD2	Methyl-CpG-binding domain protein 2
MEF2	Myocyte enhancer factor 2
MEF2ΔDBD-VP16	MEF2-VP16 with a mutant DNA-binding domain
MEF2-VP16	Constitutively active MEF2 (fused to VP16 viral transcriptional activator)
MSK1	Mitogen- and stress-activated protein kinase 1
MSN	Medium spiney neurons
MTAP2	Microtubule-associated protein 2
NAc	Nucleus accumbens
NAC1	Nucleus accumbens 1

NAD	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T-cells
Nf-κB	Nuclear factor kappaB
NK1	Neurokinin 1 (substance P)
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NRGN	Neurogranin
N-WASP	Neural wiskott-aldrich syndrome protein
p300	Histone acetyltransferase p300
p53	p53 tumor suppressor
PC12	Rat pheochromocytoma cells
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PDYN	Prodynorphin
PER1	Period 1
PFC	Prefrontal cortex
PI3-kinase	Phosphoinositide 3-kinase
pik3cg	PI3-kinase catalytic subunit gamma
PKA	Protein kinase A
PTEN	Phosphatase and tensin homolog
Rap1	RAB guanine nucleotide exchange factor 1
RapGEF6	Rap guanine nucleotide exchange factor 6
RCAN2	Regulator of calcineurin 2
RCS	Regulator of calmodulin signaling (ARPP21)
RGS9	Regulator of G-protein signaling 9
RNA	Ribonucleic acid
RNAi	RNA interference
rpd3	Yeast histone deacetylase (similar to Class I HDACs)
RXR	Retinoid X receptor
SAHA	HDAC inhibitor (suberoylanilide hydroxamic acid)
shRNA	Short hairpin RNA
sir2	Yeast histone deacetylase (similar to Class III HDACs)
SIRT1,2	Sirtuin 1,2
SSTR2	Somatostatin receptor 2
TSA	Trichostatin A (HDAC inhibitor)
TUNEL	Apoptosis assay (terminal deoxynucleotidyl transferase dUTP nick end labeling)
UBE3b	Ubiquitin-protein ligase E3B
VTA	Ventral tegmental area
WAVE1	WASP family protein 1
WAVE3	WASP family protein 3
Wnt5a	Wingless-type MMTV integration site family, member 5A

CHAPTER ONE
Introduction

Drug addiction is a debilitating psychiatric disorder that is characterized by compulsive drug seeking and taking despite severe adverse consequences (Kalivas et al., 2005; Hyman et al., 2006; Koob and Kreek, 2007). Once an individual becomes addicted to a drug of abuse, there are few effective clinical options and most addicts relapse within a short period of time. Thus, addiction research focuses on two major outstanding questions. First, what are the neural mechanisms underlying the transition from recreational drug use to a chronically addicted state? Second, what are the mechanisms responsible for the persistence of addictive behaviors even after prolonged drug abstinence? A better understanding of these mechanisms may provide clues into how we can block or even reverse the addicted state and thereby reduce the rate of relapse.

Drug-induced changes in gene expression in key brain reward regions, such as the nucleus accumbens (NAc), prefrontal cortex (PFC), and ventral tegmental area (VTA), represent one mechanism thought to contribute to both of these key questions (Kalivas et al., 2005; Hyman et al., 2006; Koob and Kreek, 2007). For example, the transcription factor ΔFosB is induced several fold in the NAc by chronic drug exposure and has been implicated in the transition to an addicted state (Hope et al., 1994; Kelz et al., 1999; McClung et al., 2004). Altered expression of specific genes, such as AGS3 (activator of G protein signaling 3) (Bowers et al., 2004) and BDNF (brain-derived neurotrophic factor) (Grimm et al., 2003), has been reported weeks after the last drug experience and manipulation of these genes in rodents regulates drug relapse behavior (Bowers et al., 2004; Lu et al., 2004; Graham et al., 2007). Genome-wide mRNA analyses have identified many more potential gene targets for drugs of abuse in distinct brain reward regions that may also contribute to their long-lasting behavioral effects (Freeman et al.,

2001; McClung and Nestler, 2003; Yuferov et al., 2003; Yao et al., 2004; McClung et al., 2005; Winstanley et al., 2007). Therefore, it has become of great interest to identify the underlying mechanisms by which chronic drug exposure promotes stable changes in gene expression and ultimately behavior. Recent evidence has suggested that epigenetic mechanisms—key cellular processes that integrate diverse environmental stimuli to exert potent and often long-lasting changes in gene expression through the regulation of chromatin structure—contribute to these drug-induced transcriptional and behavioral changes (Kumar et al., 2005; Levine et al., 2005; Renthal et al., 2007). This review discusses current progress toward understanding how epigenetic mechanisms are regulated by drugs of abuse in brain reward regions, and how such mechanisms may ultimately contribute to drug-related behaviors.

Epigenetic Mechanisms

The word "epigenetic" typically refers to a heritable phenotype not coded by DNA itself but by a cellular process "above the genome." Cellular differentiation is a classic example where epigenetic phenomena play a critical role (Feinberg, 2007; Schuettengruber et al., 2007). Since all cells in an organism contain the same genetic information, the ability to form clonal populations of distinct cell types with unique functions (e.g. neurons versus hepatocytes) is achieved by transmitting the correct transcriptional programs from parent to daughter cell. This epigenetic process is in large part coordinated through control of chromatin structure. Increasing evidence indicates that changes in chromatin structure not only mediate these heritable epigenetic phenomena (Grewal and Moazed, 2003), but also that the same types of changes in chromatin occur in mature, post-mitotic neurons (Guan et al., 2002; Tsankova et al., 2007).

Chromatin is made up of DNA and the histone proteins around which the DNA is wrapped. Histones are assembled into an octamer made up of two copies of H2A, H2B, H3, and H4 (Figure 1-1) (Luger and Richmond, 1998). These histone proteins together with DNA undergo a complex supercoiling process, which results in a highly compact structure to condense and organize what would otherwise be meters of extended DNA. This highly condensed structure means that control over gene expression occurs partly by gating access of transcriptional activators to DNA (Felsenfeld and Groudine, 2003; Li et al., 2007a). The structure of chromatin, and hence access to the DNA sequence wrapped around it, is highly regulated by post-translational modifications of histones and the DNA itself (Kouzarides, 2007). Such modifications include acetylation, phosphorylation, and methylation of histones, methylation of DNA, and copious others, with each modification either positively or negatively regulating the transcriptional activity of the underlying gene (Figure 1-1). Ultimately, dozens of potential modifications that occur at many distinct histone residues summate to determine the final transcriptional output of a given gene (Strahl and Allis, 2000).

The expansive number of combinatorial options possible enables epigenetic mechanisms to exert exquisite control over the transcriptional activity of each gene in the genome. One of the key hallmarks of epigenetic mechanisms is their potential stability within the cell. This is an important feature, since maintaining continuity of liver gene programs in hepatacytes and neural gene programs in neurons is necessary throughout the life of an individual. However, despite the stability of developmental epigenetic mechanisms *in vivo*, all types of chromatin modifications identified to date are potentially reversible and have specific enzymes or processes which mediate the addition or removal of each mark (Kouzarides, 2007).

Histone acetyltransferases (HATs) catalyze the addition of acetyl groups onto lysine residues of histone proteins. There are over a dozen known HATs, such as CBP, p300, Gcn5, and PCAF, many of which have been implicated in addiction-, stress-, or memory-related behaviors (Levine et al., 2005; Maurice et al., 2007; Oliveira et al., 2007). Recently, several transcription factors (e.g., ATF2 [activating transcription factor 2], CLOCK) have also been shown to possess HAT activity (Kawasaki et al., 2000; Doi et al., 2006).

Histone deacetylases (HDACs), which remove acetyl groups from histones, are divided into 4 classes. Class I HDACs (e.g., HDAC1, 2, 3) are ubiquitously expressed and likely mediate the majority of deacetylase activity within cells. Class II HDACs (e.g., HDAC4, 5, 9), enriched in specific tissues such as heart and brain, are larger proteins that contain both the class I deacetylase domain and an N-terminal regulatory domain that enables them to be shuttled in and out of the nucleus in a neural activity-dependent manner (Chawla et al., 2003). Class III HDACs are NAD-dependent and have been implicated in the regulation life span and metabolism (Haigis and Guarente, 2006). HDAC11 is a class IV HDAC and shares homology to both class I and class II enzymes (Yang and Seto, 2008).

Histone methyltransferases (HMTs) and demethylases (HDMs) are not only specific for the histone subunit and lysine residue (e.g., K4 vs. K9), they are even specific for the number of methyl groups they can add or remove (Kouzarides, 2007). For example, the HMT, KMT1C (G9a), is specific for histone H3K9 but only adds 1 or 2 methyl groups, with the distinct HMT, KMT1A (SUV39H1), catalyzing trimethylation of this site. Similarly, the HDM, KDM3A (JHDM2a), can demethylate 1 or 2 methyl groups on H3K9, requiring a distinct demethylase (e.g., KDM4D [JMJD2D]) to fully demethylate the trimethylated state. Thus, several enzymes

are required to move between the unmethylated and fully trimethylated states, providing an additional level of regulation and information encoded by each methyl mark.

Methylation of DNA in brain is catalyzed by three main enzymes, DNMT1, 3a, and 3b. A role in adult neural plasticity is supported by the observation that DNMT inhibitors alter behavioral effects in learning and memory paradigms (Miller and Sweatt, 2007). DNA demethylase enzymes likely exist based on reports that environmental stimuli induce the demethylation of particular genes in the brain, but the specific enzymes are not yet known (Miller and Sweatt, 2007).

Neuronal signaling is known to regulate both the addition and removal of histone acetylation, histone methylation, and DNA methylation *in vivo*, a process which most likely occurs through the enzymes discussed above (Tsankova et al., 2007).

Drug-Induced Changes in Chromatin Structure

Histone acetylation

Acetylation of histone lysine residues reduces the electrostatic interaction between histone proteins and DNA, which relaxes chromatin structure and makes DNA more accessible to transcriptional regulators (Kouzarides, 2007). Histone acetylation is best characterized on histones H3 and H4: it can occur on lysines 9, 14, 18, and 23 on the N-terminal tail of H3 and at lysines 5, 8, 12, and 16 on the tail of H4. Genome-wide studies have shown that hyperacetylation in promoter regions is strongly associated with gene activation, while hypoacetylation is correlated with reduced gene expression (Kurdistani et al., 2004; Pokholok et al., 2005). This association also exists in the brain *in vivo* in response to drugs of abuse. Acute exposure to cocaine, for example, which is known to rapidly induce the immediate early genes *c-*

fos and *fosb* in the NAc, increases histone H4 acetylation on their proximal gene promoters (Figure 1-2) (Kumar et al., 2005). Time course analysis revealed that this modification occurs within 30 minutes and disappears by 3 hours, consistent with the induction kinetics of these immediate early genes. At least for *fosb*, this increase in histone acetylation is dependent on the HAT, CBP (CREB-binding protein) (Levine et al., 2005). Interestingly, despite several control gene promoters where acute cocaine does not affect histone acetylation (β-tubulin, tyrosine hydroxylase, histone H4), acute cocaine does increase global levels of histone H4 acetylation, and histone H3 phospho-acetylation (see below), but not H3 acetylation alone, within 30 minutes (Brami-Cherrier et al., 2005; Kumar et al., 2005). Thus, global changes in histone modifications, which have been observed in learning models and in response to environmental enrichment (Levenson et al., 2004; Fischer et al., 2007), may be accounted for by a specific subset of genes.

Repeated cocaine exposure, either forced (investigator) administration or self-administration, is known to induce a distinct set of genes in the NAc (e.g. *cdk5* and *bdnf*), some of which remain elevated for days to weeks (Bibb et al., 2001; Grimm et al., 2003; McClung and Nestler, 2003; Yao et al., 2004). Consistent with such stable changes in gene expression, increased histone H3 acetylation was observed on the gene promoters of both *cdk5* and *bdnf* for 1-7 days following the final dose of cocaine (Kumar et al., 2005). Stable changes in histone acetylation and gene expression have been observed for nearly two weeks following withdrawal from cocaine self-administration in the prefrontal cortex as well. For example, *npy* (neuropeptide Y) expression was found to be upregulated and its gene promoter hyperacetylated, while *egr-1* (early growth response 1) was found to be downregulated and hypoacetylated after cocaine withdrawal (Freeman et al., 2007).

Histone phosphorylation

Histone phosphorylation is generally associated with transcriptional activation; it can be observed on the promoters of immediate early genes such as *c-fos* when they are induced after cAMP induction or glutamate treatment in cultured striatal neurons (Li et al., 2004; Brami-Cherrier et al., 2007). One of the best characterized histone phosphorylation sites is serine 10 on histone H3 (H3S10). This modification stabilizes the HAT, Gcn5, on gene promoters and antagonizes two repressive modifications—the methylation of hysine 9 on histone H3 (H3K9) and the recruitment of HP1 (heterochromatin protein 1) (Kouzarides, 2007). Since phosphorylation at H3S10 recruits a HAT, the neighboring lysine residue at H3K9 is often acetylated in concert with phosphorylation (phospho-acetylation). In the striatum, such H3 phosphorylation and phospho-acetylation are not only globally induced by acute cocaine as mentioned above, it is rapidly induced on the *c-fos* gene promoter, a point at which the gene is highly activated (Figure 1-2) (Brami-Cherrier et al., 2005; Kumar et al., 2005). Moreover, pretreatment of rats with an HDAC inhibitor prior to cocaine administration potentiates phospho-acetylation at the *c-fos* promoter and induction of *c-fos* mRNA, further illustrating the interplay between these two modifications. Interestingly, this interplay between histone phosphorylation and acetylation may occur only at certain genes in response to acute cocaine or other acute stimuli and has been implicated particularly in the induction of immediate early genes. Cocaine-induced H3S10 phosphorylation appears to be mediated by the downstream mitogen activated protein kinase, MSK1 (Figure 1-2) (Brami-Cherrier et al., 2005).

Histone methylation

Histone methylation is particularly complex, and can exist in mono-, di- (me2), or tri-methylated (me3) states, enabling each state to recruit unique co-regulators and exert distinct effects on transcriptional activity (Kouzarides, 2007). Histone methylation is also unique because each lysine residue has distinct, and often opposite, effects on transcription. For example, H3K4 is enriched mostly at promoter regions and is highly associated with gene activation, while H3K9 and H3K27 are usually associated with repression (Li et al., 2007a). However, even this is an oversimplification, as H3K9 is often found in coding regions along with H3K36 and may be involved in transcriptional elongation (Vakoc et al., 2005; Kouzarides, 2007). Such complexity is reflected in the PFC of adolescent rats, where cocaine was found to induce significant reductions in global levels of both the activating H3K4me3 mark as well as the repressive H3K27me3 mark (Black et al., 2006). One likely explanation is that these global decreases in histone methylation are occurring on distinct gene promoters. Indeed, using the gene-specific technique, chromatin immunoprecipitation (Figure 1-3A), chronic cocaine exposure increases H3K9me2 on genes where histone acetylation is not induced. Many of these hypermethylated genes are also downregulated by cocaine (see below). There is also early evidence that specific histone methyltransferases and demethylases are themselves regulated in the NAc (Maze et al., 2008).

Although enzymes exist to demethylate histones, in a mouse model of depression (social defeat stress), increased H3K27me2 on the *bdnf* promoter was observed in the hippocampus for at least a month after the final stress (Tsankova et al., 2006). This repressive mark correlated with a significant downregulation of BDNF expression. If the mice were treated with chronic antidepressants after the stress, however, increases in the activating mark, H3K4me2, occurred

on the *bdnf* promoter and BDNF expression returned to normal levels, but persistent increases in H3K27me2 remained. Although the study of histone methylation in animal models of addiction is in its early phases, these studies together have shown that histone methylation is dynamically regulated by drugs of abuse and other environmental stimuli and has the potential to stably alter gene expression *in vivo*. It will be important to identify how such long-lasting changes in histone methylation are maintained and whether these mechanisms mediate persistent behavioral deficits.

Genome wide analysis of chromatin regulation in animal models of addiction

Beyond analyzing the effect of drugs of abuse on chromatin structure at specific genes, the next important step is to characterize these drug-induced histone modifications across every gene in the genome. Using genome-wide techniques involving hybridizing immunoprecipitated chromatin to genome-wide promoter microarrays (ChIP-chip) (see Fig 2A) or to high throughput sequencing, a wealth of new information can been uncovered about epigenetic regulation in specific brain regions as well as novel gene targets that control behavioral responses to drugs of abuse. Such analyses are just now getting underway for drug addiction models.

The data one gets from such analyses are illustrated in Figure 1-3B, where histograms represent the intensity of enrichment along each chromosome (Kumar et al.). This can be "zoomed in" for each gene in the genome, as shown in Figure 1-3C, where one can overlay cocaine-induced changes in histone H3 acetylation, H4 acetylation, and H3K9me2 methylation and so on. This high resolution map of histone modifications provides a new level of insight into basic transcriptional mechanisms occurring in the brain *in vivo* in response to chronic cocaine administration. One such insight directly addresses earlier findings where a few acutely induced

genes showed selective H4 acetylation on their promoters while a few chronically induced genes displayed selective H3 acetylation (Kumar et al., 2005). Genome-wide ChIP-chip analysis reveals that while there are more H3 acetylated genes in the NAc of mice exposed to chronic cocaine, there is also a significant set of previously unrecognized, chronically-induced genes that are hyperacetylated only on H4 (Figure 1-3D) (Kumar et al.). Interestingly, this analysis showed further that only a very small subset of genes contains both H3 and H4 acetylation. This suggests that the H4 to H3 switch previously observed in both cocaine (Kumar et al., 2005) and seizure models (Tsankova et al., 2004) may be reserved for certain types of genes, such as immediate early genes.

Another striking finding from these first genome-wide studies in addiction models is that there are very few genes where cocaine induces hypoacetylation of either H3 or H4 (Kumar et al.). This suggests that active histone deacetylation may not be the most common mechanism for downregulating gene expression by cocaine. One interpretation of these results is that HDACs more commonly serve to limit gene expression (see below), while an alternative mechanism serves to actually repress genes in response to cocaine. Histone methylation may be such a repressive mechanism, as numerous genes were found to have elevated H3K9me2 methylation after chronic cocaine exposure, and many of these genes are known to be downregulated by cocaine (McClung and Nestler, 2003; Yao et al., 2004; Kumar et al.). Thus, histone methylation is an attractive candidate for a cocaine-induced mechanism to repress specific gene transcription.

Functions of chromatin remodeling in vivo

Despite the substantial progress being made in identifying chromatin modifications throughout the genome in a variety of *in vivo* model systems, many questions remain. For

example, we still do not know if histone modifications are the primary cause of changes in gene expression or if they are simply a reflection of it. Even in simple biological systems this remains a challenging question because most genetic and pharmacological tools manipulate chromatin structure genome-wide. This makes it difficult to establish direct causal relationships between altered chromatin structure and observed transcriptional changes at a specific gene locus. Importantly, a recent breakthrough has demonstrated the proof of principle that zinc finger peptides fused to a DNA methyltransferase can target that enzyme to a specific gene in cultured cells (Li et al., 2007b; Smith et al., 2008), suggesting that we may soon be able to target chromatin modifying enzymes to individual genes in the brain *in vivo*. Then, one could begin to determine whether stimulus- (e.g., cocaine-) induced histone modifications on specific gene promoters are necessary and sufficient to alter the activity of that gene *in vivo*, and ultimately if such modifications play a role in behavioral responses to drugs of abuse.

Although these tools have not yet been used in brain, there are several examples *in vivo* that suggest certain histone modifications are more than a reflection of gene expression and may serve a function in gene priming. For example, *bdnf* is highly acetylated in the NAc within 24 hours after a rat self-administers cocaine (Kumar et al., 2005), however, the steady-state levels of BNDF protein are not significantly elevated until a week of cocaine withdrawal (Grimm et al., 2003). This is an example where histone acetylation on a gene promoter precedes the induction of gene expression, and supports the idea that, at least for a subset of genes, histone acetylation may play a role in priming genes for subsequent induction. Therefore, investigating histone modifications genome-wide may provide unique insight into cocaine-induced gene regulation beyond a reflection of steady-state mRNA levels.

Role of Epigenetic Mechanisms in Drug-Related Behaviors

The identification of cocaine-induced alterations in histone acetylation, phosphorylation, and methylation in the NAc and other brain areas suggests that such modifications may be involved in regulating behavioral responses to drugs of abuse. Indeed, the first evidence for this came from studies that demonstrated that the pharmacological and genetic manipulation of certain HDACs in the NAc alters levels of histone acetylation *in vivo* and profoundly affects behavioral sensitivity to cocaine (Kumar et al., 2005). In the conditioned place preference test, in which an animal learns to associate the rewarding effects of cocaine with a specific environment, either systemic administration of sodium butyrate or trichostatin A, both non-specific HDAC inhibitors, significantly potentiates the rewarding effects of cocaine (Kumar et al., 2005). Delivery of the more specific HDAC inhibitor SAHA (suberoylanilide hydroxamic acid) directly into the NAc is sufficient to increase cocaine reward (Renthal et al., 2007). Similar potentiating effects of HDAC inhibition on drug-related behavior was observed with amphetamine and D1 agonists (Kalda et al., 2007; Schroeder et al., 2008). Consistent with the hypothesis that increased histone acetylation potentiates behavioral sensitivity to cocaine, mice that are deficient in CBP, a HAT, exhibit reduced histone acetylation on the *fosb* promoter as well as reduced sensitivity to cocaine (Levine et al., 2005). Similarly, reducing histone acetylation in the NAc by virally-overexpressing certain HDACs (HDAC4 or HDAC5, but not HDAC9) in the NAc significantly decreases cocaine place conditioning and, at least for HDAC5, this effect requires the C-terminus catalytic deacetylase domain (Renthal et al., 2007). However, class II HDACs like HDAC5 associate with class I HDACs (e.g., HDAC3) at this same C-terminus domain (Fischle et al., 2002), so the relative contribution of this interaction, vs. any

catalytic activity of class II HDACs *per se*, to the full effects of HDAC5 on cocaine reward remains unclear.

The observation that chronic cocaine exposure regulates HDAC5 in the NAc raises the exciting possibility that this class II HDAC is involved in the behavioral transitions which occur between acute and chronic cocaine exposure (e.g., recreational drug use to compulsive drug use). Specifically, chronic, but not acute, cocaine administration induces HDAC5 phosphorylation and nuclear export in the NAc, actions that block the enzyme's effects on histones. Nuclear export of HDAC5 results in histone hyperacetylation and increased mRNA expression of specific HDAC5 target genes, which would then contribute to sensitized behavioral responses to the drug (Renthal et al., 2007). An example of such a target gene is the NK1 (substance P) receptor. Consistent with this model, naïve HDAC5 knockout mice display normal rewarding responses to initial cocaine exposures, but become hypersensitive if they are previously exposed to a chronic course of cocaine (Renthal et al., 2007). Importantly, HDAC5 knockout mice also hypersensitize to other chronic, but not acute, stimuli, including chronic social defeat stress and chronic cardiac stress (Chang et al., 2004; Renthal et al., 2007). These findings, taken together with HDAC inhibitor studies in leaning and memory and depression models, suggest that histone acetylation controls the saliency of a wide variety of environmental stimuli (Levenson et al., 2004; Tsankova et al., 2006; Schroeder et al., 2007). Whether it is cocaine, stress, or memory, pharmacological and genetic manipulations that result in elevated histone acetylation appear to potentiate the respective behavioral responses.

Histone H3 phosphorylation and phospho-acetylation also appear to play key roles in drug-regulated behaviors. As discussed earlier, cocaine rapidly induces global levels of histone H3 phosphorylation and phospho-acetylation in the striatum with similar kinetics as *c-fos* mRNA

induction (Brami-Cherrier et al., 2005; Kumar et al., 2005). Moreover, *c-fos* induction appears to be highly linked to these modifications, as *c-fos* induction by acute cocaine is potentiated by HDAC inhibitors and entirely blocked by loss of the histone H3 kinase, MSK1 (Brami-Cherrier et al., 2005; Kumar et al., 2005). MSK1 is a downstream member of the MAP kinase cascade and, as mentioned earlier, is necessary for cocaine-induced H3 phosphorylation. Loss of MSK1 also reduces locomotor responses to cocaine (Brami-Cherrier et al., 2005), which is consistent with a mechanism involving dysregulation of *c-fos* (Zhang et al., 2006).

Histone methylation is also regulated by cocaine, however, the behavioral significance of this modification is still under investigation. New inhibitors of histone methyltransferases in addition to viral-mediated gene transfer are allowing this question to be directly addressed. For example, preliminary findings suggest that inhibition of a particular H3K9 histone methyltransferase, whose expression is regulated in the NAc by chronic cocaine, potentiates behavioral responses to the drug (Maze et al., 2008). Since inhibition of H3K9 methylation would be expected to enhance gene activity, these results are consistent with studies of histone acetylation (Kumar et al., 2005; Renthal et al., 2007) and indicate that manipulations that increase gene transcription generally promote behavioral adaptations to drugs of abuse.

Interplay Between Transcription Factors and Epigenetic Mechanisms

In order for environmental stimuli to regulate chromatin structure on the correct set of genes, mechanisms exist to guide the proper chromatin remodeling enzymes and transcriptional regulators to the right gene locus. Transcription factors serve as a key mechanism by which distinct gene programs are controlled since they bind to highly specific DNA regulatory sequences. These regulatory sequences—termed response elements—serve as an address, so the

cell can rapidly initiate specific gene programs that are influenced by a given set of transcription factors. While some chromatin remodeling enzymes can bind directly to a chromatin mark through specialized protein domains (Kouzarides, 2007), many others get targeted to chromatin by interacting with specific transcription factors. The transcription factor CREB (cAMP response element bindig protein), which plays an essential role in behavioral responses to cocaine (Carlezon et al., 1998), was one of the first transcription factors known to direct a chromatin modifying enzyme to gene promoters. When CREB is phosphorylated, it interacts with CREB-binding protein (CBP), a HAT that helps facilitate target gene activation by acetylating neighboring histones (Mayr and Montminy, 2001).

ΔFosB, mentioned earlier, is another key transcription factor involved in behavioral responses to cocaine. ΔFosB is a stable, truncated splice variant of the *fosB* gene that binds to AP-1 sites in the promoter regions of responsive genes (Kelz et al., 1999; McClung et al., 2004). Interestingly, ΔFosB activates certain genes (e.g., c*dk5*) (Bibb et al., 2001; McClung and Nestler, 2003; Kumar et al., 2005) and represses others (e.g., *c-fos*) (McClung and Nestler, 2003; Renthal et al.). Recent studies have found that at the *cdk5* gene, which is upregulated after chronic cocaine (Bibb et al., 2001), ΔFosB binds to its promoter and recruits transcriptional activators, such as the SWI/SNF remodeling protein BRG1 (Figure 1-4A) (Kumar et al., 2005). In contrast, at *c-fos*, which is repressed after chronic cocaine (Hope et al., 1994), ΔFosB binds to its promoter and recruits HDAC1 to deacetylate nearby histones (Figure 1-4B) (Renthal et al.). Importantly, overexpression of ΔFosB alone is sufficient both to upregulate *cdk5* and to repress *c-fos*, nicely illustrating how a single transcription factor can direct distinct chromatin modifying enzymes to specific genes. The exact mechanisms which mediate the gene-specific effects of ΔFosB remain unclear, but neighboring promoter regulatory elements that recruit distinct transcription factors

or unique post-translational modifications may contribute. Such an interplay between transcription factors and chromatin remodeling enzymes regulated by drugs of abuse may serve as an important new avenue of research and pharmaceutical development.

CHAPTER ONE FIGURES

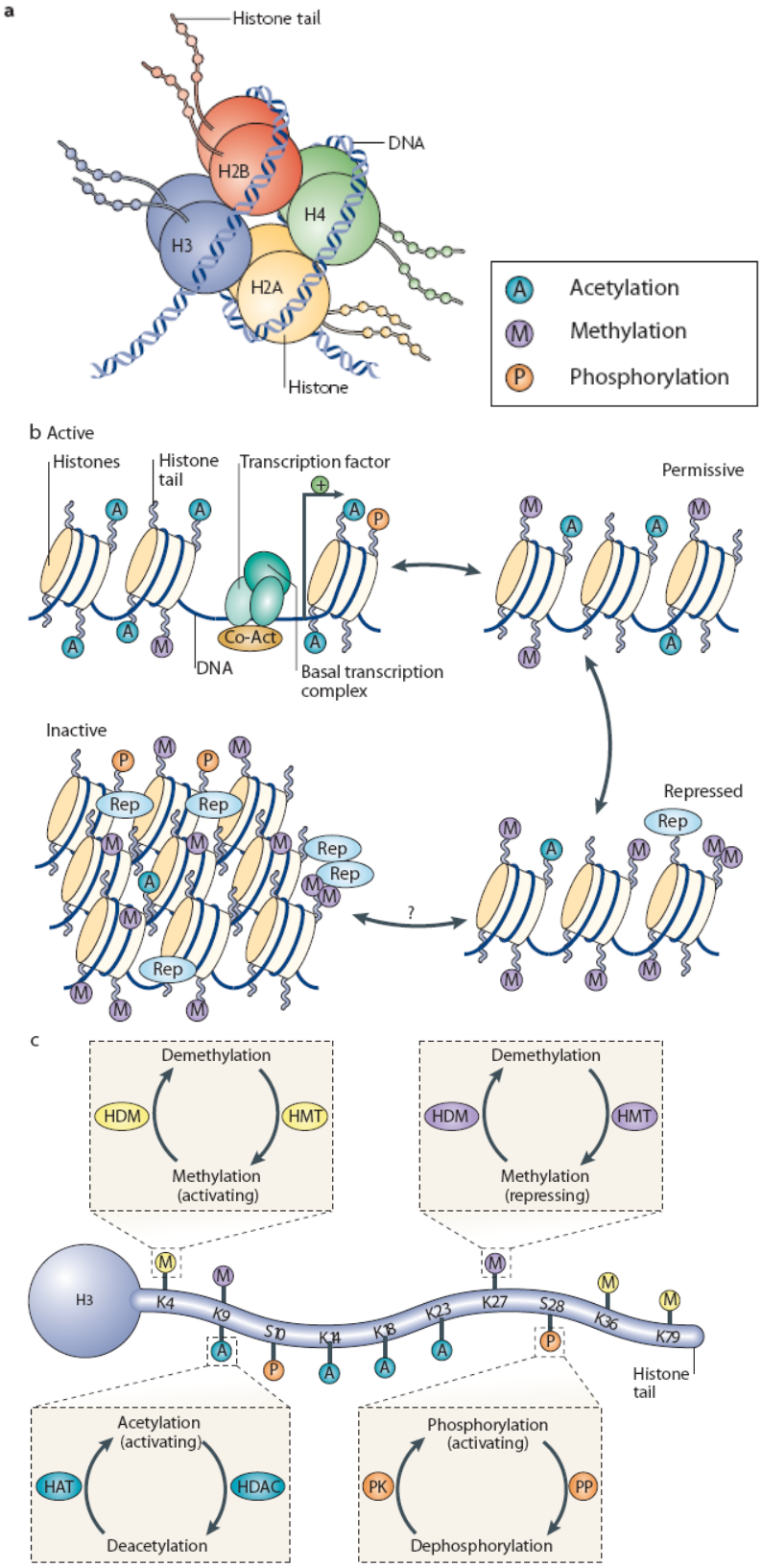


Figure 1-1 General scheme of chromatin remodeling.

A. Picture of a nucleosome showing a DNA strand wrapped around a histone octamer composed of two copies each of the histones H2A, H2B, H3 and H4. The amino (N) termini of the histones face outward from the nucleosome complex. **B**. Chromatin can be conceptualized as existing in two primary structural states: as active, or open, euchromatin (top left) in which histone acetylation (A) is associated with opening the nucleosome to allow binding of the basal transcriptional complex and other activators of transcription; or as inactive, or condensed, heterochromatin where all gene activity is permanently silenced (bottom left). In reality, chromatin exists in a continuum of several functional states (active; permissive (top right); repressed (bottom right); and inactive). Enrichment of histone modifications such as acetylation and methylation (M) at histone N-terminal tails and related binding of transcription factors and co-activators (Co-Act) or repressors (Rep) to chromatin modulates the transcriptional state of the nucleosome. Recent evidence suggests that inactivated chromatin may in some cases be subject to reactivation in adult nerve cells, although this remains uncertain. **C.** Summary of common covalent modifications of H3, which include acetylation, methylation and phosphorylation (P) at several amino acid residues. H3 phosphoacetylation commonly involves phosphorylation of S10 and acetylation of K14. Acetylation is catalysed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs); lysine methylation (which can be either activating or repressing) is catalysed by histone methyltransferases (HMTs) and reversed by histone demethylases (HDMs); and phosphorylation is catalysed by protein kinases (PK) and reversed by protein phosphatases (PP), which have not yet been identified with certainty. K, lysine residue; S, serine residue.

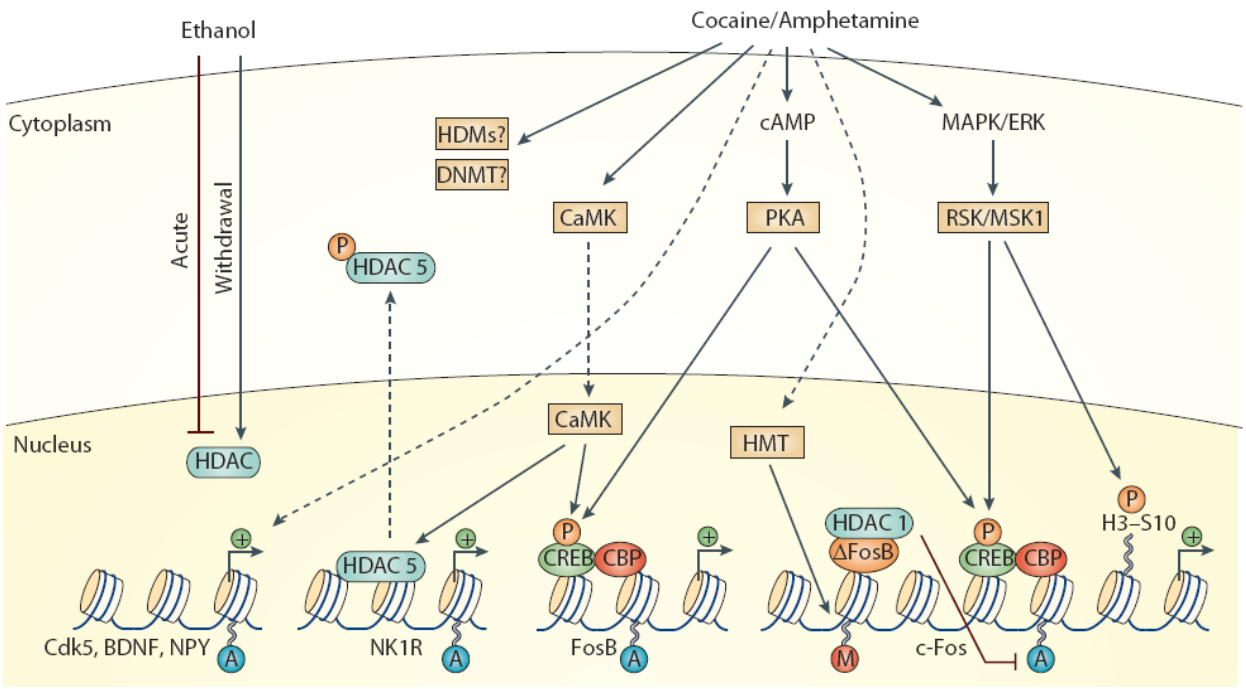


Figure 1-2. Regulation of chromatin remodeling by drugs of abuse.

Cocaine and amphetamine increase levels of cAMP in the nucleus accumbens (NAc) and activate protein kinase A (PKA). PKA then phosphorylates cAMP response element binding protein (CREB), which allows for the recruitment of the histone acetyltransferase, CREB binding protein (CBP). Examples of this are shown on the *fosb* and *c-fos* genes. Chronic cocaine or amphetamine is also known to elevate levels of ΔFosB, which can recruit histone deacetylase 1 (HDAC1) to the *c-fos* promoter and inhibit subsequent induction of the gene (see also Figure 3). This desensitization of *c-fos* also involves increased repressive histone methylation, which is thought to occur via the induction of specific histone methyltransferases. It is not yet known how cocaine regulates histone demethylases (HDM) or DNA methyltransferases (DNMTs). Cocaine also activates the mitogen activated protein kinase (MAPK) cascade, which through MSK1 can phosphorylate CREB and histone H3 at serine 10. In addition, stimulant drugs regulate Ca^{2+} levels in NAc neurons (perhaps via regulation of glutamatergic synapses from

cortical regions). This activates CaMK (calcium/calmodulin protein kinases) signaling, which, in addition to phosphorylating CREB, also phosphorylates HDAC5. This results in nuclear export of HDAC5 and increased histone acetylation on its target genes (e.g. NK1R [NK1 or substance P receptor). Several other genes have been shown to display increased acetylation on their promoters after cocaine or amphetamine exposure, including *cdk5, bdnf,* and *npy*. As well, acute ethanol has been shown to reduce histone acetylation by increasing HDAC activity, while withdrawal from chronic ethanol increases histone acetylation by reducing HDAC activity.

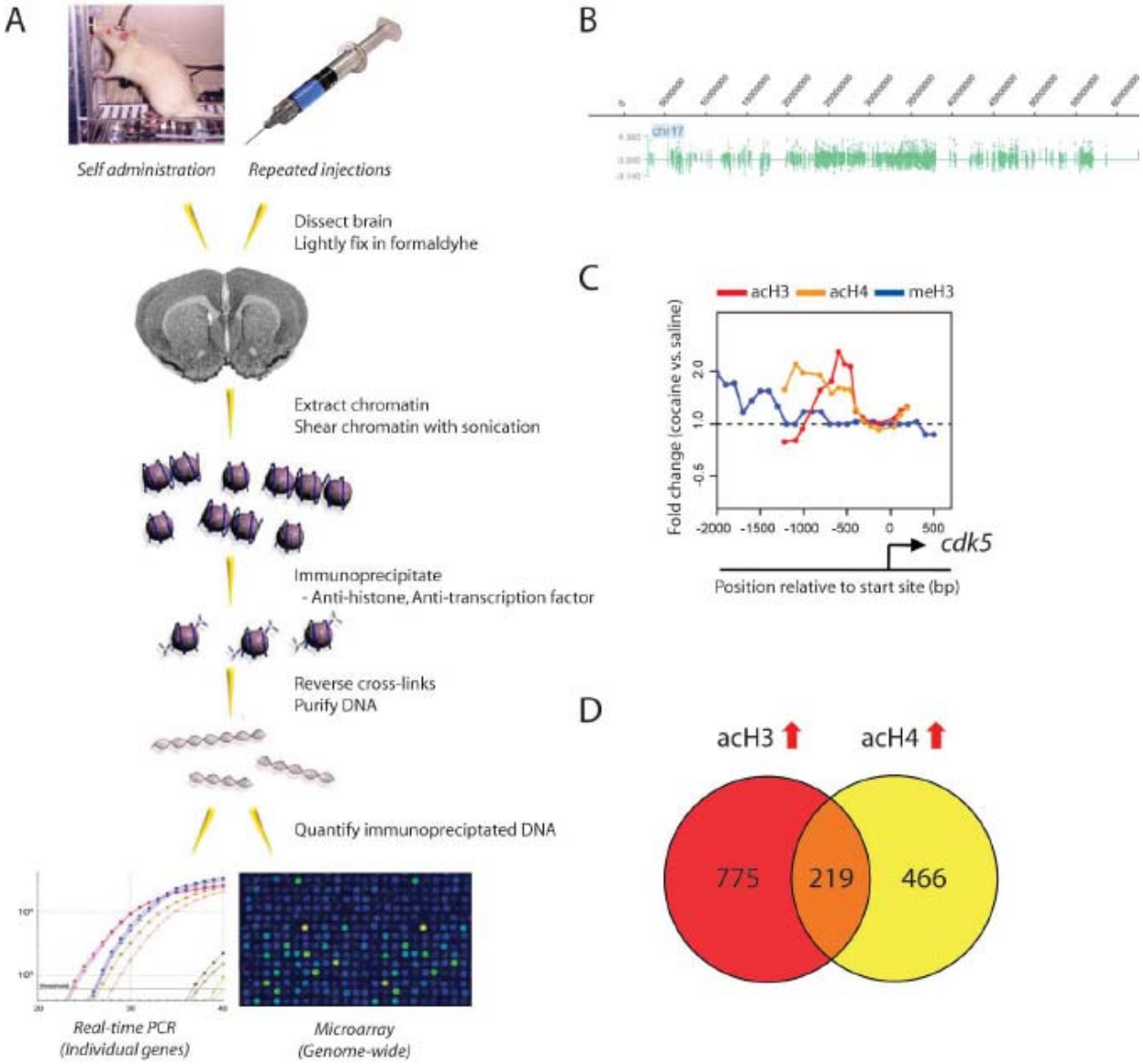


Figure 1-3. Studying chromatin regulation in brain using chromatin immunoprecipitation.

A. Schematic of the chromatin immunoprecipitation (ChIP) protocol. ChIP is a technique used to quantify the amount a specific DNA sequence occupied by a given histone modification or transcription factor. The technique involves lightly fixing the tissue or cells with formaldehyde to cross-link DNA with the associated histone and other chromatin-bound proteins. The cross-linked chromatin is then sonicated into ~500bp fragments and immunoprecipitated with an

antibody raised against a specific histone modification or transcription factor. The immunoprecpitated DNA is then reverse cross-linked from associated proteins and purified. Specific regions of this DNA can then be directly quantified by real time PCR to determine how much of that DNA was immunoprecipitated in a drug-treated vs. saline-treated animal. The final purified DNA can also be amplified for downstream use in genome-wide analysis techniques such as microarrays (ChIP-chip) or next generation sequencing (ChIP-seq). **B**. ChIP-chip data are typically displayed as a enrichment profile across each chromosome. For example, acetylated H3 (acH3) binding on chromosome 17 from the nucleus accumbens (NAc) of a cocaine-treated mouse is displayed. One can then compare the enrichment profiles between cocaine- and saline-treated mice to determine the fold difference of acH3 on a specific chromosomal region. **C**. The chromosome-wide data shown in B can be zoomed in to display each gene in the genome. Displayed here is the *cdk5* gene promoter in the NAc and the fold difference between cocaine- and saline-treated mice for acH3 (red), acH4 (orange), and methylated H3 (meH3) (blue). **D**. The fold differences between cocaine- and saline-treated mice can be quantified for each gene and analyzed for statistical significance. The genes can then be compared and displayed, for example, using Venn diagrams to show how many genes are commonly regulated between two conditions. Shown here are the number of genes in the NAc on which cocaine commonly or uniquely increases acH3 and acH4 binding.

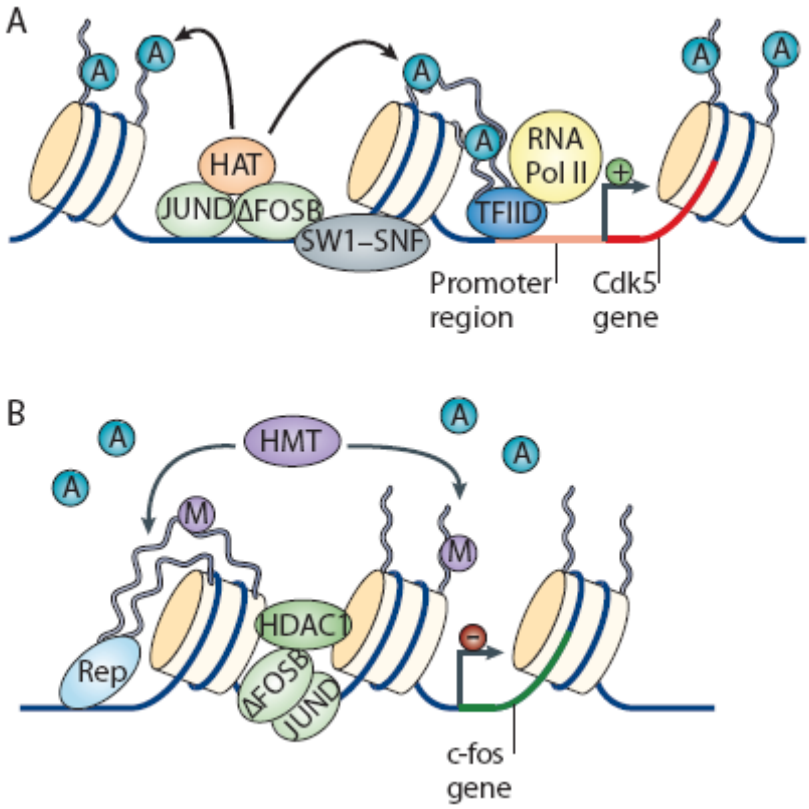


Figure 1-4. Gene-dependent recruitment of chromatin remodeling enzymes.

A. Cocaine- and amphetamine-induced increases in ΔFosB in the nucleus accumbens (NAc) are known to activate transcription of the *cdk5* gene. This involves binding of ΔFosB to the *cdk5* promoter and recruitment of the SWI-SNF ATP-dependent chromatin remodeling complexes and histone acetyltransferases (HATs). Histone deacetylase (HDACs) are not present on the *cdk5* promoter, which permits significantly higher levels of acetylated histone H3 after chronic cocaine exposure. **B**. Cocaine- and amphetamine-induced increases in ΔFosB also acts as a transcriptional repressor at a different gene locus, *c-fos*. After repeated stimulant exposure, the *c-fos* gene is desensitized in the NAc and much more weakly induced by subsequent drug exposures. This involves the binding of ΔFosB to the *c-fos* gene promoter, and recruitment of HDAC1 to reduce histone acetylation and gene activity. In concert with HDAC1, chronic drug exposure increases the levels of the repressive histone methyltransferase (KMT1a, SUV39H1)

and levels of histone H3K9 methylation on the *c-fos* promoter. Together, these enzymes and histone modifications serve to repress *c-fos* gene activity through a mechanism involving ΔFosB.

CHAPTER 2
Histone Deacetylase 5 Epigenetically Controls Behavioral Adaptations to Chronic Emotional Stimuli

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 (Nestler et al., 2002; Hyman et al., 2006), 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 which 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 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 hyper-adaptation 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 non-specific 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 2-1A). 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 2-1B). However, the mRNA levels of these HDACs, including HDAC5, were unaffected by cocaine administration (Figure 2-1C 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 2-1D). No change in HDAC5 phosphorylation was observed 24 hours after an acute or chronic cocaine injection. The phospho-specific HDAC5 band observed via Western blotting was only present, as expected, in cytoplasmic fractions (Supplemental Figure 2-S1A), 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 (Supplemental Figure 2-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 2-1E). 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 which 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*

(Supplemental Figure 2-S2). Such translocation of HDAC5 out of the nucleus is thought to block HDAC5 function, since only its nuclear form can deacetylate histones (Zhang et al., 2002; Czubryt et al., 2003). 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 2-2A). 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 which received HSV-GFP injections (Figure 2-2C). 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 MEF2 (myocyte enhancing factor-2)-interacting domain, which has been shown to be important for Class II HDAC function in non-neural cells (Lu et al., 2000). As

shown in Figure 2-2C, 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 2-2B). 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 2-2C). 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 2-2C), 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 (Supplemental Figure 2-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 one week with

higher, sensitizing doses of cocaine prior to conditioned place preference training and subsequently assessed their sensitivity to cocaine reward (Figure 2-3A). We found that prior cocaine exposure sensitizes HDAC5 knockout mice to the rewarding effects of cocaine significantly more than wildtype littermate control mice (Figure 2-3B). This increased response to chronic cocaine was not evident in HDAC9 knockout animals (Figure 2-3C).

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 wildtype 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 2-3D). Notably, expressing HDAC5 in the NAc of wildtype mice during pre-exposure to chronic cocaine did not affect cocaine reward as it did when expressed during the conditioning phase (i.e., Figure 2-2C). 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 which 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 hyper-adapt 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 wildtype 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 4 mice for each replicate.

We first compared the set of genes differentially expressed between HDAC5 knockouts treated with chronic cocaine and wildtypes treated with chronic cocaine, since this is the condition in which behavioral differences in cocaine reward were observed. This analysis identified 1,616 significantly regulated genes in the NAc (>1.2 fold, $P < 0.05$). One interesting observation from this comparison is that a vast majority of these 1,616 genes are not significantly different between cocaine naïve knockout and wildtype mice (Supplemental Figure 2-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 Supplemental Figure 2-S4B and covered in the Discussion.

To identify the most biologically relevant genes from this large gene set, we filtered the 1,616 genes for the ones which were also significantly regulated by cocaine in the knockout

(knockout cocaine vs. 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 which most closely match the behavioral phenotype. We identified a set of 172 genes that satisfied these criteria (Figure 2-4A). The enrichment of this set of 172 genes is highly 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 2-4C).

Since these 172 candidate genes were identified without regard to whether they were up- or down-regulated, 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 vs. wildtype mice (row 1) as they were by cocaine- vs. saline-treated knockout mice (row 2, Figure 2-4B). This pattern indicates that the gene expression differences observed between cocaine-exposed knockouts and wildtypes 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 vs. cocaine-naïve wildtype controls (row 3, Figure 2-4B). 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 which 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 vs. wildtype mice, while expression levels of these genes are normal in naïve 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 2-4C). In HDAC5 knockout mice, cocaine induced RapGEF6, a guanine nucleotide exchange factor that activates Rap1 and can promote ERK signaling (Lee et al., 2002; Kuiperij et al., 2003), which is a crucial pathway for cocaine reward (Lu et al., 2006). Also regulated by 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 which 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 which are known to regulate cocaine reward (Supplemental Figure 2-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 2-4C, Supplemental Figure 2-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 2-5A). We confirmed that its mRNA was elevated in cocaine-treated knockout mice (Figure 2-5B), that its promoter was hyper-acetylated under these conditions (Figure 2-5C), and that blockage of this receptor, via systemic administration of the NK1R antagonist RP-67580, attenuated cocaine reward (Figure 2-5D). 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, for the first time, 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 (Erb

et al., 1996; Ahmed and Koob, 1997; Koob and Kreek, 2007). We utilized social defeat stress, a chronic stress paradigm which 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 *Hdac*5 mRNA levels in the NAc (Figure 2-6A) without altering the phosphorylation state or subcellular distribution of HDAC5 (Supplemental Figure 2-S5). Thus, chronic stress, like chronic cocaine, reduces HDAC5 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 2-6B), 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 since HDAC5 knockout mice develop more severe social avoidance after chronic social defeat stress as compared to their wildtype littermate controls (Figure 2-6C). Defeated HDAC5 knockout mice also became anhedonic to the natural rewarding effects of sucrose, another hallmark of a depressive-like condition, while defeated wildtype mice of this background did not show this response (Figure 2-6D). In contrast, knockout and wildtype 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 (Supplemental Figure 2-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 which 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 non-neural cells suggest that phosphorylation of Ser259 (along with a cooperatively regulated site at Ser498), provide docking sites for 14-3-3 proteins, which mediate the export of HDAC5 out of the nucleus (McKinsey et al., 2000b; McKinsey et al., 2000a). The kinetics of this phosphorylation are also notable, as HDAC5 phosphorylation was observed 30 minutes after repeated cocaine administration but returned to normal by 24 hours. 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 minutes (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 non-neural 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 wildtype 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 vs. cocaine-exposed wildtype 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 wildtype mice, but are not significantly altered in the knockout in the cocaine-naïve 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 Supplemental Figure 2-S4) including dopamine receptor signaling (Nazarian et al., 2004), ATF2/CREB signaling (Carlezon et al., 2005), NF-κB (Ang et al., 2001; Russo et al., 2004), NFAT (Mermelstein, 2007), cytoskelatal 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 wildtype response to chronic cocaine, HDAC5 is phosphorylated and exported out of the nucleus to permit controlled activation of its downstream target genes (Figure 2-7). Within 24 hours, 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, naïve 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, 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 2-7). This dual action in cocaine and stress is reminiscent of the opioid peptide, dynorphin, which also regulates behavior in animal models of depression (Pliakas et al., 2001; Newton et al., 2002) 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 2-7). 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 pro-depressant 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 (Shirayama et al., 2002; Eisch et al., 2003; Carlezon et al., 2005; Berton and Nestler, 2006).

Together, our data draw fascinating parallels to observations of HDAC5 function in cardiac tissue. The hearts of naïve 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 wildtype 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 hyper-sensitivity in animal models of chronic but not acute drug addiction and stress. These findings for the first time 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 (CPP). 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 2-2, 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 minutes 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 boxes (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 (sec) on cocaine-paired side minus the time spent on the saline paired side (sec). 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 non-physical aggressive interaction to continue

for 24hrs. Social interaction with a novel mouse was measured 24 hours after the last defeat. Acute defeat consisted of a single 10min defeat session with social interaction measured 24 hours 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 hours 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 minutes 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 500bp. 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 non-specific DNA binding. The chromatin was eluted from the beads and cross-links were removed overnight at 65 degrees 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).

Microarrays and analysis

Total NAc RNA was pooled from 4 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 2-4B 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 ± s.e.m. ($^{*}P \leq 0.05$; $^{**}P < 0.01$).

SUPPLEMENTAL METHODS

Mice

All mice used in this study were adult males tested between the ages of 9 and 14 weeks. They were housed on a 12-hr light-dark cycle with access to food and water access *ad libitum*. C57BL/6 mice were obtained from Jackson Laboratory. HDAC5 and HDAC9 knockout mice were generated as described previously (Zhang et al., 2002; Chang et al., 2004). They were bred from het x het crosses and housed with their littermate controls. All procedures were in accordance with the Institutional Animal Care and Use (IACUC) guidelines.

Injections

All injections were performed once daily at the same time each day. For mRNA and protein studies, mice receiving chronic and acute cocaine were treated at the same time as their

saline controls. Mice treated with cocaine chronically, including for the microarray studies, received 7 daily cocaine injections (20 mg/kg), acutely, as 6 saline injections and 1 cocaine injection, and saline controls received 7 saline injections. Mice were then sacrificed 30 min, 1 or 24 hours later. Chronic imipramine (20 mg/kg) was administered daily for 28 days, while acute imipramine was given only once; both groups were sacrificed 24 hours after the last dose.

Intra-NAc mouse cannulations

Mice examined for conditioned place preference to cocaine (4.5 mg/kg) with SAHA continuously infused into the NAc. Mice were surgically implanted with two subcutaneous Alzet minipumps (Durect Corp., Cupertino, CA) and bilateral guide cannula (Plastics One, Roanoke, VA) targeting the NAc. The day before surgery, two cannulae (28 gauge stainless steel, 5.7 mm projection) mounted within a single pedestal assembly were filled with SAHA (100 μM) or 5% Hydroxypropyl Beta Cyclodextrin vehicle (CTD, Inc., High Springs, FL) and affixed to a small mini-pump that was also filled with either SAHA or vehicle, respectively. For the NK1R experiment, minipumps were filled with 1 μM RP 67580 (in 0.1% DMSO) and CPP was performed at 10mg/kg cocaine. Each mini-pump was activated 12 hrs before implantation via incubation at 40°C. Activation of each mini-pump initiated the continuous delivery of 0.25 μl/hr for the next 14 days. Each mini-pump was positioned just under the skin on the mouse's back. Cannulae placements were accomplished by drilling two small holes above the area of the NAc and by their simultaneous delivery at the coordinates: AP + 1.25 mm; ML +1.0 mm; DV – 4.5 mm (from dura). The pedestal assembly was cemented in place with Loctite skull adhesive (Henkel, Rocky Hill, CT). Mice were allowed four days of recovery from surgery before the start of place conditioning.

Slice pharmacology

Acute NAc punches were prepared and subjected to pharmacological manipulation as described previously (Sahin et al., 2006; Nguyen et al., 2007). Briefly, male C57BL/6 mice were killed by decapitation, and their brains were rapidly removed and placed in cold, oxygenated Krebs buffer (124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.5 mM $CaCl_2$, 1.5 mM $MgSO_4$ and 1.25 mM KH_2PO_4, pH 7.4). Bilateral 15 gauge punches of NAc were transferred to net wells (Costar) containing 3 ml of either normal Krebs buffer or a depolarizing Krebs solution (109.25 mM NaCl, 18.75 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 3 mM $CaCl_2$ and 1.25 mM KH_2PO_4, pH 7.4) depending on experimental conditions. Punches were allowed to recover in their respective buffers at 30°C under continual oxygenation (95% O_2/5% CO_2) for 60 minutes. Punches were subsequently incubated for 60 minutes in either normal Krebs buffer, depolarizing Krebs buffer, or a depolarizing Krebs buffer containing 50 μM of the KN-93 (Sigma), which inhibits CaMKII and certain other CaMKs. Punches were then transferred to microfuge tubes, frozen on dry ice, and stored at -80 °C until further analysis by Western blotting.

Nuclear extraction

NAc was dissected and subjected to nuclear extraction as previously described with some modifications (Ogita et al., 2002). Briefly, tissues were homogenized in 200 μl of homogenizing buffer containing 10 mM Tris-HCl buffer, pH 7.5, 0.32 M sucrose, 5 mM dithiothreitol (DTT), and ~10 mM of both protease inhibitors (Roche, complete Mini, EDTA-free) and phosphatase inhibitors (Sigma phosphatase inhibitor cocktail 1 and 2). Tissues were homogenized using a

Dounce homogenizer, followed by centrifugation at 3700 rpm for 10 minutes at 4°C. Pellets (P1 fractions) and supernatants (S1 fractions) were used to prepare the nuclear and cytosolic fractions respectively. S1 fractions were subjected to centrifugation at 7500 rpm for 7 minutes at 4°C, and supernatants were collected as cytosolic fractions. P1 fractions further suspended in 200 µl of homogenizing buffer containing 10 mM Tris-HCl buffer, pH 7.5, 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol (DTT), and ~10mM of both protease and phosphatase inhibitors, followed by a 10 minute incubation on ice, and centrifugation at 3700 rpm for 10 minutes at 4°C. Pellets were then suspended in 100 µl of homogenizing buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 400 mM NaCl, 5 mM DTT, 0.5% Nonidet P-40, and the aforementioned protease and phosphatase inhibitors. Suspensions were kept on ice for 30 minutes and then centrifuged at 15000 rpm for 5 minutes at 4°C. Supernatants were collected as nuclear fractions. Lamin was used as a positive control for exclusively nuclear staining and β-tubulin was used as a positive control for exclusively cytoplasmic staining.

Behavior

Conditioned place preference (CPP). For the NK1R experiment, 1.5 mg/kg RP-67580 (in 1% DMSO) or vehicle was injected i.p. 15 minutes before each conditioning session. Details on the intra-NAc delivery of SAHA are discussed above. The accelerated paradigm used in Figure 2-2 was designed to conduct all the conditioning during the peak of viral expression (days 3-4 post-op) (Barrot et al., 2002). Therefore, two days after sterotactic surgery in which an HSV vector was delivered bilaterally into the NAc, mice received two pairings per day over two consecutive days. The morning pairing was saline and the afternoon pairing was 5 mg/kg of cocaine. For the group receiving Trichostatin A (4 mg/kg mg/kg i.p. in 10% DMSO) or vehicle, mice were

treated during both AM and PM pairings 30 minutes prior to each conditioning phase. The mice were then tested for their conditioned preference the subsequent day.

Sensitization-CPP. Mice were given 15 mg/kg cocaine injections daily in their home cages for 7 days. Three days after the last cocaine injection, mice were pre-tested in CPP boxes for any pre-existing bias. A standard CPP paradigm was then followed as described above at 2.5 mg/kg for the HDAC5 knockout mice and 1.25 mg/kg for HDAC9 knockout mice. These doses were chosen as a threshold dose from the dose-response run in naïve mice. For the HDAC5 rescue experiment, a slightly accelerated protocol was developed to accommodate the brief overexpression of the HSV. The next day after stereotactic surgery during which HSV-GFP or HSV-HDAC5 was delivered, daily cocaine was given at 20 mg/kg for a total of 5 days to allow for viral overexpression during the cocaine treatment. This accelerated paradigm resulted in slightly higher baseline CPP scores. Three days later the mice were pre-tested and underwent CPP (2.5 mg/kg) as described above.

Elevated plus maze. Mice were placed in the center of an elevated plus maze in low lighting and their movements were recorded by a videotracking system (Ethovision 3.0, Noldus, Leesburg, Virginia). The arms were 33 cm x 5 cm, with 25 cm tall walls on the closed arms. The total time spent in open and closed arms was recorded over five minutes.

Open field. Using a video tracking system (Ethovision 3.0, Noldus, Leesburg, Virginia), the activity and total time spent within the center or periphery of an open field box (72cm diameter), was measured for each mouse over five minutes.

Sucrose Preference. Singly housed controls and socially defeated mice were given two bottles, one containing water and the other 2% sucrose. Over 6 days, the bottles were alternated every 24 hours to avoid bottle or location bias. The first two days both bottles contained water to

habituate the mice to using two bottles. The third and fourth days, both bottles contained 2% sucrose, in order to expose mice to the reward prior to the test. On the last two days, the mice could choose between a water bottle and a sucrose bottle; the data from the final day was used as a measure of sucrose preference (Barrot et al., 2002).

Forced swim test. The forced swim test was performed similarly as originally described (Porsolt et al., 1977). Mice were placed into a 4 L glass beaker filled with 3 L of room temperature water. A video camera recorded behavior over 6 min. An observer blind to genotype scored immobility during the last 4 min of the test.

CHAPTER TWO FIGURES

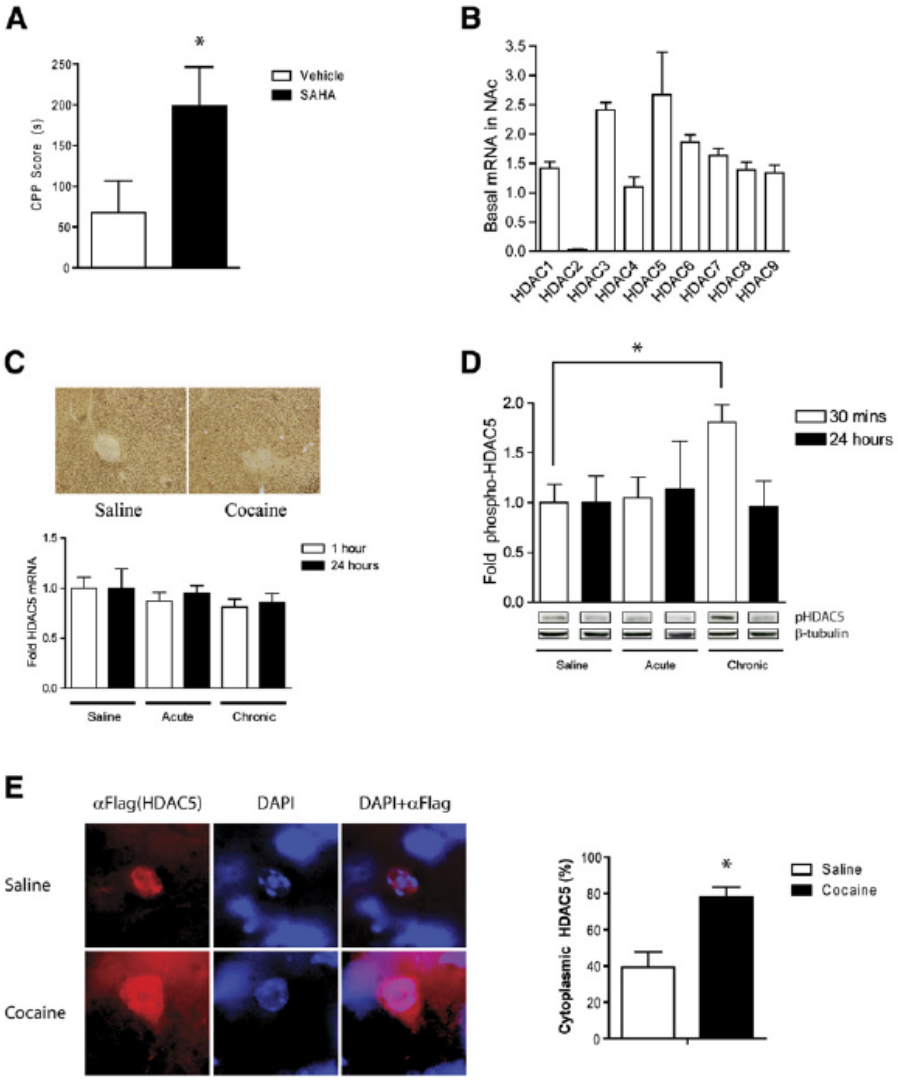


Figure 2-1: Cocaine induces HDAC5 phosphorylation and nuclear export in the nucleus accumbens.

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 8 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 hours 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 minutes 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 hours [$F(2,19) = 0.27$, $P > 0.05$, $n = 4$].

E. Representative image of chronic cocaine shuttling HDAC5 out of the nucleus 30 minutes 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, 3 mice per group, Student's t-test).

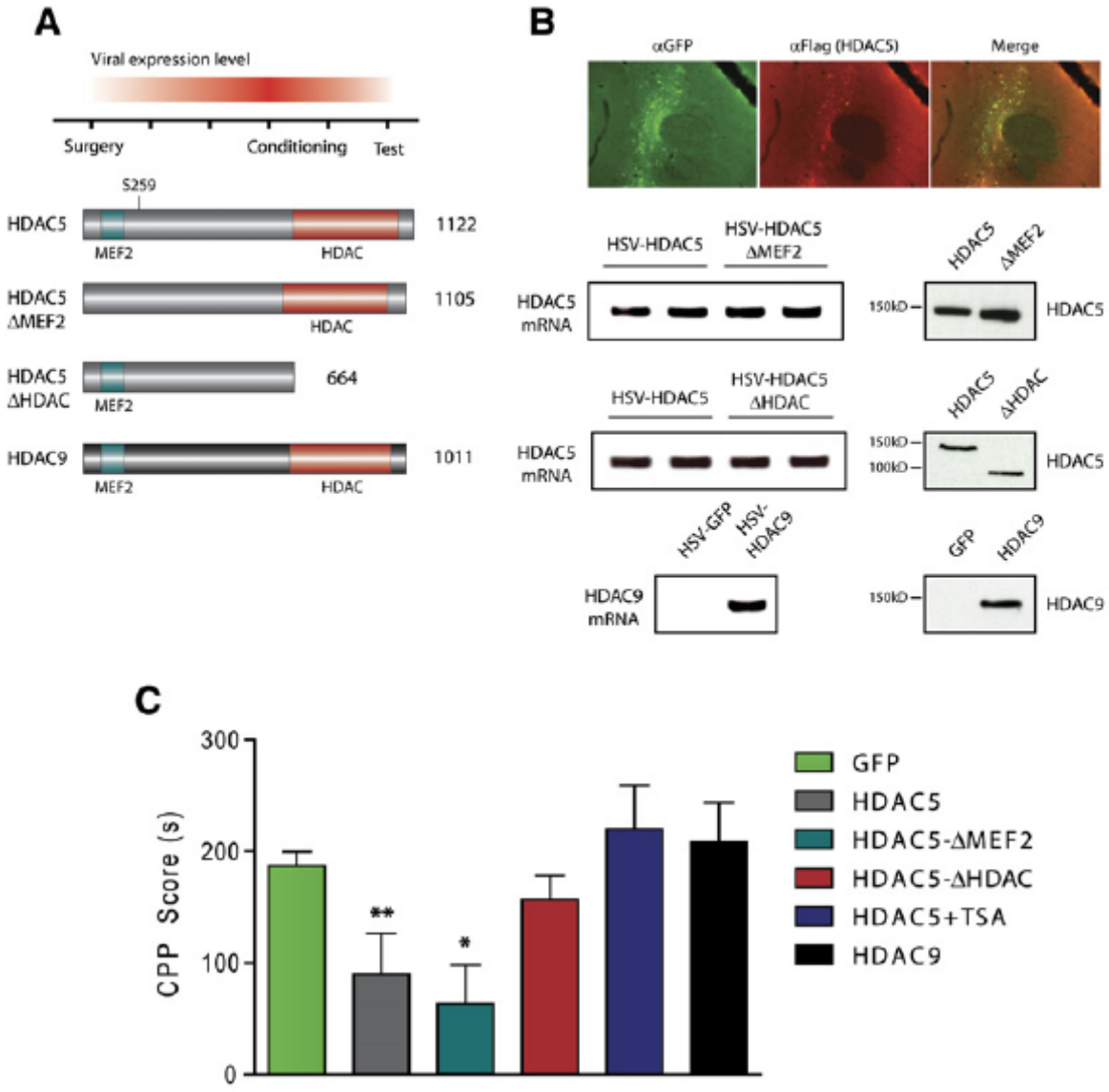


Figure 2-2: Viral-mediated expression of HDAC5 regulates cocaine reward.

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 co-expressed in all neurons infected with HDAC5 (top). Expression levels of human HDAC5 mRNA from wildtype 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 wildtype 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 wildtype (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].

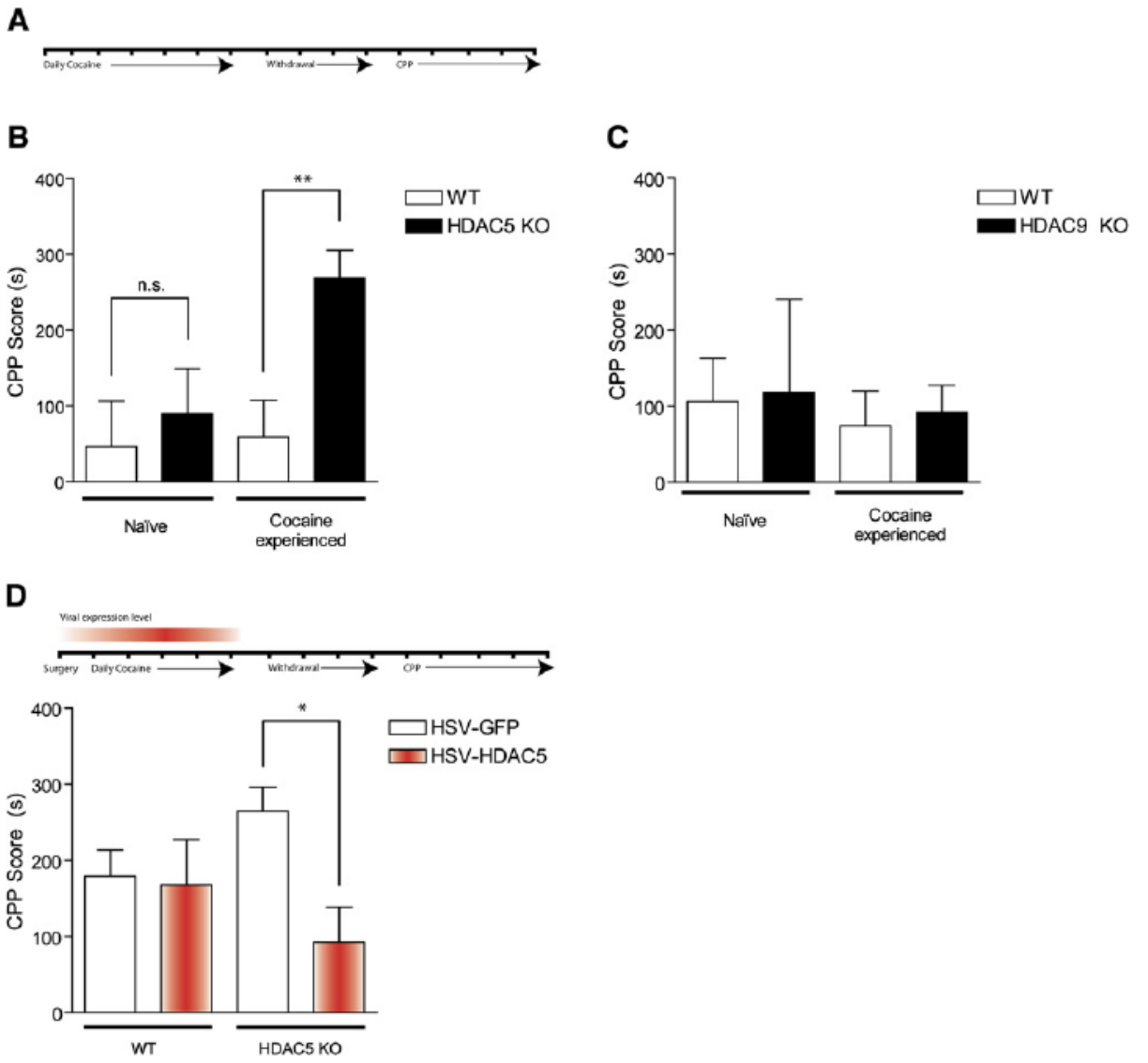


Figure 2-3: Mice lacking HDAC5 differentially adapt to chronic cocaine exposure.

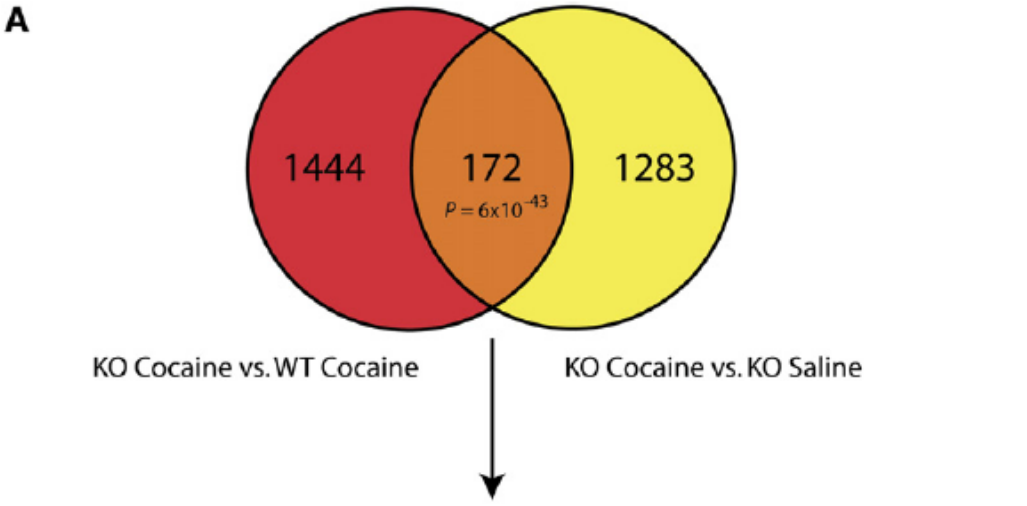
A. Behavioral paradigm to assess the effect of prior cocaine exposure on subsequent cocaine reward in HDAC5 knockout (KO) and wildtype (WT) mice. Each tick mark represents one day.

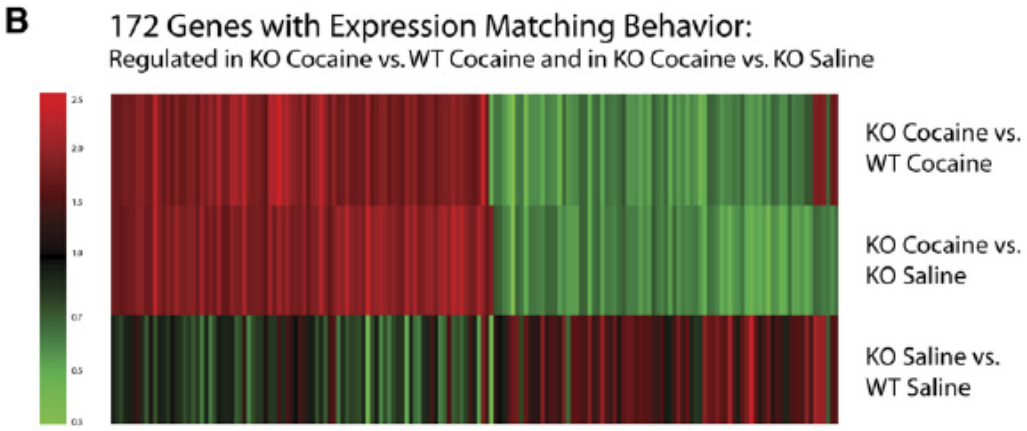
B. HDAC5 KO mice and littermate controls were given daily cocaine injections for 7 days, and then tested for cocaine CPP after 4 days of withdrawal. While cocaine-naïve HDAC5 KO mice do not display a greater preference for cocaine than their littermate controls, after prior exposure to cocaine, HDAC5 KO develop a significant hyper-sensitization 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: naïve WT vs. naïve HDAC5 knockout, $P > 0.05$, n = 13, cocaine-experienced WT vs. cocaine-experienced HDAC5 knockout, $^{**}P < 0.01$, $n = 18$).

C. Neither cocaine-naïve 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 naïve HDAC5 KO mice and their littermate controls rescues the hyper-sensitization 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 vs. HSV-HDAC5 in HDAC5 KO mice $^{*}P < 0.05$, $n = 7 – 9$) .





C Examples of Genes Regulated by Cocaine in KO vs. WT

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, subfamily 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 2-4: 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 wildtype (WT) littermate controls that received either chronic cocaine or saline and were

sacrificed 24 hours 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 vs. cocaine-treated WT mice (left) and cocaine treated HDAC5 KO vs. saline-treated HDAC5 KO (right). There were 172 genes which 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 vs. cocaine-treated WT (row 1), cocaine-treated HDAC5 KO vs. saline-treated HDAC5 KO (row 2), and saline-treated HDAC5 KO vs. 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 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.

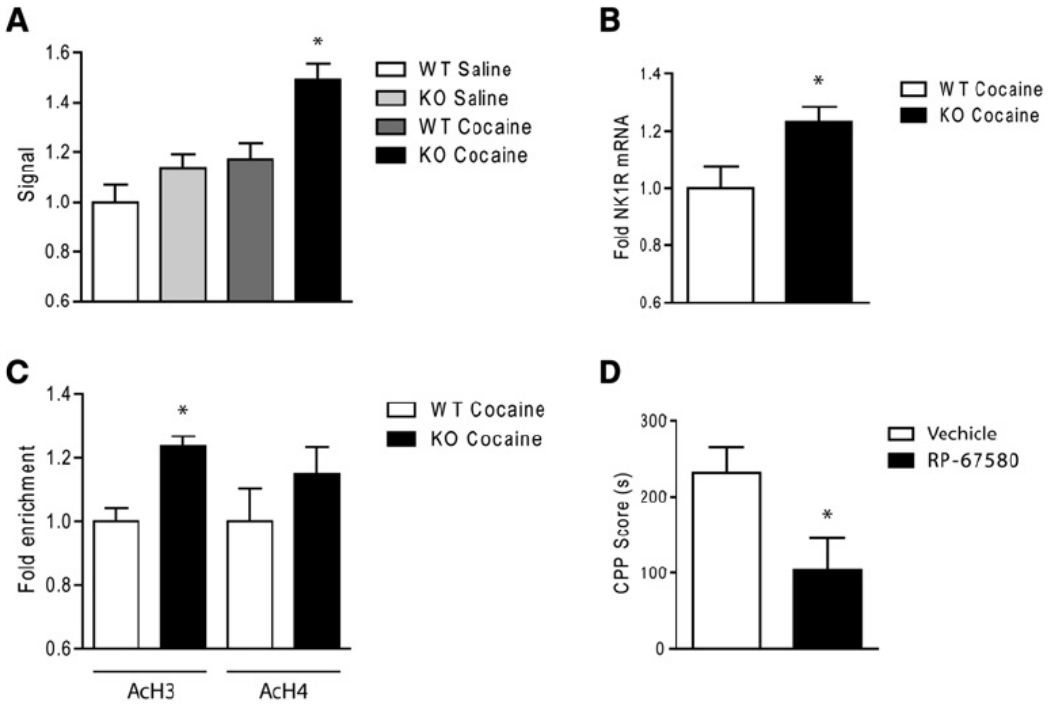


Figure 2-5: Microarray target, NK1 receptor (NK1R), regulates cocaine reward.

A. Microarray expression data of NK1R normalized to wildtype (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) vs. mice treated with vehicle ($P < 0.05$, $n = 8$).

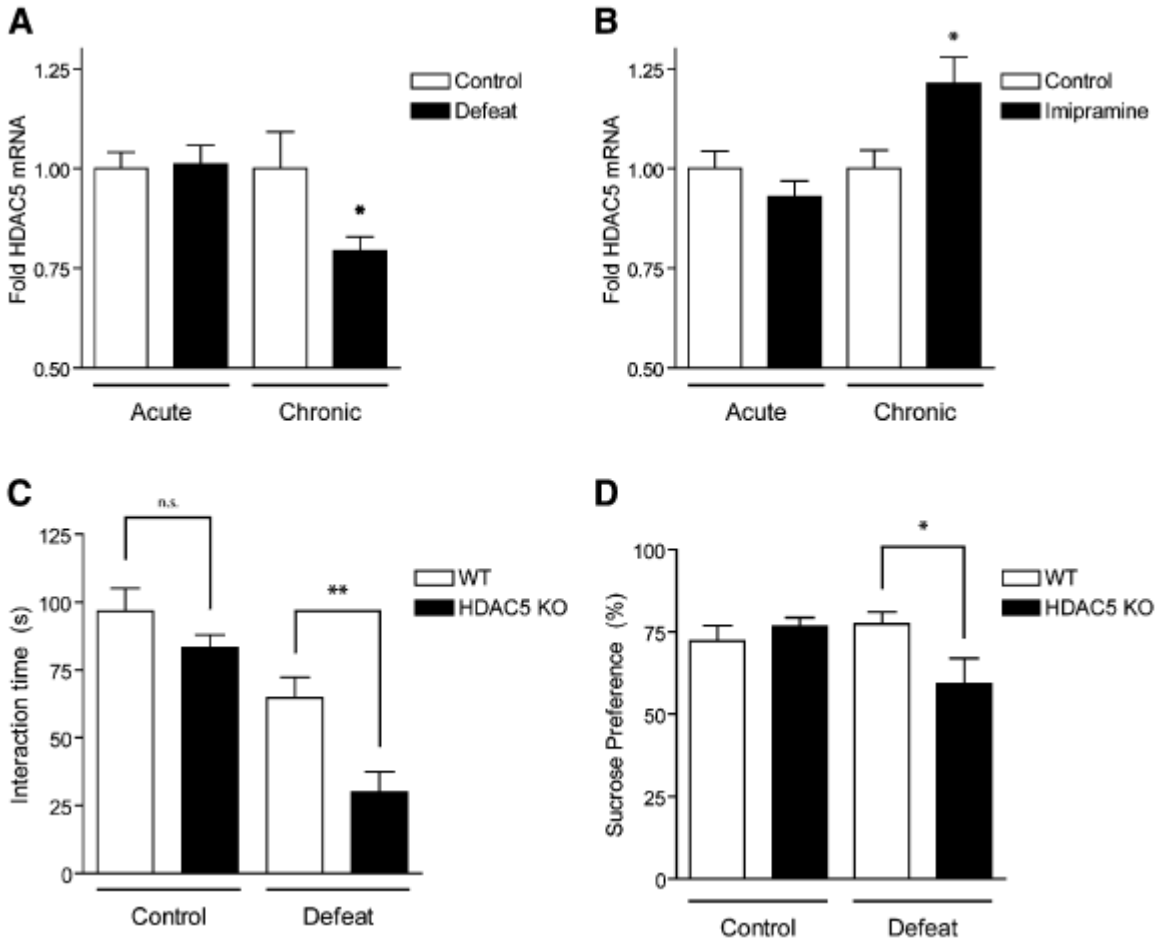


Figure 2-6: HDAC5 regulates adaptation to chronic stress.

A. Mice underwent acute (1 day) or chronic (10 days) of social defeat stress and were sacrificed 24 hours 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 4 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 hours after the last defeat. While naïve, unstressed HDAC5 knockout (KO) and wildtype (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 vs. control KO, $P > 0.05$, defeated WT vs. defeated KO, $^{**}P < 0.01$, n = 9-11].

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

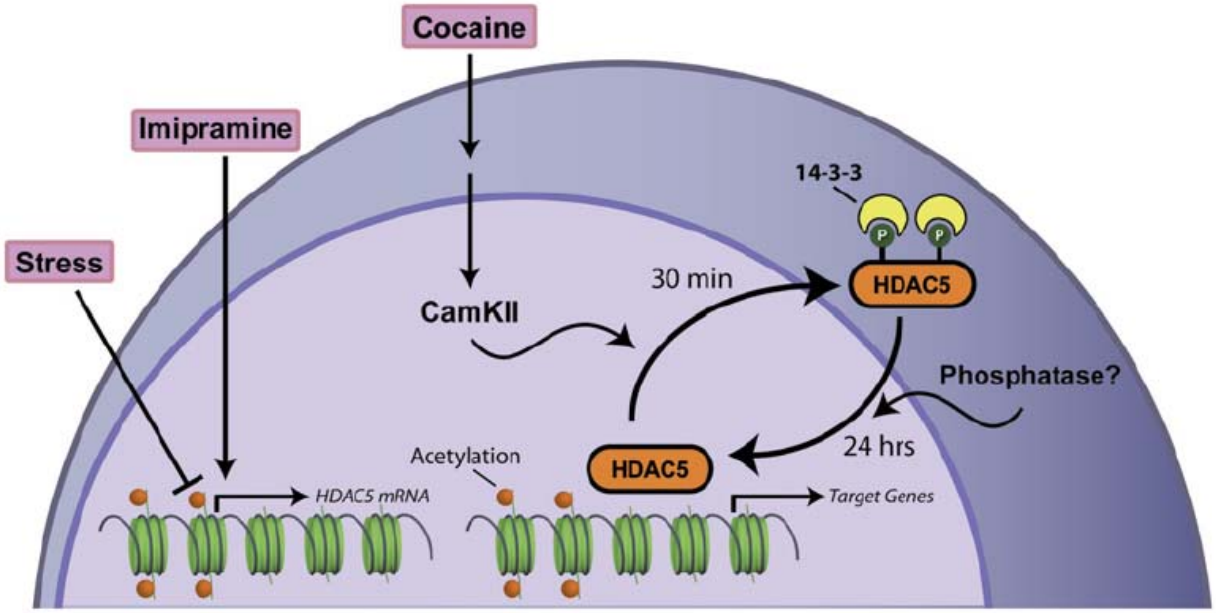
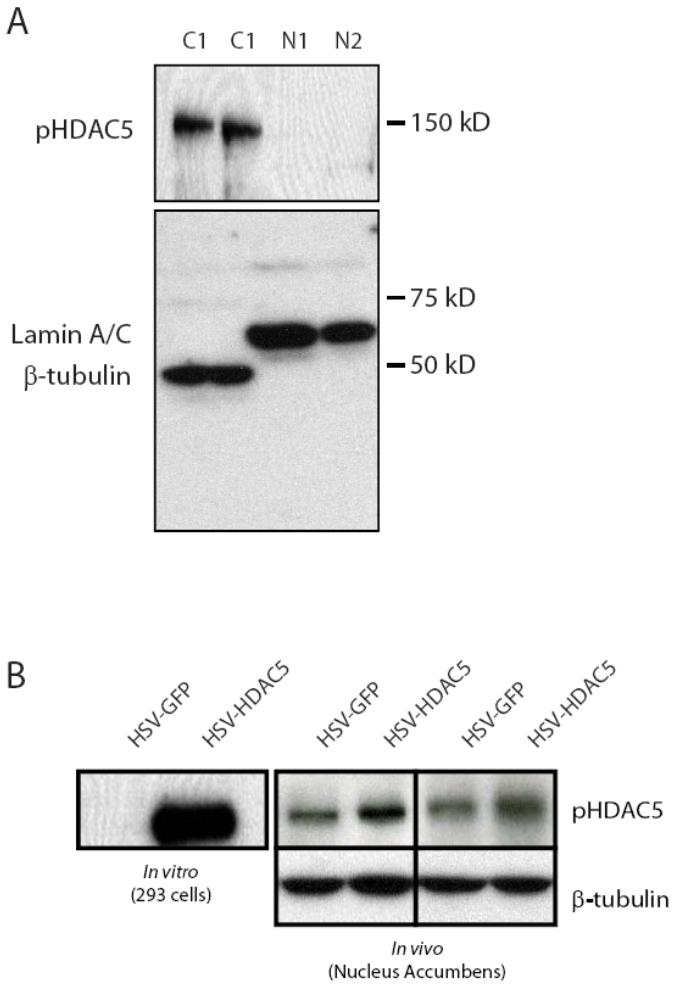


Figure 2-7: Model of HDAC5 action in the NAc in cocaine and stress responses.

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 hours 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.

CHAPTER TWO SUPPLEMENTAL FIGURES



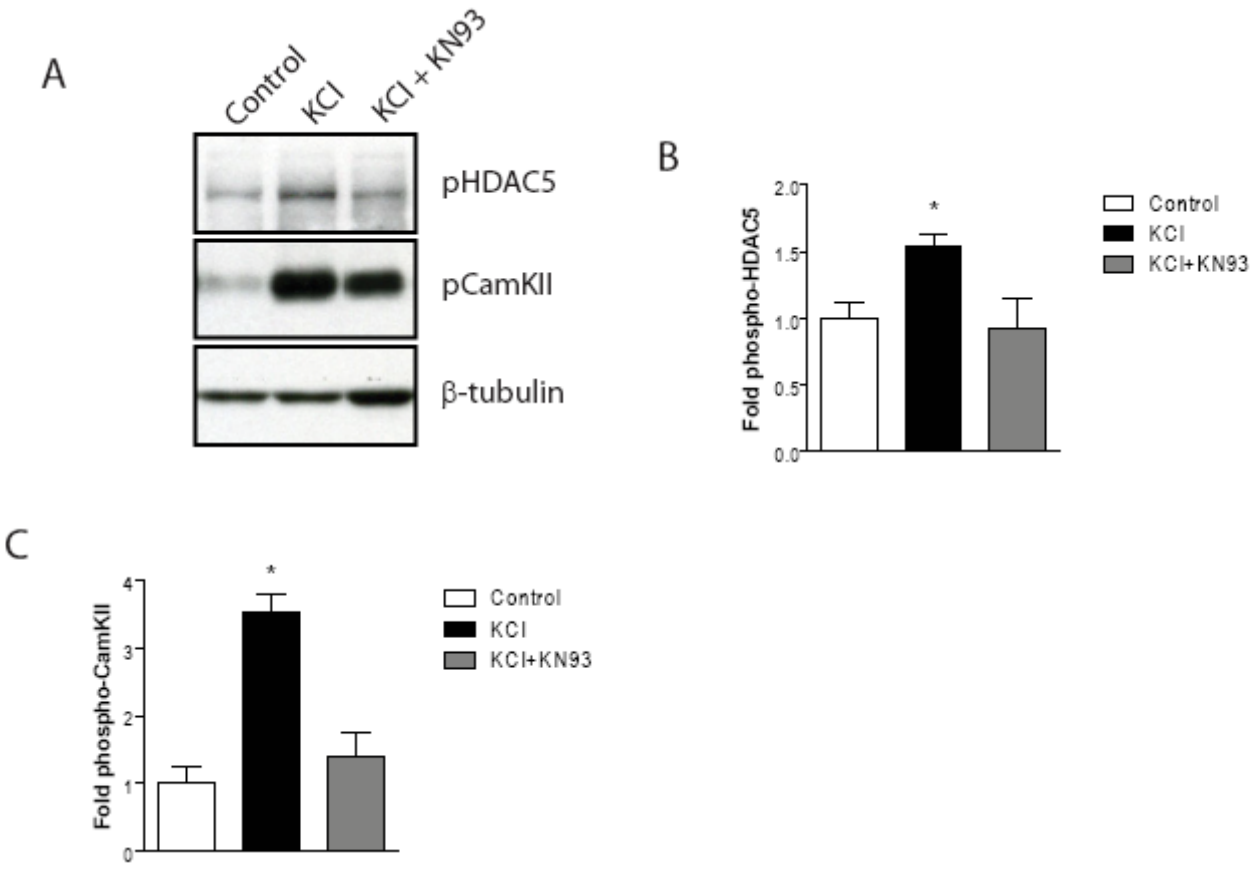
Supplemental Figure 2-S1: Characterization of HDAC5 phosphorylation in the NAc.

A. Nuclear extractions demonstrate that phosphorylated HDAC5 is only present in the cytoplasmic fraction since no immunoreactivity is observed in the nuclear fractions. As positive controls for our subcellular fractions, we used lamin, which is only expressed in the nucleus, and β-tubulin, which is mainly expressed in the cytoplasm.

B. Virally overexpressed HDAC5 can be phosphorylated *in vitro* and *in vivo*. *Left*: In 293 cells infected with HSV-HDAC5, a dramatic increase can be observed in levels of phospho-HDAC5. No such effect is seen in cells infected with HSV-GFP. *Right:* Mice received intra-NAc

injections of HSV-HDAC5 or HSV-GFP and were then treated with chronic cocaine. Thirty minutes after the last cocaine dose, mice overexpressing HDAC5 have higher levels of the phosphorylated protein.

Blots shown in the figure are representative of at least 3 replicates.

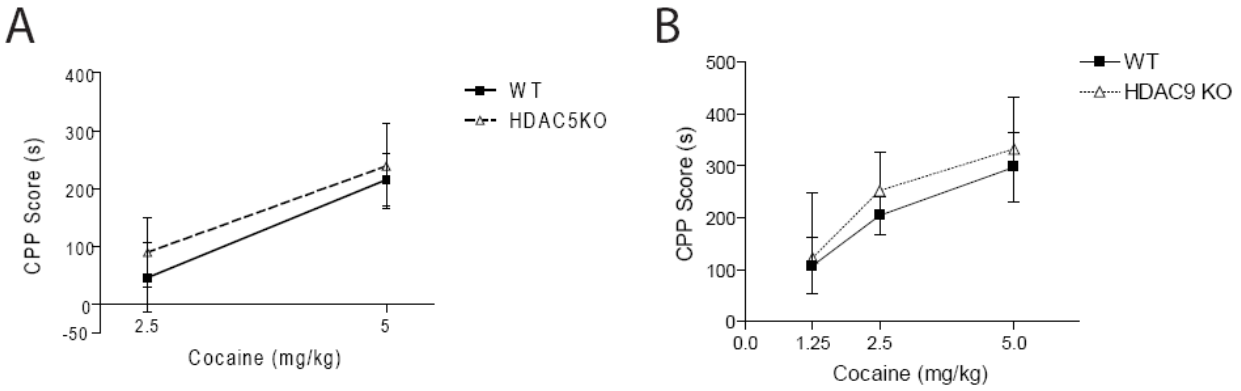


Supplemental Figure 2-S2: CaMKII is necessary for depolarization-induced HDAC5 phosphorylation in the NAc.

A. Representative Western blot depicting freshly dissected NAc punches incubated in either Krebs buffer, Krebs + KCl 18.75 mM, or Krebs + KCl + KN-93 (50 μM). See Supplemental Methods for composition of Krebs buffer. HDAC5 phosphorylation is induced by KCl and inhibited by the CaMKII inhibitor, KN-93. The autophosphorylation site of CaMKII is also induced by KCl and inhibited by KN-93. The loading control, β-tubulin, demonstrates equal loading protein levels in each lane.

B. Quantification of the phospho-HDAC5 signals shows a significant increase in phosphorylated HDAC5 by KCl-induced depolarization, which is completely blocked by the CaMKII inhibitor, KN-93 (*$P < 0.05$, $n = 5$).

C. As a control for CaMKII activity, we blotted for the autophosphorylation site (Thr 286) of CaMKII. Quantification of CaMKII phosphorylation showed a significant increase after KCl that is significantly attenuated by the CaMKII inhibitor, KN-93 (*$P < 0.05$, $n = 5$).

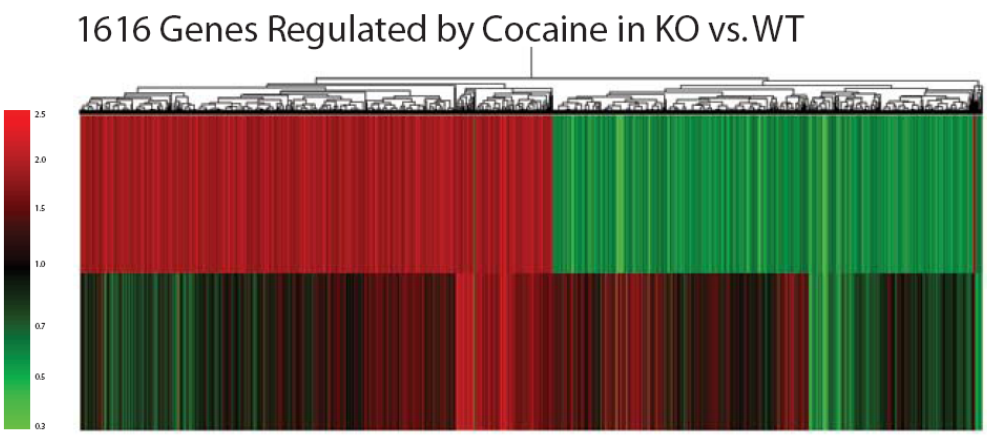


Supplemental Figure 2-S3: Cocaine place preference in drug naïve HDAC5 knockout mice.

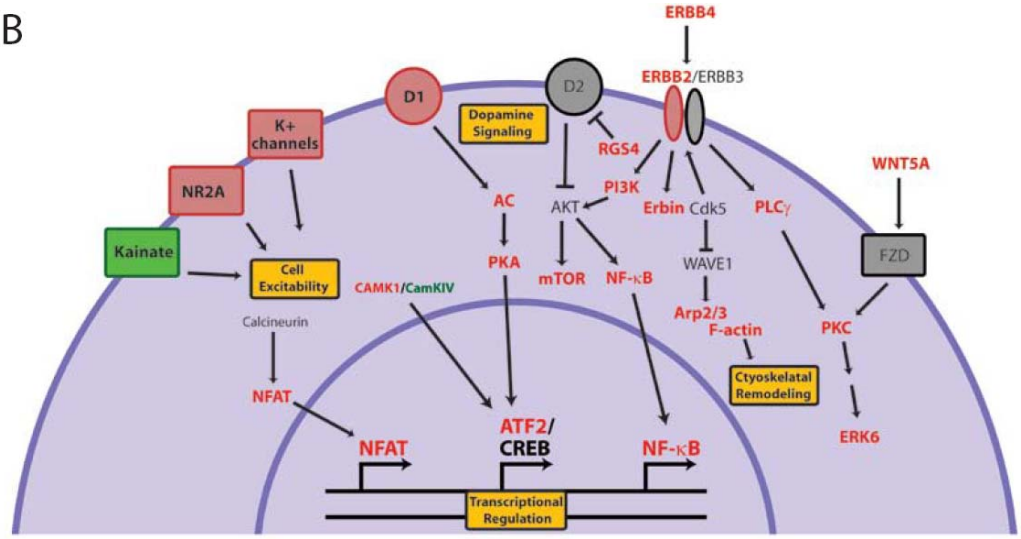
A. Conditioned place preference to cocaine was performed at 2.5 and 5 mg/kg doses of the drug in HDAC5 knockout (KO) mice and their wildtype littermate controls. No significant differences were observed between genotype ($P > 0.05$, n = 15-17).

B. Conditioned place preference to cocaine was performed at 1.25, 2.5, and 5 mg/kg doses of the drug in HDAC9 KO mice. No significant differences were observed between genotype ($P > 0.05$, $n = 5 – 11$).

A



B



C

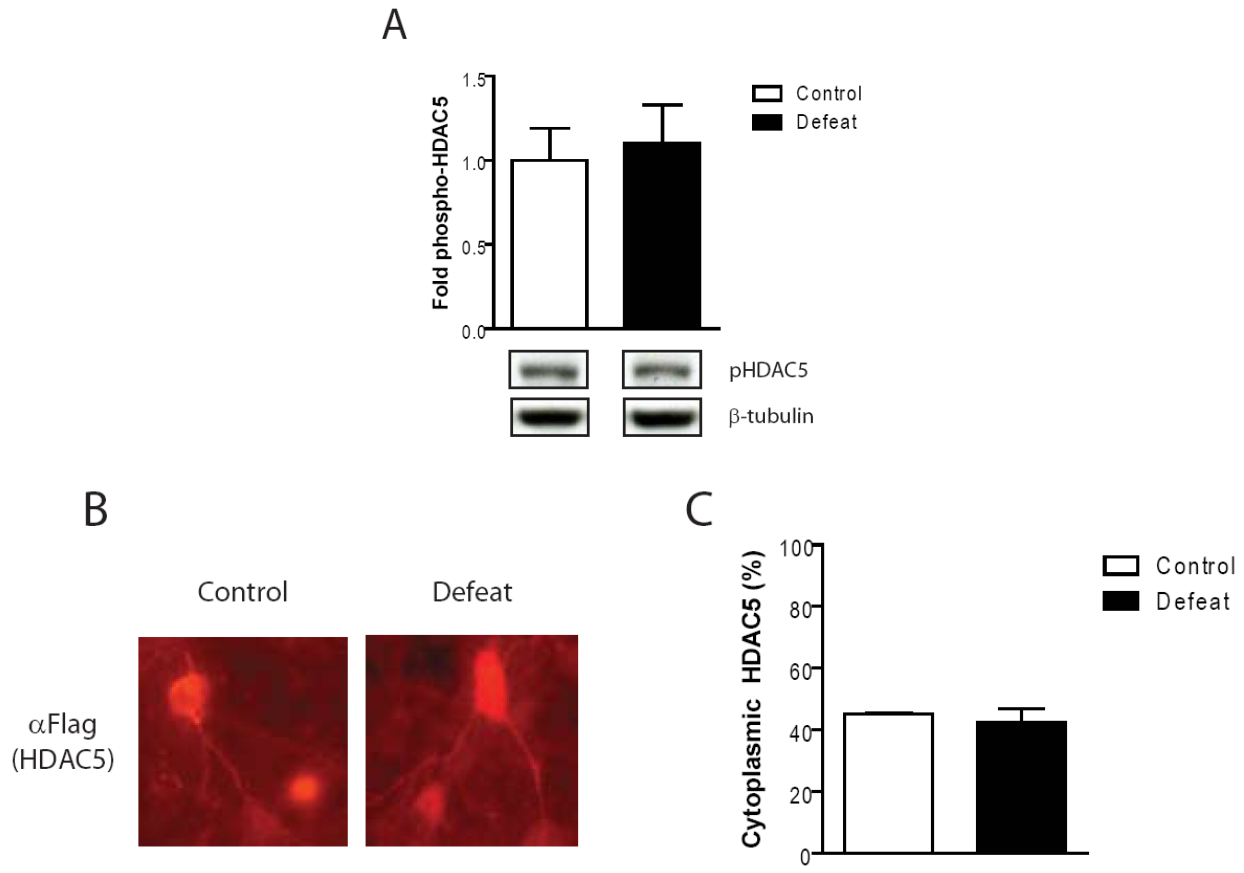
	WT Cocaine		KO Cocaine		
	Average	StError	Average	StError	P-value
Gnb4	1.00	0.14	1.45	0.17	<0.05
Erbb2ip	1.00	0.18	2.03	0.19	<0.01
Grin2A	1.00	0.02	1.32	0.03	<0.0001
Kcnk4	1.00	0.03	1.50	0.07	<0.001
Kcnq5	1.00	0.06	1.23	0.07	<0.05
NK1R	1.00	0.04	1.24	0.03	<0.01
ADAM1	1.00	0.07	1.32	0.11	<0.05
Prm1	1.00	0.01	1.34	0.11	<0.05
RapGEF6	1.00	0.19	1.43	0.19	<0.05
SUV39H1	1.00	0.09	1.46	0.06	<0.01
RGS20	1.00	0.28	1.21	0.18	>0.05
Cuedc1	1.00	0.18	1.58	0.23	<0.05
Abca5	1.00	0.18	1.37	0.10	<0.05

Supplemental Figure 2-S4: Microarray analysis of cocaine-induced gene expression in the NAc of HDAC5 knockout (KO) mice.

A. Heatmap of all significantly regulated genes (1.2 fold, $P < 0.05$) between cocaine-treated KO vs. wildtype (WT) mice. The vast majority of these genes is not altered in cocaine-naïve KO vs. WT mice, and hence mirror the cocaine reward phenotype exhibited by the mutants.

B. Ingenuity analysis of the significantly regulated genes between cocaine-treated KO and WT mice reveals several molecular pathways known to regulate neural plasticity and, in many cases, cocaine reward. See text for discussion. Red indicates an upregulation in the knockouts, green indicates a downregulation in the knockouts, and black indicates no change.

C. Table of acetylated histone H3 (AcH3) ChIP data from cocaine-treated knockout and cocaine-treated wildtype mice. For each promoter studied, data were normalized to the wildtype. Average AcH3 enrichment, standard error of the mean, and the Student's t-test *P*-value are shown for each gene.

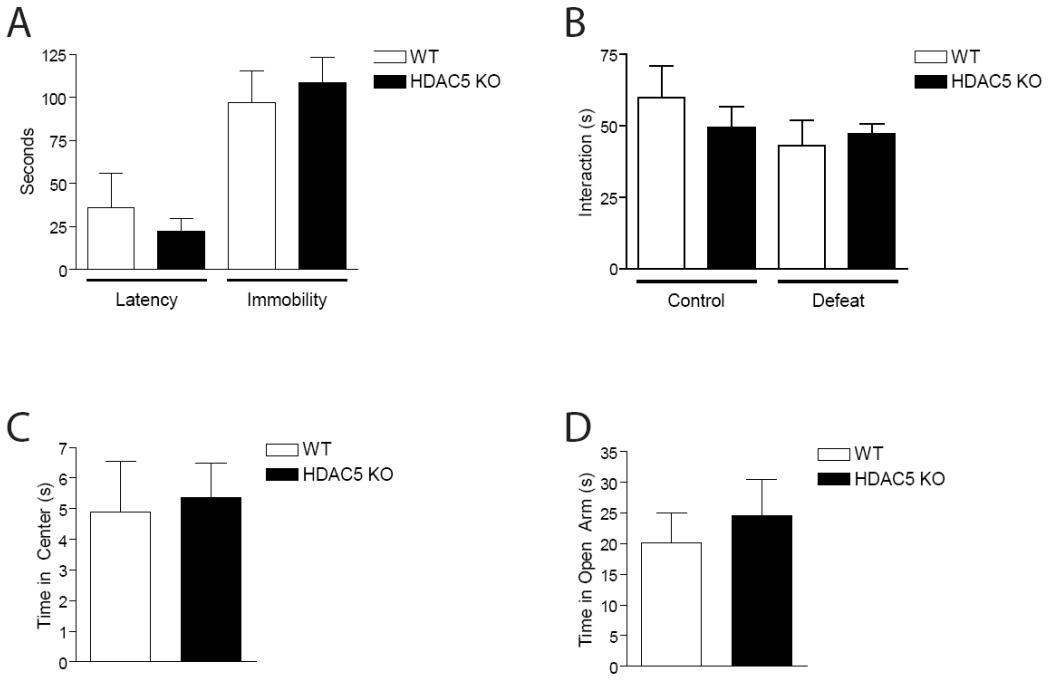


Supplemental Figure 2-S5: Chronic social defeat does not regulate the phosphorylation or nuclear localization of HDAC5.

A. Chronic (10 days) social defeat does not regulate HDAC5 phosphorylation ($P > 0.05$, $n = 6$).

B. Representative staining of Flag-tagged HSV-HDAC5 infected neurons in socially defeated mice.

C. Quantification of cells containing cytoplasmic HDAC5 shows no difference between defeated mice and control mice ($P > 0.05$, $n = 4$ mice/group).



Supplemental Figure 2-S6: Normal responses of HDAC5 KO mice in acute stress and anxiety tests.

A. Forced swim test shows no significant differences between HDAC5 knockout (KO) mice and their littermate wildtype (WT) controls in either the latency to immobility or the total time spent immobile ($P > 0.05$, $n = 12$).

B. Acute social defeat stress does not significantly reduce social interaction with a novel target in either the WT or HDAC5 KO mice. Moreover, there is no significant difference in the response to acute defeat between genotype, as was seen with chronic social defeat stress ($P > 0.05$, $n = 5 – 9$).

C. Open field test reveals that both WT and HDAC5 KO mice spent similar amounts of time in the center of the arena ($P > 0.05$, $n = 11$).

D. Elevated plus maze shows that both WT and HDAC5 KO mice spent similar time in the open arm of the maze ($P > 0.05$, $n = 11$).

CHAPTER THREE
Genome Wide Analysis of Epigenetic Regulation by Cocaine

SUMMARY

Changes in gene expression contribute to the long-lasting regulation of the brain's reward circuitry seen in drug addiction, however, the specific genes regulated and the transcriptional mechanisms underlying such regulation remain poorly understood. Here, we used chromatin immunoprecipitation coupled with promoter microarray analysis to characterize genome-wide epigenetic changes in the mouse nucleus accumbens, a crucial brain reward region, after repeated cocaine administration. Our findings reveal several interesting principles of gene regulation by cocaine and of the role of ΔFosB and CREB, two prominent cocaine-induced transcription factors, in this brain region. The findings also provide novel and comprehensive insight into the molecular pathways – including a new role for sirtuins (*Sirt1* and *Sirt2*) – that are altered by cocaine in the nucleus accumbens.

INTRODUCTION

Repeated use of addictive drugs such as cocaine causes long-lasting changes in the brain's reward circuitry, a key component of which is the nucleus accumbens (NAc). Accordingly, a major goal in the field has been to uncover the molecular mechanisms underlying addiction-associated neuroadaptations in this brain region. It has been hypothesized that one such mechanism of drug-induced neuroadaptations is the regulation of gene expression (Hyman et al., 2006), and there have been numerous studies that have documented altered expression of genes, through candidate gene approaches or through DNA expression arrays, in the NAc (Freeman et al., 2001; McClung and Nestler, 2003; Yao et al., 2004). As well, several transcription factors have been shown to be altered in the NAc after chronic cocaine exposure, including ΔFosB (a Fos family protein) (Hiroi et al., 1997; McClung et al., 2004), CREB (cAMP response element binding protein) (Carlezon et al., 2005) , NFκB (nuclear factor κB) (Ang et al., 2001), NAC1 (nucleus accumbens 1) (Mackler et al., 2000), Egr1-3, (early growth response factors 1-3) (O'Donovan et al., 1999), and GR (glucocorticoid receptor) (Deroche-Gamonet et al., 2003), and in several instances this regulation has been related directly to the behavioral abnormalities that characterize an addicted state. Among these transcription factors, ΔFosB and CREB are of particular interest. ΔFosB is induced uniquely by chronic cocaine exposure and persists for several weeks after cessation of the drug due to the unusual stability of the protein (McClung et al., 2004). Moreover, increasing evidence supports the view that induction of ΔFosB increases rewarding responses to, and incentive motivation for, cocaine (Hiroi et al., 1997; Kelz et al., 1999; Colby et al., 2003; McClung and Nestler, 2003). In contrast, CREB activity is induced in response to acute or chronic cocaine administration and serves a homeostatic role by reducing sensitivity to drug reward and mediating negative emotional symptoms during drug withdrawal

(Carlezon et al., 2005; Choi et al., 2006).

However, all investigations of cocaine-induced changes in gene expression to date have focused by necessity on measures of mRNA levels, and alterations in the steady levels of an mRNA may not reflect transcriptional regulation of the encoding gene. Recent advances in chromatin biology have made it possible for the first time to extend this level of knowledge to direct examination of transcriptional mechanisms. Thus, we now know, largely from studies of non-neural tissue, that the state of activation or repression of a gene is reflected in the covalent modifications of histone proteins in the gene's vicinity (Felsenfeld and Groudine, 2003; Hake et al., 2004). Increased acetylation of histone H3 or H4 is highly predictive of gene activation, while increased methylation of H3 at K9 or K27 (Lys9 or 27) is predictive of gene repression. Chromatin immunoprecipitation (ChIP), where tissue is lightly fixed to crosslink DNA with histones and other DNA-binding proteins and then immunoprecipitated for a protein of interest (e.g., acetylated H3), can be used to assess the extent to which a given gene is associated with these markers of activation or repression. For example, we recently demonstrated by use of ChIP that chronic administration of cocaine induces histone acetylation, in the NAc, at the promoters of several genes whose mRNA levels are known to be induced under these conditions (Kumar et al., 2005; Renthal et al., 2007), and we directly implicated ΔFosB in mediating some of these effects by carrying out ChIP for this transcription factor (Kumar et al., 2005).

ChIP represents a powerful advance in establishing regulation of gene transcription by cocaine, but traditional methods are still limited by the analysis of individual genes of interest one at a time. Moreover, increasing evidence indicates that regulation of a gene is mediated by many types of coincident alterations of histones and related modifications (Kouzarides, 2007). These considerations argue for the importance of analyzing the immunoprecipitated DNA, not

for an individual gene, but genome-wide using ChIP on chip assays, to gain a global view of genes that show markers of activation or repression after cocaine. In the present study, we mapped the genomic effects of chronic cocaine in the NAc by performing ChIP on chip for acetylated and methylated histones and for ΔFosB and phospho-CREB (the activated form of this transcription factor). The findings provide fundamentally novel insight into cocaine's regulation of gene expression in this brain reward region.

RESULTS

Cocaine regulation of histone acetylation and methylation in the NAc

In order to extend our previous ChIP study (Kumar et al., 2005), which examined the effect of repeated cocaine administration on histone acetylation at particular gene promoters of interest in the NAc, we mapped the entire NAc genome from mice treated chronically with cocaine (20 mg/kg/day for 7 days, *i.p.*) or saline by performing ChIP with antibodies directed against polyacetylated H3 (K9 and K14), polyacetylated H4 (K5, K8, K12, and K16), or dimethylated H3 (K9 and K27). ChIP's were performed on independent NAc samples, with each sample pooled from ~8 mice. These histone modifications have been widely shown to reflect the state of gene activation or repression (Felsenfeld and Groudine, 2003; Kouzarides, 2007). The immunoprecipitated DNA, as well as input (total) DNA, was sheared and then repaired, amplified, and labeled with a fluorescent dye with the use of ligation-mediated PCR (Sikder et al., 2006). Cy5-labeled immunoprecipitated DNA and Cy3-labeled total DNA were mixed and hybridized to MM8 NimbleGen promoter microarrays (Madison, WI) featuring over 50,000 unique curated entries for ~20,000 genes. Highly rigorous statistical analyses of these promoter

chips (3.1 standard deviation cutoff corresponding to $p<0.001$; see Supplemental Methods in Supplementary Information) provided a unique view of cocaine-regulated gene targets and the regulatory mechanisms in this critical reward region of the brain.

Repeated cocaine administration induced hyperacetylation of histone H3 at 994 gene promoters in NAc and hypoacetylation at 82 gene promoters compared to saline-treated controls (Figure 3-1A). Similarly, repeated cocaine caused hyperacetylation of histone H4 at 685 gene promoters and hyopacetylation at 122. It is interesting to note that several fold more genes showed H3 or H4 hyperacetylation after chronic cocaine compared to hypoacetylation. This is consistent with our earlier findings, from DNA expression arrays, that the predominant effect of cocaine is gene activation (McClung and Nestler, 2003; Renthal et al., 2007), and with more recent findings that reductions in histone acetylation block cocaine's behavioral effects while increases in acetylation augment cocaine action (Kumar et al., 2005; Renthal et al., 2007). A striking finding from the present study is that a relatively small number of gene promoters showed altered acetylation of both H3 and H4: approximately 13% of the promoters that exhibited altered histone acetylation in response to chronic cocaine showed alterations in both H3 and H4 (see Figure 3-1A; this is also illustrated in the heat maps shown in Figure 3-1C). This observation is interesting, in that it suggests that acetylation of one histone is sufficient to induce transcription in the NAc *in vivo*, consistent with cell culture data (Kurdistani et al., 2004; McCool et al., 2007). Cocaine-induced changes in H3 acetylation occurred at a larger number of genes than H4 acetylation, consistent with our earlier demonstration that chronic cocaine induces selective H3 acetylation at several candidate gene targets (*FosB, Cdk5* [cyclin-dependent kinase 5]*, Bdnf* [brain-derived neurotrophic factor]), with no induction of H4 acetylation observed (Kumar et al., 2005). On the other hand, we observed increased H4 acetylation at several

hundred gene promoters in the present study, which underscores the importance of a genome-wide investigation.

Numerous genes, shown in previous studies to be upregulated in NAc after chronic cocaine, were found in our ChIP on chip data to be associated with hyperacetylation of H3 or H4. Examples of such genes are listed in Table 3-1. Importantly, many of these individual genes have been directly related to aspects of neural and behavioral plasticity associated with cocaine exposure (see below). These observations provide important validation for our ChIP on chip approach and highlight the likely significance of the >1000 other genes that show similar markers of activation in this study.

The genome-wide acetylation pattern of H3 and H4 shows interesting features (Figure 3-1B). Levels of acetylated H3 and H4 were maximal between –500 to +200 bp, with acetylated H3 forming a bimodal peak and acetylated H4 forming a single peak over the transcriptional start site. Notably, these overall patterns were not significantly altered by chronic cocaine administration, indicating that cocaine increases the amount of acetylation on specific promoters (Figure 3-1A), but not the distribution of acetylation within promoters genome-wide. Representative examples of cocaine-regulated gene promoters illustrate both prototypical and less common patterns of H3 and H4 acetylation (Figure 3-4D). The gene encoding CART (cocaine- and amphetamine-regulated transcript) showed significant hyperacetylation of both H3 and H4 at an overlapping region of its promoter, each of which was very similar to the genome-wide average shown in Figure 3-1B. Two other genes that are upregulated by cocaine (Sivam, 1989; Fosnaugh et al., 1995) and display acetylation patterns similar to the genome-wide average are *Arc* (activity regulated cytoskeletal-associated protein) and *Pdyn* (prodynorphin). Distinct from CART, however, the upregulation of ARC and PDYN by cocaine are associated with

significant acetylation of a single histone only, H3 or H4, respectively. *Per1* (period 1, a circadian gene) is also upregulated by cocaine (McClung and Nestler, 2003) and is only hyperacetylated significantly on H4, but it displays a pattern of acetylation distinct from the genome-wide average. While the acetylation peak closest to the transcription start site overlaps with the region of ΔFosB binding (Table 3-1 and data not shown), the significance of the more upstream peak is unknown and may represent a region of novel regulatory elements. However, most of the genes regulated by cocaine show only one peak of altered acetylation, suggesting a single regulatory region important for its cocaine-induced activity. Another illustrative gene is *Cdk5*), which shows selective hyperacetylation of H3 that correlates with cocaine-induced upregulation of its transcription (Bibb et al., 2001). Moreover, the selective increase in acetylated H3 at the *Cdk5* promoter after chronic cocaine is consistent with our previous findings using quantitative ChIP (Kumar et al., 2005) and supports the quality of our genome-wide study. In addition to the genes highlighted here, we identified numerous other cocaine-regulated genes that display significant alterations in histone acetylation on their promoters (see Table 3-1). Examples include *Adcy3* (adenylyl cyclase 3), *Adora1* (adenosine receptor A_1), *Drd3* (dopamine receptor D_3), *Egr3* (early growth response transcription factor 3), *Rgs9* (regulator of G protein signaling 9), and *Sstr2* (somatostatin receptor 2). Each of these genes has been implicated in cocaine action in the NAc (see Table 3-1). Taken together, a clear pattern emerges from these findings (Figure 3-1 and Table 3-1): cocaine-induced increases in acetylation of either H3 or H4 correlates strongly with elevated gene expression in the NAc in vivo.

Methylation of H3 at K9 and K27 is implicated not only in silencing heterochromatin, but has also been shown to repress genes in transcriptionally active euchromatin (Kouzarides, 2007). It was, therefore, of interest to characterize this marker of gene repression in NAc after chronic

cocaine. We focused on dimethyl-K9/K27, because preliminary ChIP experiments revealed that these modifications were the most dynamically regulated in the NAc by cocaine. A key advantage of this analysis is that it provides a highly novel look at genes that are largely repressed or silenced. By contrast, conventional DNA expression arrays are greatly limited by their relative inability to reliably study mRNA's expressed at low levels.

We found that chronic cocaine altered levels of H3 dimethyl-K9/K27 at the promoter regions of 1107 genes in the NAc compared to saline-treated controls (Figure 3-1A). Interestingly, the genome-wide distribution of this methylation mark occurred on a much broader promoter span (from -1600 to +500 bp) compared to that observed for histone acetylation (Figure 3-1B), similar to the pattern of histone methylation observed in yeast (Sinha et al., 2006). Although the genome-wide distribution did not change, much like histone acetylation, after chronic cocaine exposure, cocaine increased histone methylation levels at several fold more genes than it decreased. This finding suggests that cocaine represses the expression of many genes, despite the predominant effect of cocaine being gene activation (McClung and Nestler, 2003; Renthal et al., 2007). Moreover, just as few genes showed significant changes in acetylation of both H3 and H4, there was minimal (~3%) overlap between genes that showed altered histone acetylation and those that showed altered H3 methylation after chronic cocaine (see Figure 3-1A,C). These findings reveal that, for the vast majority of cocaine-regulated gene promoters in the NAc, activation or repression involves independent alterations in histone acetylation or methylation (see Figure 3-1C). Moreover, relatively few cocaine-regulated genes were associated with reductions of histone acetylation or methylation, suggesting that the most common mechanisms of cocaine-induced gene regulation in the NAc involve net increases in histone acetylation (gene activation) or methylation (gene repression) rather than net histone

deacetylation or demethylation. For example, the gene encoding the K^+ channel subunit, Kcnv2, which is downregulated by cocaine (Renthal et al., 2007), showed increased H3 dimethyl-K9/K27 at its promoter without a change in histone acetylation, while prodynorphin and ARC, which are upregulated by cocaine, showed increased acetylation on their promoters without any changes in methylation (see Figure 3-1D). Although few genes showed reduced H3 methylation after chronic cocaine exposure, at certain genes, such as the gene encoding adenosine deaminase (*Ada*), which is upregulated by cocaine (McClung and Nestler, 2003), hypomethylation does appear to have functional significance (see Table 3-1). As well, the gene encoding CART shows a trend for hypomethylation at the same site where it displays significant hyperacetylation, but also a trend for hypermethylation further upstream; although the functional significance of this latter change is not known, a similar pattern was observed at other promoters, including Cdk5, and illustrates the complex patterns of histone methylation induced on gene promoters by chronic cocaine.

Overall, the vast majority of cocaine-induced genes, including most of those in Table 3-1, show increased histone acetylation after chronic cocaine and undetectable or barely detectable levels of H3 dimethyl-K9/K27 (Figure 3-1C). This suggests that these genes are not in a silenced or repressed state under normal conditions, and that the critical step in their induction by cocaine is not derepression but rather the generation of an active transcription factor which increases gene transcription.

Role of ΔFosB in the genomic effects of cocaine in the NAc

ΔFosB has been shown to play an important role in addiction: overexpression of the transcription factor in the NAc increases an animal's responses to the rewarding effects of

cocaine and of morphine, while overexpression of a dominant negative antagonist, termed ΔcJun, causes the opposite effects (Hiroi et al., 1997; Kelz et al., 1999; McClung and Nestler, 2003; Peakman et al., 2003; Zachariou et al., 2006). A previous DNA expression array study identified genes in the NAc whose expression is altered upon overexpression of ΔFosB or ΔcJun (McClung and Nestler, 2003). However, this study could not provide information as to which of these genes are direct targets for ΔFosB or regulated indirectly via other mechanisms. To address this question, we carried out ChIP for ΔFosB in mice treated with chronic cocaine or saline. Chronic cocaine significantly altered ΔFosB binding to 1674 promoters (Figure 3-2A). The number of promoters where ΔFosB binding increased after cocaine (1189) was more than double that of promoters (485) where ΔFosB binding fell after cocaine, which is consistent with the several fold induction of ΔFosB levels under these conditions (McClung et al., 2004). The finding that chronic cocaine administration decreased ΔFosB binding to a significant number of genes is surprising given the higher levels of ΔFosB. Although the magnitude of decreased ΔFosB binding is mostly very small, and hence not apparent in the heat maps which use a 1.2 fold cutoff (Figure 3-2C), the findings raise the possibility of a previously unappreciated, albeit less common, mode of ΔFosB regulation by cocaine.

Prior ChIP and related studies in cultured cells for other transcription factors, such as CREB, have shown that many sites along the genome where these proteins bind exert no apparent functional effect (Impey et al., 2004; Zhang et al., 2005). Therefore, we analyzed the genes that showed cocaine-induced changes in ΔFosB binding for those that also showed cocaine-induced changes in epigenetic markers of gene activation or repression. Roughly 10% of the genes that show increased or decreased ΔFosB binding in response to chronic cocaine exhibited coincident changes in histone acetylation or methylation (Figure 3-2A). This figure is

similar to that reported for other transcription factors in terms of the fraction of transcription factor binding sites along the genome that appear functionally relevant (Zhang et al., 2005). Many of the genes that display altered ΔFosB binding have previously been implicated in cocaine action (see Table 3-1). Moreover, the large majority of these genes appear to be bona fide targets for ΔFosB, since levels of their mRNA's are altered upon inducible overexpression of ΔFosB or ΔcJun in the NAc (Fig 2C), which provides important validation of our ChIP on chip data. Interestingly, increased binding of ΔFosB to gene promoters is associated with either increased or decreased mRNA expression, consistent with its complex role as both a transcriptional activator and repressor depending on the gene in question (McClung and Nestler, 2003).

Figure 3-2D illustrates ΔFosB binding across the promoters of six representative genes after chronic cocaine. One ΔFosB target gene, *Gpsm1* (AGS3, activator of G-protein signaling 3) is upregulated for weeks after cocaine exposure in the NAc and has been shown to be critically involved in behavioral responses to cocaine (Bowers et al., 2004). The stability and persistence of ΔFosB during weeks of drug withdrawal further support its role as a key regulator of AGS3 transcription during this period. ΔFosB also binds to *Drd1* (dopamine D_1 receptor) and *Cnr1* (cannabinoid CB_1 receptor), two key G-protein coupled receptors in NAc that mediate behavioral responses to cocaine (Chaperon et al., 1998; Ben-Shahar et al., 2007). ΔFosB may promote D_1 receptor transcription, since the receptor is significantly upregulated after chronic cocaine (Ben-Shahar et al., 2007); however, to the best of our knowledge, chronic cocaine has not yet been shown to regulate CB_1 receptor expression in the NAc, a possibility which now warrants direct investigation. Cocaine also increases ΔFosB binding to *Wasf1* (WAVE 1, Wiskott-Aldrich syndrome protein family member 1), a key Cdk5 substrate that regulates dendritic spine

plasticity in the NAc and thus may be involved in mediating the ability of cocaine/ΔFosB/Cdk5 to induce structural changes in NAc neurons (Robinson and Kolb, 2004; Kim et al., 2006). The *Per1* gene displays a cocaine-induced increase in ΔFosB binding as well (Table 3-1), consistent with previous findings that overexpression of ΔFosB increases PER1 expression in the NAc (McClung and Nestler, 2003), and that *Per1* mutant mice show reduced cocaine reward (Abarca et al., 2002). Together, cocaine induction of ΔFosB in the NAc regulates numerous gene targets, which now offer new understanding of the complex mechanisms by which this transcription factor causes sensitized drug reward.

ΔFosB displayed significant binding to a broad region of promoters genome-wide (-1500 to +250 bp) (Figure 3-2B), similar to that of H3 dimethyl-K9/K27. As with histone acetylation and methylation, chronic cocaine did not significantly affect the average location of ΔFosB binding genome-wide. ΔFosB, like all Fos family transcription factors, is thought to dimerize with a Jun family protein to form an active AP1 complex which binds to AP1 sites present in responsive genes (Morgan and Curran, 1995; Rylski and Kaczmarek, 2004). Indeed, the promoters for the genes encoding AGS3, GBP2 (GTP-binding protein 2), RCAN2 (regulator of calcineurin 2), and the D_1 receptor contain a consensus AP1 site (TGA[G/C]TCA) at or near the peak of altered ΔFosB binding (Figure 3-2D). However, a majority of genes that showed altered ΔFosB binding after chronic cocaine (e.g., those for the CB_1 receptor, WAVE1, PER1, ATF1 [activating transcription factor 1], and EGR3), including the majority that displayed coincident changes in histone acetylation or methylation, do not contain consensus AP1 sites at or near the ΔFosB binding region. This is despite the several lines of evidence that most of these genes are up- or downregulated in the NAc upon ΔFosB overexpression (see Figure 3-2C) (McClung and Nestler, 2003). To better understand what regulatory sequences ΔFosB binds to in the NAc after

chronic cocaine, we performed bioinformatic sequence analysis of the most common bindings sites *in vivo*. We found that the most common ΔFosB binding site is highly divergent from the consensus AP1 site (Figure 3-4D). The explanation for this surprising difference between *in vitro* and *in vivo* ΔFosB binding sites is unknown, but may be related to the ability of AP1 complexes to bind to other transcriptional regulators (Morgan and Curran, 1995; Chinenov and Kerppola, 2001).

Role of CREB in the genomic effects of cocaine in the NAc

As stated earlier, repeated cocaine administration induces CREB activity in the NAc, which then feeds back and attenuates the rewarding effects of cocaine (Carlezon et al., 1998; Carlezon et al., 2005). Cocaine activates CREB by increasing its phosphorylation at Ser133. According to a generally accepted scheme, CREB, which is bound to responsive genes at CRE (cAMP response element) sites whether it is phosphorylated or not, recruits transcriptional co-activators when CREB is phosphorylated and this increases gene transcription (Mayr and Montminy, 2001). Therefore, to gain insight into the transcriptional actions of CREB in the NAc after chronic cocaine, we carried out ChIP for phospho-CREB followed by promoter array assays. We found that cocaine increased phospho-CREB binding to 1279 promoters, while decreased levels of CREB binding were observed at 482 promoters (Figure 3-3A). As with ΔFosB, it is surprising to identify promoters that show reduced phospho-CREB binding under conditions which are known to robustly induce phospho-CREB levels. Roughly 13% of the genes that show cocaine-induced changes in phospho-CREB binding display coincident increases in histone acetylation, a marker of gene activation (Figure 3-3A). Interestingly, another 7% of genes that show increased phospho-CREB binding exhibit coincident increases in H3

dimethyl-K9/K27, a marker of repression, which is counter to the general view that CREB acts solely as a transcriptional activator. These findings are, however, consistent with our previous results that overexpression of CREB can repress certain genes in the NAc, while a dominant negative mutant of CREB can increase expression of those genes (McClung and Nestler, 2003). Among the genes that show cocaine-induced alterations in phospho-CREB binding are many that have previously been implicated in cocaine and CREB action, such as *Nrgn* (neurogranin) and *Gpsm1* (AGS3) (McClung and Nestler, 2003; Bowers et al., 2004) among other examples given in Table 3-1.

The genome-wide pattern of phospho-CREB binding is equivalent under basal and cocaine-treated conditions, and reveals a relatively sharp peak between -500 and +200 bp; however, there is also moderate binding of phospho-CREB beyond -500 bp up to -1500 bp (Figure 3-3B). As with ΔFosB, patterns of phospho-CREB binding are complex with respect to changes in histone modifications. This is illustrated for six representative gene promoters in Figure 3-3D. Some genes show changes in phospho-CREB binding that correspond to regions that also show changes in histone modifications, whereas phospho-CREB binding and histone modifications do not overlap at many other promoters. Three gene promoters illustrated in Figure 3-3D, *Mbd2* (Methyl-CpG-binding domain protein 2), *Kcne3* (voltage-gated K^+ channel, Isk-related subfamily, member 1), and *Ube3b* (ubiquitin protein ligase E3B), contain consensus CRE sites (TGACGTCA) which coincide with the peak area of increased phospho-CREB binding. As with ΔFosB, however, only a minority of all the genes that exhibit altered levels of phospho-CREB binding after chronic cocaine contain CRE sites at or near the peak region of phospho-CREB binding. Nevertheless, a large majority of these would appear to be bona fide CREB targets: the heat maps in Figure 3-3C illustrate that most genes that show increased or decreased

phospho-CREB binding after chronic cocaine also display altered levels of mRNA expression when CREB is inducibly overexpressed in the NAc and opposite changes when the dominant negative mutant mCREB is inducibly expressed in this brain region (McClung and Nestler, 2003). The other genes depicted in Figure 3-3D showing increased phospho-CREB binding after chronic cocaine are *Gpsm1* (AGS3), *Gabrb1* ($GABA_A$ receptor β1), and *Nrgn*, each of which has been implicated in cocaine action in the NAc (Gerasimov et al., 2001; McClung and Nestler, 2003; Bowers et al., 2004). Since many genes where cocaine regulated phospho-CREB binding do not have a consensus CRE site at or near the binding site, we determined the most common regulatory sequence to which phospho-CREB binds in the NAc in vivo after chronic cocaine. Figure 3-4D shows that the most common phospho-CREB binding sequence differs substantially from the consensus CRE site identified from *in vitro* studies. These findings are consistent with previous work which reported that nearly 30% of phospho-CREB target genes do not contain a consensus CRE site in their promoter region (Zhang et al., 2005), and suggest as with ΔFosB, highly complex mechanisms of gene regulation in vivo.

Comparison of ΔFosB- and CREB-mediated regulation of the genomic effects of cocaine in the NAc

Our laboratory previously investigated the contributions of ΔFosB and CREB to cocaine action by performing gene expression microarrays on NAc of bitransgenic mice that inducibly overexpress the respective proteins, or their dominant negatives, in this brain region (McClung and Nestler, 2003). We found that ΔFosB and CREB overexpression showed similarity in gene profiles after short-term ΔFosB overexpression, a condition that is similar to the cocaine paradigm used in this study. A major limitation of this prior study, however, was its inability to

address which genes are direct ΔFosB or CREB targets. Using ChIP on chip, we identified roughly 15% overlap between the cocaine-induced ΔFosB- and phospho-CREB-bound genes (Figure 3-4A,B), and many of these 168 overlapping genes, including *Nrgn* and *Gabrb1*, were also significantly regulated in ΔFosB and CREB overexpression studies (McClung and Nestler, 2003). Some of the new promoters where we find enriched binding of both ΔFosB and CREB are those for the genes encoding AGS3, the D_1 receptor, the CCK_A receptor (cholecystokinin A receptor), IkBε (inhibitor of kappaB), and actin. Many of these ΔFosB and phospho-CREB gene targets have been implicated previously in cocaine responses [e.g., AGS3 (Bowers et al., 2004), CCK_A receptor (Massey et al., 1994), and D_1 receptor (Robledo et al., 1992)], and likely contribute to the potent action of these two transcription factors on cocaine-induced behaviors (McClung et al., 2004; Carlezon et al., 2005). The ~15% overlap we observed here in ΔFosB and CREB binding is not as strong as that found in our earlier overexpression study (McClung and Nestler, 2003). This may reflect a temporal disconnect between transcription factor binding and changes in steady-state mRNA levels, for example, phospho-CREB or ΔFosB may bind but co-activators are not immediately recruited. It may also reflect indirect effects of CREB and ΔFosB overexpression on gene regulation.

Consensus AP1 and CRE sites differ by one base pair only, and it has been shown that AP1 and CREB dimers can bind to the other's site. We were therefore interested in how ΔFosB and CREB bound to the promoters of genes which they both regulate. Representative examples of the 168 overlapping genes where cocaine increases ΔFosB- and phospho-CREB-binding are shown in Figure 3-4C and illustrate that on many promoters ΔFosB and phospho-CREB indeed seem to bind to the same region (e.g., D_1 receptor, $GABA_A$ receptor, and AGS3). However, there are also examples where cocaine induction of ΔFosB and phospho-CREB binding occurs at

distinct regions of the gene promoter (e.g., *Nrgn*), representing distinct regulatory regions to which each transcription factor binds. To better understand the relationship between ΔFosB and phospho-CREB regulatory elements, we performed bioinformatic sequence analysis on the most commonly enriched sequences for ΔFosB and phospho-CREB binding after chronic cocaine. Consistent with the consensus AP1 and CRE sites differing by only one nucleuotide, we found that ΔFosB and phospho-CREB binding sites in the NAc of cocaine-treated mice had several common features (Figure 3-4D). Surprisingly, however, neither of these sites closely resembles the consensus AP1 or CRE sites identified from in vitro studies, as discussed earlier. Nevertheless, it appears from the heatmaps in Figs. 2C and 3C, that ΔFosB and phospho-CREB can alter transcription when bound to these novel sites.

Behavioral validation of a novel target of cocaine action

Thus far, our discussion has highlighted examples of known cocaine, ΔFosB, or phospho-CREB targets that show altered association with acetylated or methylated histones or altered ΔFosB or phospho-CREB binding in our ChIP on chip studies. However, the studies also provide rich lists of many novel genes, not heretofore implicated in cocaine action, which show robust and highly significant markers of activation or repression in response to chronic cocaine. It was, therefore, important to evaluate the predictive power of these gene lists for revealing fundamentally new insight into the molecular pathophysiology of cocaine action in the NAc. One gene family was of particular interest: the sirtuins. Sirtuins–also referred to as SIRT's (silent information regulator of transcription)–are categorized as Class III NAD-dependent histone deacetylases, which in addition to deacetylating histones, also deacetylase other cellular proteins, such as tubulin, p53 (a tumor suppressor transcription factor), and NF-κB (nuclear factor kappa

B) (Denu, 2005). Sirtuins are highly conserved from bacteria to mammals–7 forms have been identified in mouse and human–and have been implicated in diverse processes, including cell morphology, growth, and transformation, apoptosis, general metabolism, and aging, although very little is known about their function in the nervous system. Our ChIP on chip studies of chronic cocaine action in the NAc identified significant hyperacetylation of histone H3 at the *Sirt2* promoter, indicating a potential role in cocaine responses (Figure 3-5A, Table 3-1). We first confirmed that cocaine increases levels of acetylated H3 binding at *Sirt2* in an independent ChIP experiment (Figure 3-5B) and that this was associated with increased *Sirt2* mRNA expression in the NAc (Figure 3-5B). We also observed a significant increase in cocaine-induced ΔFosB binding at the *Sirt2* promoter in an independent ChIP experiment. This is important because ΔFosB was not significantly enriched on *Sirt2* in the ChIP-chip analysis at the rigorous significance cutoff we used (3.1 SD ~ $p < 0.001$). However, at a significance cutoff of $p < 0.01$, ΔFosB binding was induced significantly on the *Sirt2* promoter by cocaine. Moreover, we found significant cocaine-induced H3 acetylation of a related sirtuin, *Sirt1*, at the $p < 0.01$ cutoff, a finding which we validated in an independent ChIP experiment and showed is associated with a significant increase in *Sirt1* mRNA levels (Supplemental Figure 3-S1). Together, these data illustrate the predictive quality of our ChIP on chip analyses and suggest that we are underestimating the number of cocaine-regulated promoters at our rigorous statistical cutoff (3.1 SD ~ $p < 0.001$).

The cocaine regulation of histone acetylation and ΔFosB binding to *Sirt1* and *Sirt2* in the NAc prompted us to examine directly whether these sirtuins are involved in behavioral responses to cocaine. This was made possible by available pharmacological agents that stimulate or inhibit sirtuin function. We administered the sirtuin activator, resveratrol, systemically since this drug is

known to penetrate the brain after systemic administration (Baur and Sinclair, 2006). As shown in Figure 3-5C, resveratrol significantly augmented the rewarding effects of cocaine in the place conditioning assay, where animals learn to prefer a cocaine-paired environment. Conversely, direct intra-NAc administration of the sirtuin inhibitor, sirtinol, caused the opposite effect and suppressed cocaine reward (Figure 3-5C). It is interesting to note that inhibition of Class I and Class II HDAC's in the NAc dramatically increase cocaine reward (Kumar et al., 2005; Renthal et al., 2007), while inhibition of Class III HDAC's (sirtuins) reduces it. Given the role of sirtuins in regulating tubulin, NF-κB, and several other proteins involved in neuronal plasticity, it is possible that these downstream effectors are more important contributors than histone deacetylation to sirtuin action on cocaine reward. As well, it is interesting to speculate that cocaine regulation of SIRT1 and SIRT2 could contribute to cocaine-induced dendritic remodeling of NAc neurons (Robinson and Kolb, 2004). Taken together, these findings demonstrate the ability to translate ChIP on chip data of epigenetic and transcriptional regulation by cocaine to identify new molecular pathways involved in cocaine action.

Molecular pathway analysis of the "cocaine transcriptome"

To gain a more global view of the genes regulated by cocaine in the NAc, we analyzed our gene sets with Ingenuity molecular pathway analysis software (see Figure 3-S2). The most significantly regulated molecular pathways in terms of altered histone acetylation were cAMP signaling, long term potentiation or depression, RXR signaling, and neurotrophin signaling (e.g., ERK/MAP kinase and PTEN/PI-3-kinase/Akt) pathways. Each of these pathways are known to play integral roles in the cellular and behavioral responses to chronic cocaine, and their enrichment in our analyses supports the predictive quality of this study. Notably, chronic cocaine

also regulated several glutamate receptors and downstream calcium signaling molecules, including subunits of NMDA and AMPA receptors, metabotropic receptors, and HOMER3. The upregulation of glutamate signaling is consistent with previous reports, which demonstrate that chronic cocaine increases the sensitivity of NAc neurons to glutamate (Kalivas, 2004). Examples of these highly regulated pathways are shown in Figs. 6A and 6B. The panels illustrate the profound and complex effects of cocaine on second messenger- and growth factor-regulated molecular pathways in the NAc, and how the individual genes in these pathways are influenced epigenetically by alterations in histone acetylation or methylation. The panels also identify numerous proteins in these pathways that show cocaine-induced alterations in ΔFosB or phospho-CREB binding at the regulatory regions of their genes, which again supports the important influence of these transcription factors in cocaine action.

Among the most significant findings revealed by such molecular pathway analyses are new signaling pathways and molecules not heretofore appreciated in cocaine action. Examples include protein ubiquitination, Toll-like receptor signaling, and FGF signaling. Indeed, protein ubiquitination signaling is known to regulate dendritic spine morphology and neuronal function (Pak and Sheng, 2003), and therefore warrants further investigation in responses to cocaine. Less is known about Toll-like receptor signaling in the brain, however, recent studies have found that similar molecules associated with the innate immune system have pronounced effects on synaptic plasticity in neurons and therefore may play an important role in cocaine action (Huh et al., 2000).

In addition to identifying novel pathways involved in cocaine action, we have gained insight into the layers of complex regulation that control these pathways. For most of the pathways we investigated, chronic cocaine promotes the expression of an intricate network of

activating and inhibiting molecules. This is surprising because we know that many of these same pathways are functionally upregulated several fold after chronic cocaine. For example, in the NF-κB pathway, there is increased acetylation on the genes coding both the transcriptional activator, p50, and its inhibitor, IκB (see Figure 3-6B). However, we know that NF-κB activity is upregulated in the NAc after chronic cocaine (Ang et al., 2001), suggesting that the increased acetylation on IκB may serve to limit the primary activation of p50 and its upstream activators, TNFR and TRAF1. Similar complex regulation is seen for the dopamine/cAMP pathway. Cocaine is known to functionally upregulate cAMP signaling, but simultaneously increases histone acetylation on the genes for the Gi-coupled D_3 receptor and an inhibitor of Gi-signaling, RGS9. Similarly, adenylyl cyclase 3 (AC3) shows reduced acetylation, while PKA subunits show increased acetylation, yet we know that chronic cocaine induces net increases in cAMP signaling in the NAc. The NF-κB and cAMP pathways exemplify the intricate cocaine-induced regulation of intracellular signaling in the NAc, and therefore underscore the importance of genome-wide studies which permit the parallel assessment of multiple molecular components of each pathway.

DISCUSSION

This study provides a genome-wide assessment of epigenetic and transcriptional alterations in the NAc in response to repeated cocaine administration. Among the many genes that show regulation by cocaine are a large number reported in previous studies to show altered mRNA or protein expression in the NAc after chronic cocaine (see examples in Table 3-1). For instance, the cocaine-induced genes that encode, ARC, CART, CDK5, NFκB, dynorphin, σ-

opioid receptor, and Period 1 and 2 (Freeman et al., 2001; McClung and Nestler, 2003; Yao et al., 2004) (see also Supplemental References in Supplementary Information) were shown in the present study to exhibit increased acetylated H3 or H4, with either attenuated or unchanged methylation of H3 at K9/K27. Genes that are known to be downregulated by cocaine, such as the voltage-gated potassium channel Kv8.2 and the microtubule associated protein MTAP2 (Renthal et al., 2007), were associated with increased methylation of H3 at K9/K27 (Table 3-1). For each type of histone modification, cocaine induced increases in acetylation or methylation at many more genes than it induced decreases, which can be observed in the heatmaps in Figs. 1C, 2C, and 3C as more red (upregulated) genes than green (downregulated) genes. Although there are a few examples where reductions in acetylation or methylation are associated with respective changes in gene expression, our data suggest that chronic cocaine more commonly regulates transcription by either increasing histone H3 or H4 acetylation (to elevate mRNA levels), or by increasing histone H3 dimethyl-K9/27 (to reduce mRNA expression). There are also a subset of genes which are highly regulated at the chromatin level but show no detectible change in steady-state mRNA expression. For example, mRNA's for HDAC4 and myocyte-enhancer factors 2A and 2D (MEF2A and MEF2D) are not altered by cocaine (data not shown), but their promoters are dramatically altered after cocaine treatment by histone modifications and/or transcription factor binding. These are particularly interesting examples given the potent influence of HDAC4 (Kumar et al., 2005) and of MEF2 (Pulipparacharuvil et al., 2007) on cocaine responses in the NAc, and may illustrate a new layer of regulation not previously appreciated. *Bdnf* is a good example where hyperacetylation of its promoter (Kumar et al., 2005) does not correlate with an immediate increase in steady state BDNF levels, however during cocaine withdrawal, levels of BDNF protein are significantly elevated (Grimm et al., 2003). Similarly, gene expression

microarray analyses of rats after extinction from cocaine self-administration found MEF2D significantly increased in the NAc (Personal Communication, D.W. Self). Thus, histone acetylation at certain genes may represent a priming mechanism to facilitate subsequent gene induction. Taken together, our study corroborates numerous established molecular targets of cocaine action in the NAc, and demonstrates the power of ChIP on chip assays to uncover in a comprehensive manner the genomic targets through which cocaine induces neural and behavioral plasticity in this critical brain reward region.

Likewise, results of the present study provide novel insight into the target genes through which ΔFosB and CREB contribute to the genomic effects of cocaine. Several genes previously identified as targets of ΔFosB and CREB, inferred from DNA expression array studies of mice overexpressing the transcription factors or their dominant negative antagonists in the NAc (McClung and Nestler, 2003), were identified in this study. Examples include neurogranin, period 1, $GABA_A$ receptor subunits, and MEF2C, to name a few. The present findings thereby indicate that many of the ΔFosB and CREB target genes determined through overexpression studies are indeed direct, physiological targets for these transcription factors in the NAc in vivo. There are also fewer examples, such as NF-κB, for which ΔFosB is necessary and sufficient for its induction in the NAc (Ang et al., 2001), but where cocaine-induced changes in ΔFosB binding on its promoter were not detected in our ChIP on chip analyses. Although there have been no prior genome-wide ChIP on chip studies for FosB or ΔFosB, we should note that many of the phospho-CREB-bound genes we observed to be regulated by cocaine (e.g., MEF2C, phosphodiesterase 6b [PDE6B], frizzled homologue 2 [FZD2]) were also identified as CREB targets by previous CREB ChIP on chip studies (Zhang et al., 2005; Tanis et al., 2007). Although it is known that CREB targets differ dramatically from cell type to cell type (Cha-Molstad et al.,

2004; Zhang et al., 2005) underscoring the importance of genome-wide assessments of CREB binding in multiple brain regions and experimental conditions, observing the same gene regulated by CREB from cultured cells to brain gives us high confidence in the predictive quality of these data.

Of all the genes in the NAc that show markers of activation or repression after chronic cocaine, roughly 10% exhibit altered levels of ΔFosB binding (Figure 3-2A) and roughly 15% exhibit altered levels of phospho-CREB binding (Figure 3-3A). This is interesting in that our previous DNA expression array study found that similar fractions of genes regulated in the NAc after 5 days of cocaine were also regulated upon ΔFosB overexpression or CREB overexpression, respectively (McClung and Nestler, 2003). The number of cocaine-regulated genes influenced by ΔFosB increased to >25%, whereas that for CREB decreased to 5%, after 4 weeks of cocaine administration, which demonstrates the importance of performing the genome-wide studies reported here after longer periods of cocaine exposure.

As stated, the dramatic cell type differences that have been reported for the genomic targets of a given transcription factor between even two types of cultured cells (Cha-Molstad et al., 2004; Zhang et al., 2005) means that an absolutely crucial next step in the field of epigenetics is to define modes of epigenetic regulation that occur in the brain in vivo. Indeed, well beyond identifying lists of genes that show interesting patterns of epigenetic regulation by cocaine, results of the present study reveal several novel principles by which cocaine regulates gene expression in the NAc of behaving animals. Among the lessons revealed are that most cocaine-regulated genes show altered acetylation either of histone H3 or of H4, with changes at H3 predominating, and alterations either in histone acetylation or in histone methylation, but only rarely both modifications together on the same gene. Another striking lesson is that cocaine (at

least after 7 days) decreased binding of ΔFosB and of phospho-CREB to a significant number of promoters despite inducing several fold more of both factors, and that the reductions in binding are functionally relevant given coincident changes in markers of gene activation or repression. Moreover, while ΔFosB has been shown previously to exert complex transcriptional effects, activating or repressing gene transcription depending on the gene promoter involved, findings of the present study, and of a previous study examining gene expression changes in the NAc upon inducible overexpression of CREB (McClung and Nestler, 2003), suggest similarly complex transcriptional effects of phospho-CREB, which heretofore has been seen primarily as a transcriptional activator.

There have been numerous studies of cocaine regulation of gene expression in the NAc and other brain regions by DNA expression arrays, and this research has revealed large numbers of transcripts that are altered in response to cocaine administration. The ChIP on chip studies reported here offer several advantages that complement these earlier reports: for example, ChIP on chip enables an examination of genes expressed at low levels (for which DNA expression arrays are poorly suited), and avoids confounds of RNA degradation and amplification. Importantly, however, the coordinated use of both approaches promises to identify a smaller set of genes in which the field can place greater confidence as being bona fide targets of cocaine, and also focuses on those targets that are altered via direct transcriptional regulation. Moreover, this work begins to describe the specific epigenetic mechanisms underlying these cocaine-induced transcriptional changes and reveals fundamentally new insight into the genome-wide patterns of chromatin regulation by cocaine in the NAc. Together, this new insight has led to the identification of a novel family of genes involved in the behavioral responses to cocaine, the sirtuins, and provides other fundamentally new targets for the development of more effective

treatments of cocaine addiction.

EXPERIMENTAL PROCEDURES

Animals and reagents

Male C57/BL/6 mice 10-12 weeks old were obtained from Jackson Laboratory and housed on a 12-hour light-dark cycle with access to food and water *ad libitum*. Mice were injected i.p. with cocaine (20 mg/kg, Sigma) or saline once per day for 7 days. Twenty-four hours after the last dose, nucleus accumbens (NAc) was punch dissected from mice as described previously (Kumar et al., 2005).

Chromatin immunoprecipitation and microarray analysis

Chromatin immunoprecipitation was performed for acetylated histone H3 and H4, methylated histone H3 lysine 9 and 27, ΔFosB, and pCREB as described previously (Kumar et al., 2005) with minor modifications. Immunoprecipitated DNA was amplified via ligation-mediated PCR and hybridized to Nimblgen mouse MM8 promoter arrays. See Supplementary Information for detailed methods of promoter microarray analysis. Quantitative ChIP was performed as described above except that the immunoprecitated DNA was directly quantified by quantitative real-time PCR. For validating cocaine regulation of H3 acetylation at *Sirt2*, we used the following promoter primers: fwd GAAGTCCAGCATCGGAAATC, rev GTGGGCGCCCTTAGTCTT. For validating ΔFosB binding at *Sirt2*, we used: fwd GCACAAAAACCAGACCCAAA, rev TTGTACCCGGGATTTTCTTG. For validating cocaine regulation of H3 acetylation at *Sirt1*, we used the following promoter primers: fwd

TCTGGCAAGCGTGACTTTAC, rev ACAGAAGGGAGAGTGGTTGG.

RT-PCR

NAc punches were homogenized in Trizol (Invitrogen, Carlsbad, CA) and processed according to the manufacturer's instructions. Purified RNA was reverse transcribed using Bio-Rad iScript Kit (Hercules, CA). cDNA was quantified with real-time PCR using SYBR Green (Applied Biosystems, Foster City, CA). Each reaction was run in triplicate and analyzed using the ΔΔCt method as described previously (Tsankova, et al., 2006). The primers used for the validation of *Sirt2* were: fwd TCCACTGGCCTCTATGCAAAC, rev CGTGTCTATGTTCTGCGTGTAG. We quantified the cocaine regulation of *Sirt1* using the primers fwd TTGGCACCGATCCTCGAAC, rev CCCAGCTCCAGTCAGAACTAT. The normalizing primer used was GAPDH (Kumar et al., 2005).

Conditioned place preference

A standard 4 day conditioning paradigm to cocaine was used as described previously (Kumar et al., 2005). Briefly, mice received either a systemic injection of resveratrol (20mg/kg, i.p.) or vehicle and were conditioned to 5mg/kg cocaine. A separate cohort of mice was implanted with osmotic minipumps that delivered 50μM sirtinol into their NAc at a rate of 0.25μL/hr. After 4-5 days of recovery, conditioned place preference was performed with 10mg/kg of cocaine (See Supplementary Information for detailed methods).

SUPPLEMENTAL METHODS

Genomic and epigenomic profiling using ChIP and ChIP on chip

Fresh NAc punches were processed for ChIP as described (Kumar et al., 2005) with minor modifications. Briefly, punches were cross-linked and lysed, and the chromatin was fragmented by sonication to an average length of 700 bp. For each sample, bilateral NAc punches were pooled from ~8 mice. Aliquots of sonicated chromatin were incubated with 4 μg of the following polyclonal antibodies for 8 hr at 4°C: anti-acetylated K9/K14 H3 (cat. #06-599, Upstate/Millipore, Billerica, MA,); anti-acetylated K5/K8/K12/K16 H4 (cat. #06-866, Upstate); anti-dimethyl K9/K27 H3 (cat. #ab7312, Abcam, Cambridge, UK); anti-FosB(C-terminus) antibody generated at UT Southwestern; anti-pan FosB/ΔFosB (cat. #SC048, Santa Cruz Biotechnology, Santa Cruz, CA); and anti-phospho- CREB (cat. #06-519, Upstate). Note that for ΔFosB ChIP, chromatin was first immunoprecipitated with anti-FosB (C-terminus) to precipitate any full-length FosB-bound chromatin, followed by anti-pan FosB/ΔFosB which then precipitates ΔFosB-bound chromatin. These conditions have been shown to selectively precipitate ΔFosB-bound chromatin (Kumar et al., 2005). Immunprecipitates were reverse cross-linked, and both the immunoprecipitated (enriched) and total (input or non-enriched) DNA from each treatment group was amplified and labeled with a fluorescent dye (Cy5 and Cy3) with the use of ligation-mediated-polymerase chain reaction (Sikder et al., 2006). Samples of Cy5-labeled immunoprecipitated DNA and Cy3-labeled input DNA were mixed and hybridized to NimblGen (Madison, WI) MM5 or MM8 mouse promoter microarrays (2-3 biological replicates per condition).

Analyses of ChIP on chip data and gene expression data

The $\log_2$ ratios of signal intensities between the experimental samples and control genomic DNA were calculated, scaled by Tukey Bi-weight method, and median-normalized. The

signals from replicates were averaged and then a moving average of three adjacent probes within the promoter region of each gene (Kim et al., 2005) was used to stabilize the signal. We used the floor method to handle background noise by treating all negative values as zero. Negative values represent background noise of unenriched genes and were therefore not biologically relevant. The probes that showed no binding strength in both saline and cocaine conditions were removed, and the differences between cocaine and saline treatments were used to calculate cocaine-induced changes. The probes at which cocaine changes binding intensity by 3 standard deviations (corresponding to $p < 0.001$ under normal assumptions) were identified as significantly regulated. Certain genes had both significant cocaine-induced increases and decreases in binding of histone modifications or transcription factors at distinct promoter regions. These genes were included in the gene lists as both upregulated and downregulated to avoid averaging out the information, as were genes that were validated in independent tissue samples. To derive the overall spatial pattern of H3 and H4 acetylation, H3 methylation, ΔFosB binding, and pCREB binding locations relative to transcriptional starting sites, we used mpeak (Zheng et al., 2007) software to determine the location of signal peaks. The empirical densities of the peak location were plotted and compared between saline and cocaine treatments. The heat maps show the signal strength averaged across probes for each gene. The genes were rearranged using a standard hierarchical clustering method (Genespring). The heat maps show genes that were significantly regulated by cocaine (increase or decrease) and with a fold change greater than 1.2. In order to compare signals across different histone modifications and transcription factors (e.g. heatmaps, promoter plots), the signal intensities were scaled using the ΔFosB data as a reference. Published Affymetrics gene expression data (McClung & Nestler, 2005; and

unpublished) were preprocessed with RMA method (Irizarry et al., 2003) and subjected to quantile-quantile normalization.

Conditioned place preference (CPP)

The development of cocaine place conditioning was assessed over 6 consecutive days as described previously (Rahman et al., 2003). On day 1, each mouse was allowed 30 min of habituation to all three quadrants of a CPP chamber, and the duration of time spent in each quadrant was recorded. On days 2-5, mice were paired to one side of the test chamber after cocaine (5 mg/kg, ip) or saline administration exactly 30 min after an initial pre-injection of resveratrol (20 mg/kg ip, dissolved in 5% hydroxypropyl β-cyclodextrin vehicle; CTD, Inc., High Springs, FL) or vehicle alone. On day 6, no injections were given and mice were assessed for side preferences during unrestricted access to all three quadrants of the test chamber. For experiments with sirtinol, a similar paradigm was used except that mice were surgically implanted with two subcutaneous Alzet® minipumps (Durect Corp., Cupertino, CA) and with bilateral guide cannula (Plastics One, Roanoke, VA) targeting the NAc under ketamine/xylazine anesthesia. One day prior to surgery, two cannulae (28 gauge stainless steel, 5.7 mm projection) mounted within a single pedestal assembly were filled with sirtinol (50 μM) or 5 hydroxypropyl β–cyclodextrin vehicle, and each pedestal within the assembly was separately affixed via vinyl tubing to a mini-pump, each also filled with either sirtinol or vehicle, respectively. Mini-pumps were activated 12 hrs before implantation by incubating them at 40°C, initiating the continuous delivery of 0.25 μl/hr for 14 days. The surgical procedure began by making an incision above the skull, and the subcutaneous area between the scapulae was spread to create an area for positioning the mini-pumps. Subsequently, each mini-pump was positioned subcutaneously on

the mouse's back. Cannulae placements were made possible by drilling two small holes above the area of the NAc, and by the delivery of the cannula from bregma AP + 1.5; ML +1.0; DV – 5.4. The pedestal was fixed to the skull with Loctite® skull adhesive (Henkel, Rocky Hill, CT). The entire infusion system was secured under the skin by closing the incision around the head-mount using Vetbond™ tissue adhesive (3M, St. Paul, MN) and surgical staples. Mice were allowed four to five days to recover from surgery before beginning the place conditioning procedure to cocaine as above, except with a higher cocaine dose (10 mg/kg).

CHAPTER THREE FIGURES

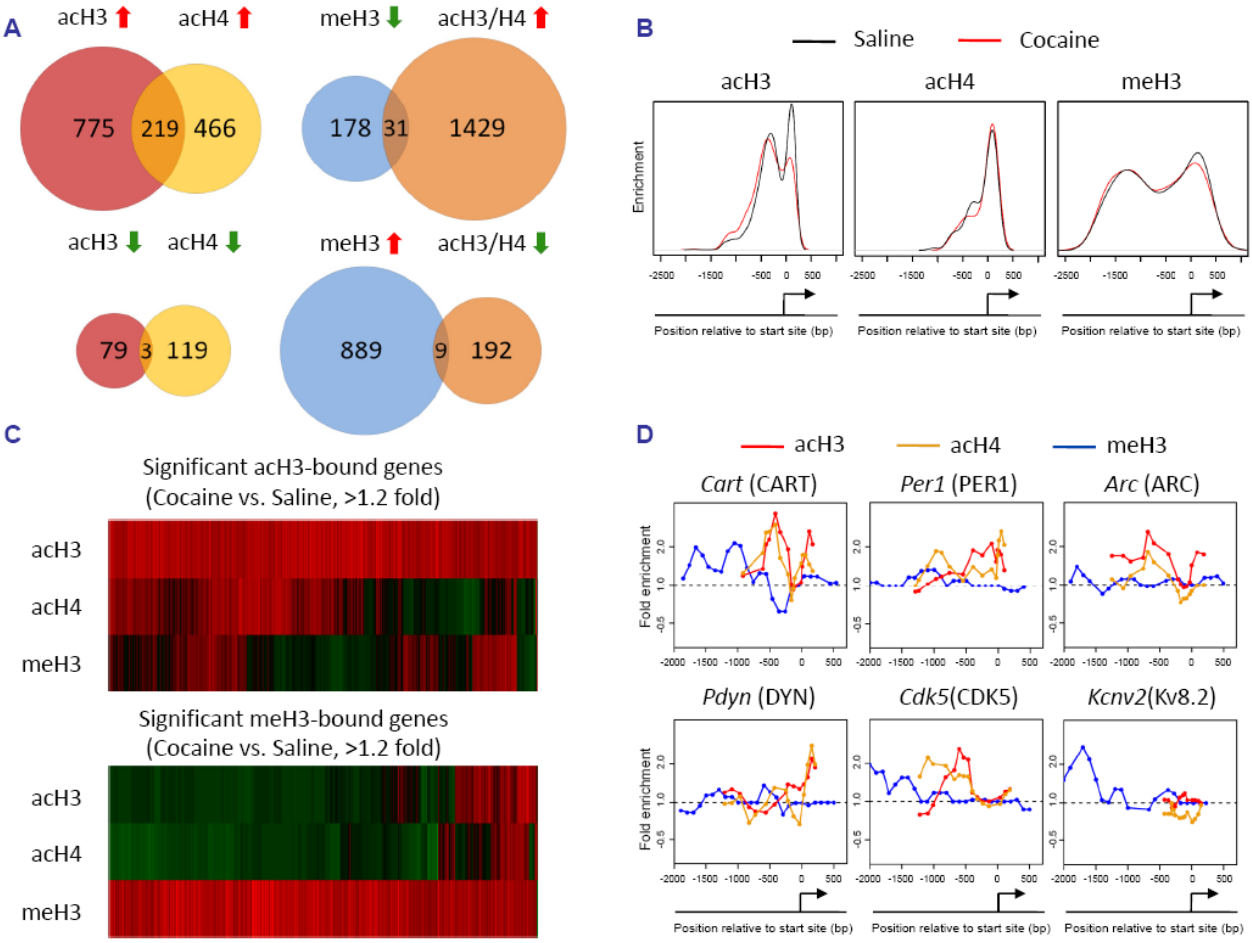


Figure 3-1. Regulation of histone acetylation and methylation at gene promoters in the NAc by chronic cocaine.

A. Venn diagrams of genes that show altered levels of H3 or H4 acetylation and H3 methylation (dimethyl-K9/K27) binding after chronic (7 days) cocaine. **B**. Genome-wide pattern of H3 and H4 acetylation and H3 methylation at gene promoters under control and cocaine-treated conditions. **C**. Heat maps illustrate relationships between cocaine regulation of histone acetylation vs. methylation at gene promoters in the NAc. Top heat maps: Only a small subset of

genes that show significant changes (red, upregulation; green, downregulation) in acetylated H3 binding after cocaine show similar changes in acetylated H4 binding, whereas many fewer still show changes in H3 methylation. Bottom heat maps: Very few of the genes that show significant changes in methylated H3 binding after cocaine show changes in H3 or H4 acetylation. **D**. Patterns of cocaine-induced changes in H3 and H4 acetylation and H3 methylation at six representative gene promoters previously implicated in cocaine action.

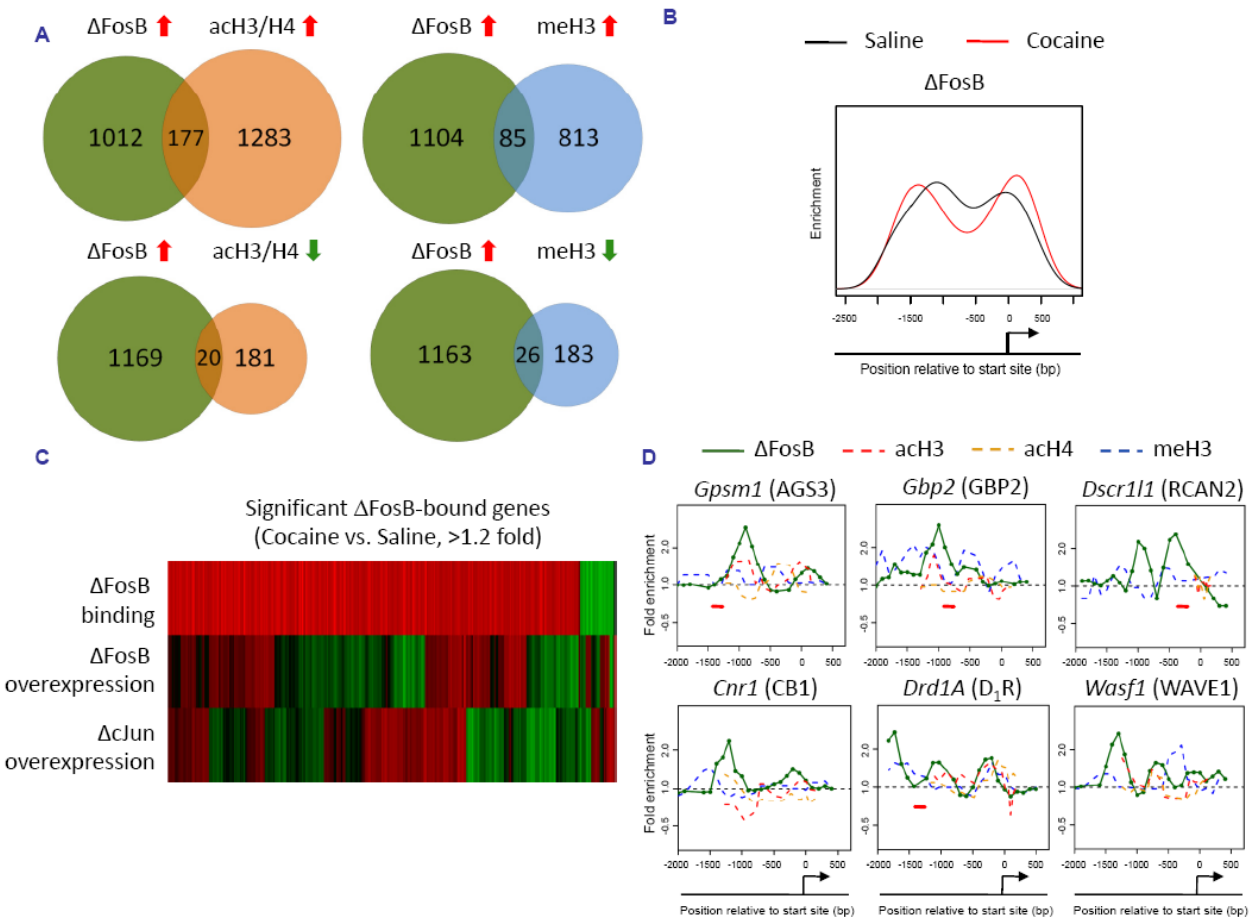


Figure 3-2. Regulation of ΔFosB binding at gene promoters in the NAc by chronic cocaine. **A.** Venn diagrams of genes that show altered levels of ΔFosB binding, or of H3 or H4 acetylation or H3 methylation, after chronic (7 days) cocaine. **B**. Genome-wide pattern of ΔFosB binding at gene promoters under control and cocaine-treated conditions**. C.** Heat maps illustrate comparison of cocaine-induced ΔFosB binding on gene promoters in the present study, and how expression of the encoded mRNA's is regulated upon inducible overexpression of ΔFosB or its dominant negative antagonist ΔcJun in the NAc (McClung and Nestler, 2003). **D**. Patterns of cocaine-induced changes in ΔFosB binding at six representative gene promoters. Coincident changes in histone acetylation and methylation are also shown. Short bold red lines under x-axes indicate positions of consensus AP1 sites.

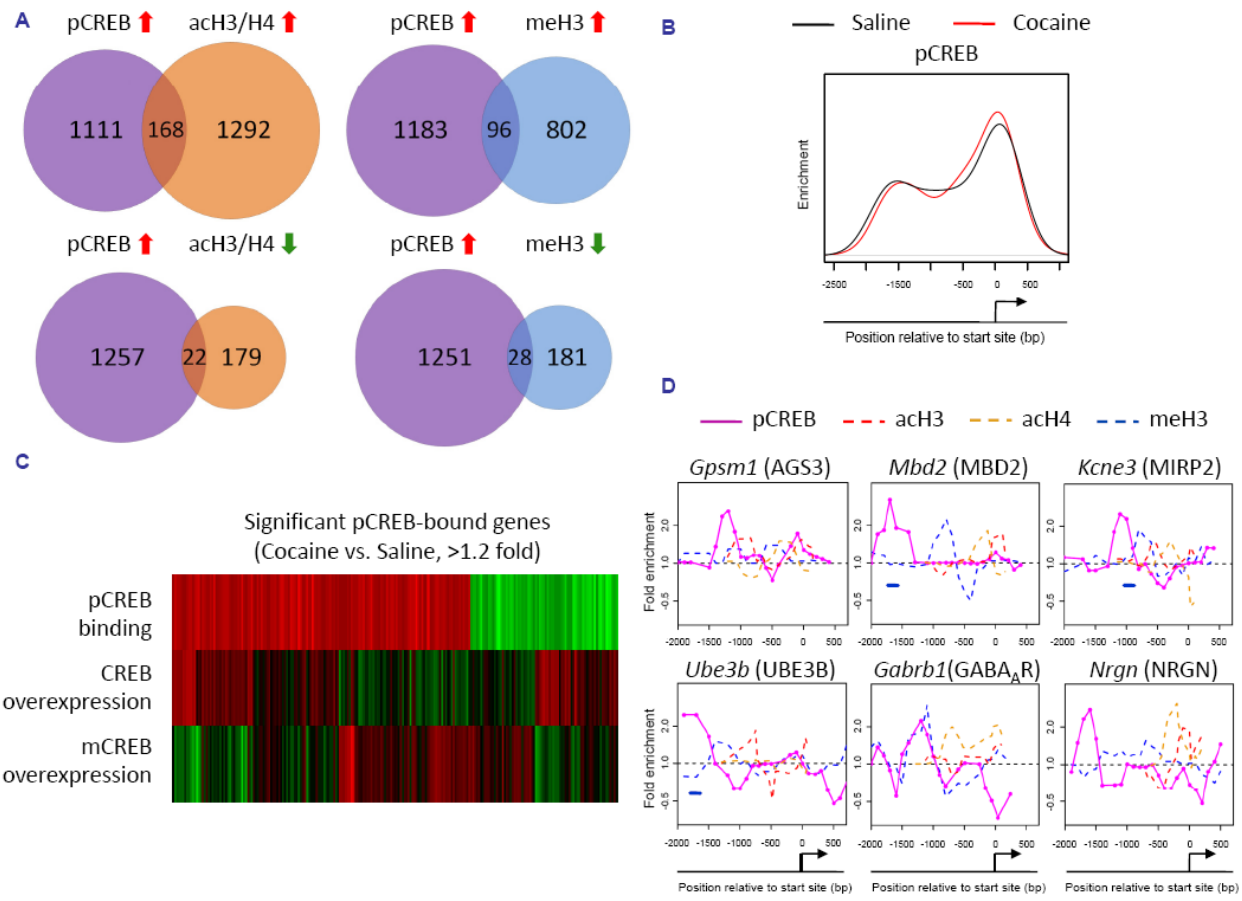


Figure 3-3. Regulation of phospho-CREB binding at gene promoters in the NAc by chronic cocaine.

A. Venn diagrams of genes that show altered levels of phospho-CREB binding, or of H3 or H4 acetylation or H3 methylation, after chronic (7 days) cocaine. **B**. Genome-wide pattern of phospho-CREB binding at gene promoters under control and cocaine-treated conditions. **C**. Heat maps illustrate comparison of cocaine-induced phospho-CREB binding on gene promoters in the present study, and how expression of the encoded mRNA's is regulated upon inducible overexpression of CREB or its dominant negative antagonist mCREB in the NAc (McClung and Nestler, 2003). **D**. Patterns of cocaine-induced changes in phospho-CREB binding at six representative gene promoters. Coincident changes in histone acetylation and methylation are also shown. Short bold blue lines under x-axes indicate position of consensus CRE sites.

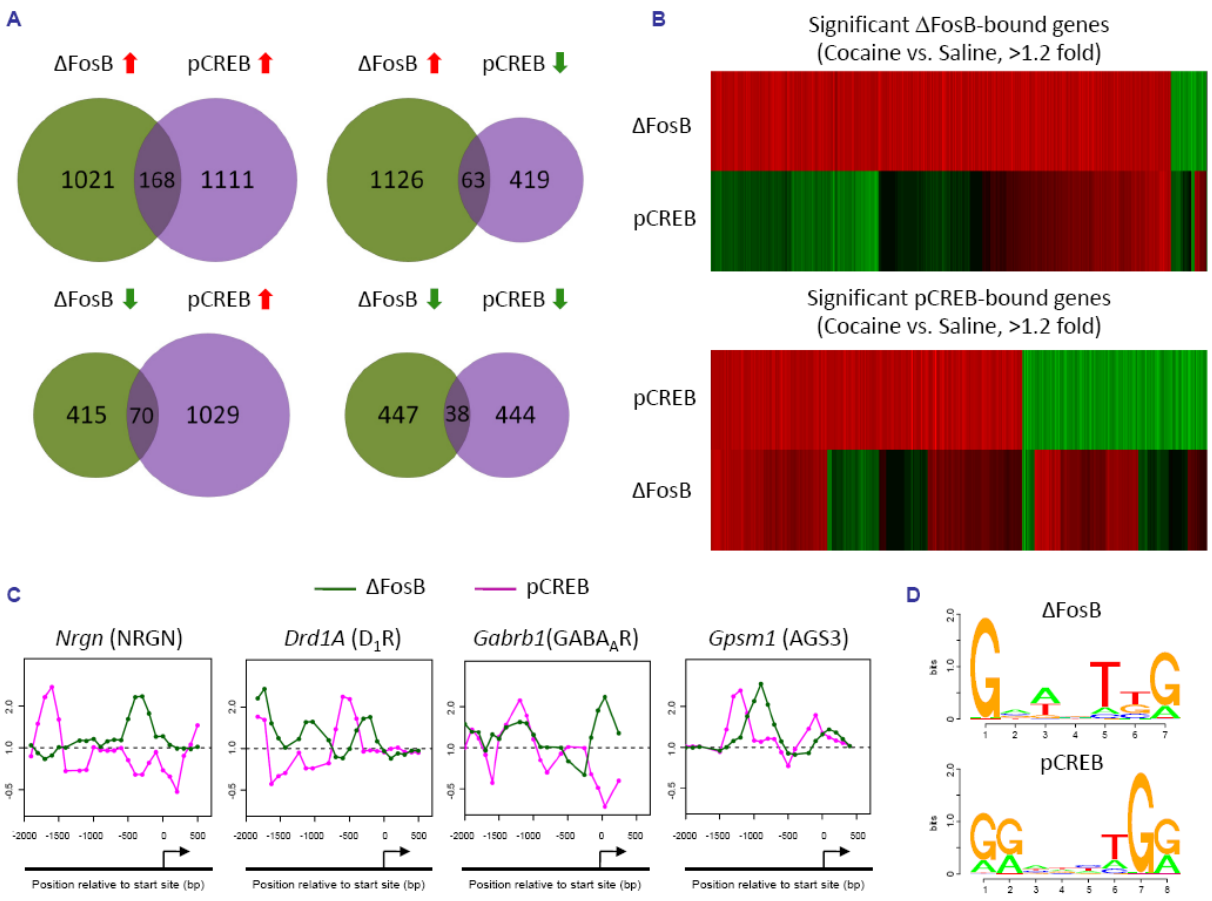


Figure 3-4. Comparison between ΔFosB- and CREB-regulated gene promoters in the NAc after chronic cocaine.

A. Venn diagrams of genes that show cocaine-induced changes in ΔFosB or phospho-CREB binding after chronic (7 days) cocaine. **B**. Heat maps illustrate comparison of cocaine-induced ΔFosB and CREB occupancy at gene promoters. At some gene promoters, cocaine increases ΔFosB and phospho-CREB binding, but most of the promoters display increased ΔFosB but decreased or no change in phospho-CREB binding (see the top heatmap), or increased phospho-CREB binding and decreased or no change in ΔFosB binding (bottom heat map). **C**. Patterns of cocaine-induced changes in ΔFosB and CREB binding at four representative gene promoters. **D**. Sequence logos of the most common 7 nucleotide binding sequences for ΔFosB (top) or 8 nucleotide binding sequences for phospho-CREB (bottom).

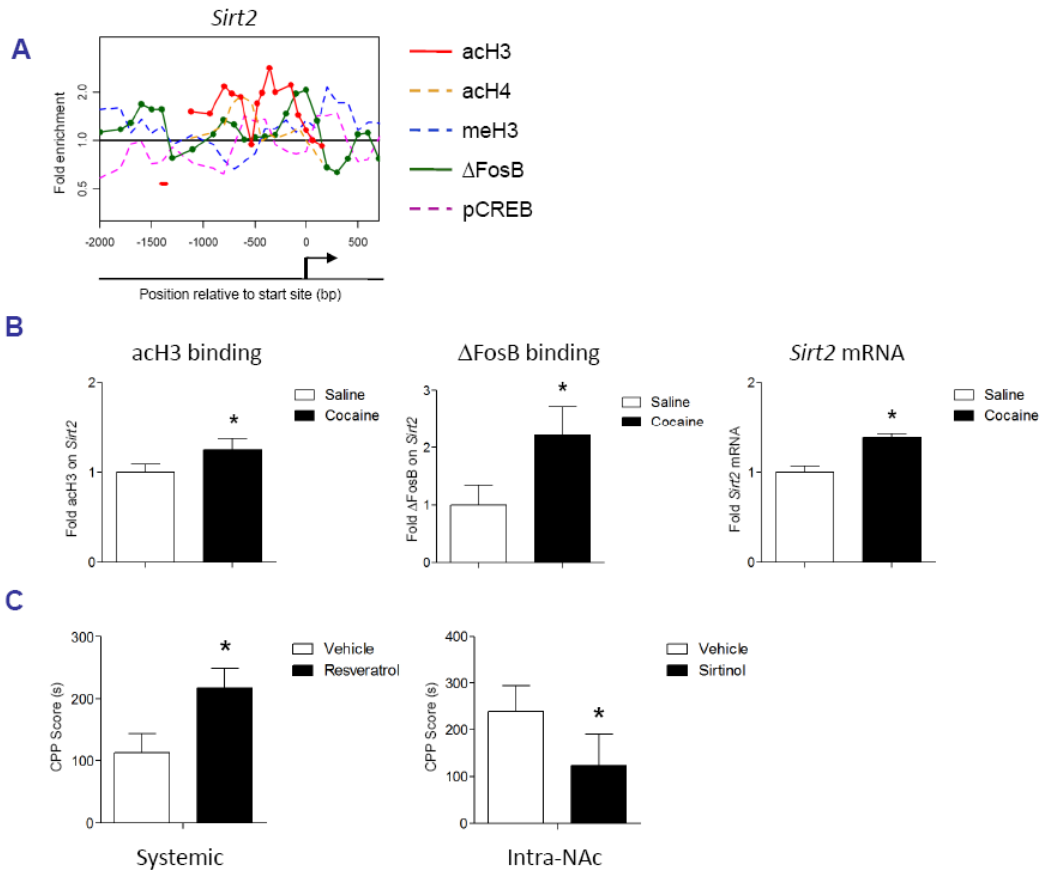


Figure 3-5. Validation of sirtuins as a novel target for cocaine.

A. Changes in histone H3 and H4 acetylation, H3 methylation, and ΔFosB and phospho-CREB binding at the Sirt2 gene promoter after chronic (7 days) cocaine. **B**. Cocaine-induced increases in H3 acetylation (left) and ΔFosB binding (middle) on the Sirt2 gene promoter were confirmed in an independent ChIP experiment. This epigenetic regulation is associated with a significant increase in Sirt2 mRNA levels in the NAc (right) ($p < 0.05$, n = 3-6). **C**. Systemic administration of the Sirtuin agonist, resveratrol (20 mg/kg ip, dissolved in 5% hydroxypropyl β-cyclodextrin vehicle) increases the rewarding effects of cocaine (5 mg/kg) in the conditioned place preference (CPP) paradigm (left). Intra-NAc injection of the sirtuin antagonist, sirtinol (50 µM in 5% hydroxypropyl β-cyclodextrin) decreases the rewarding effects of 10 mg/kg cocaine (right). Data are expressed as mean ± s.e.m. (n=9-12 in each group), *$p < 0.05$ by t-test.

FIGURE 3-6A

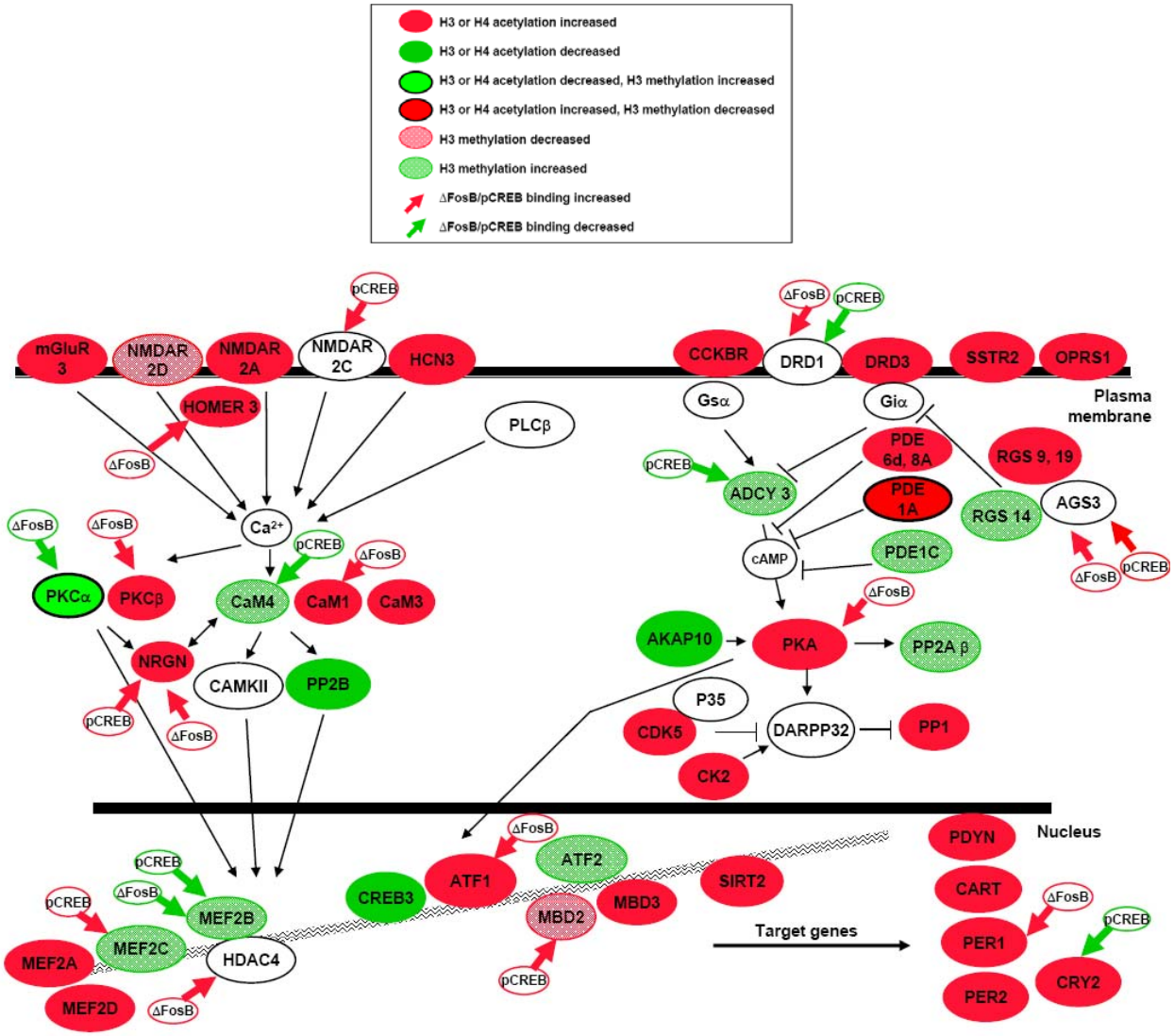

H3 or H4 acetylation increased
H3 or H4 acetylation decreased
H3 or H4 acetylation decreased, H3 methylation increased
H3 or H4 acetylation increased, H3 methylation decreased
H3 methylation decreased
H3 methylation increased
ΔFosB/pCREB binding increased
ΔFosB/pCREB binding decreased
mGluR 3
NMDAR 2D
NMDAR 2A
NMDAR 2C
HCN3
pCREB
HOMER 3
ΔFosB
PLCβ
Ca2+
PKCα
PKCβ
CaM4
CaM1
CaM3
NRGN
CAMKII
PP2B
CCKBR
DRD1
DRD3
SSTR2
OPRS1
ΔFosB
pCREB
Plasma membrane
Gsα
Giα
PDE 6d, 8A
RGS 9, 19
ADCY 3
PDE 1A
RGS 14
AGS3
cAMP
PDE1C
AKAP10
PKA
PP2A β
P35
CDK5
DARPP32
PP1
CK2
Nucleus
MEF2B
MEF2C
MEF2A
MEF2D
HDAC4
CREB3
ATF1
ATF2
MBD2
MBD3
SIRT2
Target genes
PDYN
CART
PER1
PER2
CRY2

FIGURE 3-6B

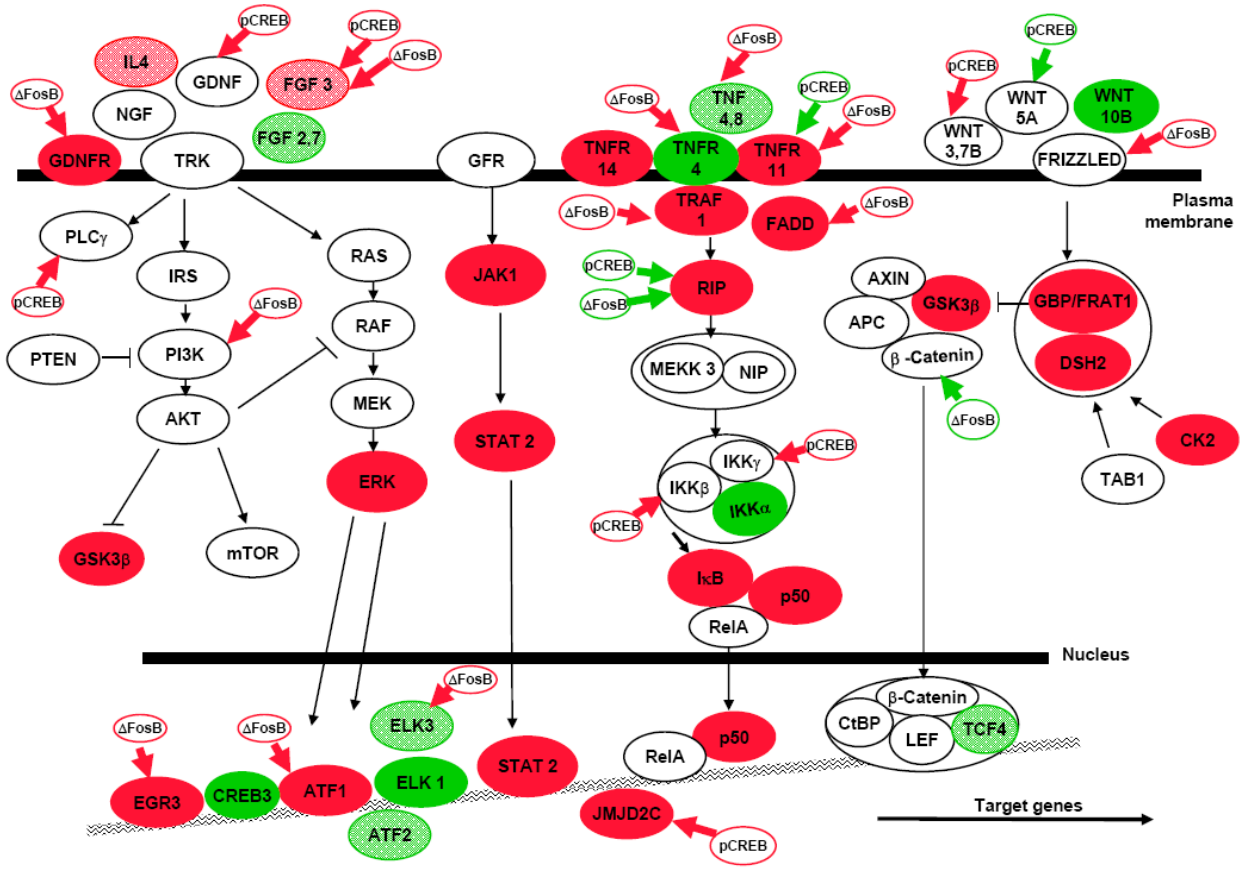
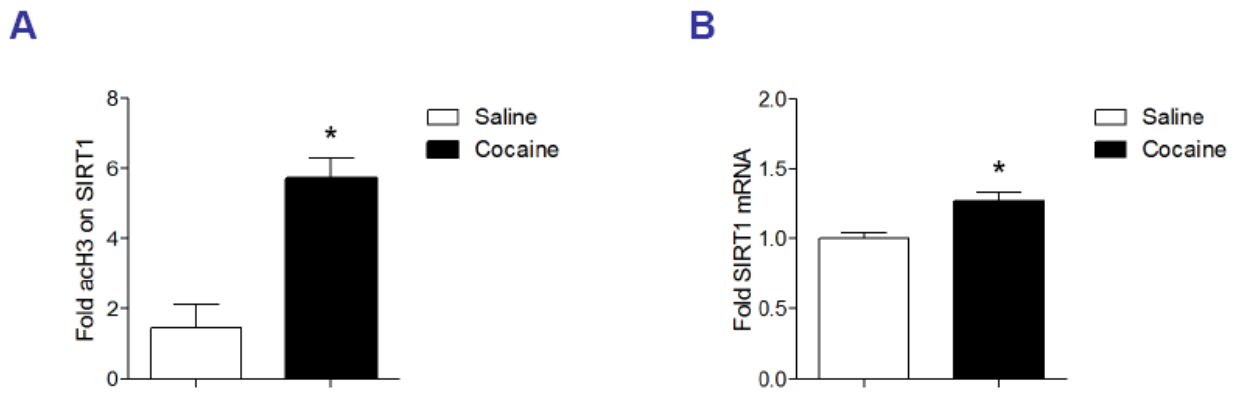


Figure 3-6. Molecular pathway analysis of the genomic effects of cocaine in the NAc. The molecular changes in the NAc of chronic cocaine-treated mice were analyzed by ChIP on chip for changes in acetylated H3 and H4, H3 dimethyl-K9/K27, ΔFosB, and phospho-CREB binding. Rigorous statistical analysis ($p<0.001$) was followed by Ingenuity molecular pathway examination. The Key defines the different types of regulation shown in the figure. **A**. Regulation of second messenger-regulated pathways, and **B**. Regulation of growth factor-regulated pathways. Red indicates modifications associated with gene activation (increased histone acetylation or decreased methylation); green, gene repression (decreased histone acetylation or increased methylation). Red arrows indicate increased TF binding while green

arrow indicates decreased binding. See Supplementary Information for definitions of the abbreviations used in the figure.

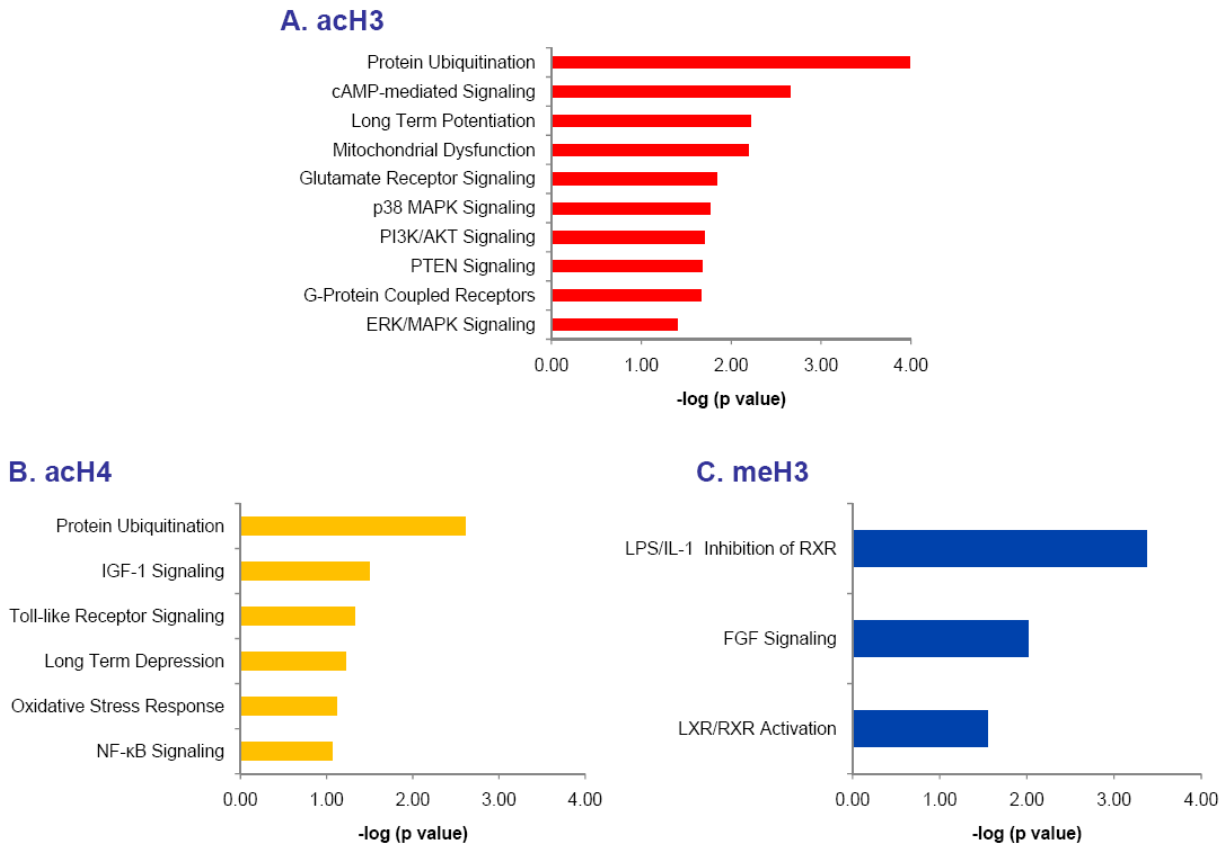
CHAPTER THREE SUPPLEMENTAL FIGURES



Supplemental Figure 3-S1. Cocaine regulation of *Sirt1*.

A. Chronic (7 days) cocaine significantly increases H3 acetylation at the *Sirt1* promoter in the NAc as measured by ChIP on an independent cohort of cocaine-treated mice ($p < 0.05$, $n = 3\text{-}6$).

B. Chronic cocaine significantly increases *Sirt1* mRNA levels in the NAc ($p < 0.05$, $n = 7\text{-}8$).



Supplemental Figure 3-S2. Molecular pathway analysis of cocaine-regulated gene promoters in the NAc.

The figure shows the top signaling pathways significantly affected in the NAc by cocaine-induced epigenetic mechanisms, as analyzed by Ingenuity pathway software. Several of these pathways are thought to contribute to cocaine action in the NAc. **A.** Significantly enriched pathways for acetylated histone H3 (acH3). **B.** Significantly enriched pathways for acetylated histone H4 (acH4). C. Significantly enriched pathways for methylated histone H3 (meH3).

TABLE 3-1. Chromatin changes on cocaine-regulated genes

Symbol	Name	acH3	acH4	meK9/27	ΔFosB	pCREB	Expression[a]	Ref[a]
Actl6B	Actin-like 6B	↑					↑	1
Ada	Adenosine deaminase			↓			↑	1
Adora1	Adenosine receptor A1	↑			↑		↑	2
Arc	Activity regulated cytoskeletal-associated protein	↑					↑	3
Atf1	Activating transcription factor 1		↑		↑		↑	*b*
Cacna1c	Ca^{2+} channel, voltage-dependent, L type, alpha 1C	↑			↑		↑	4
Card15	Caspase recruitment domain 15		↓				↓	5
Cart	Cocaine and amphetamine regulated transcript	↑	↑				↑	6,7
Cckbr	Cholecystokinin B receptor		↑					8
Cdk5	Cyclin dependent kinase 5	↑					↑	9
Cry2	Cryptochrome 2		↑			↓	↑	
Drd3	Dopamine receptor 3		↑				↑	10
Edg2	Lysophosphatidic acid receptor 2			↑		↑	↓	5
Erg3	Early growth response 3	↑			↑		↑	11
Galk1	Galactokinase 1		↑	↑			↑	12
Gfra4	GDNF family receptor alpha 4	↑	↑		↑			13
Hist1h4h	Histone H4		↑				↑	5
Igfbp6	Insulin-like growth factor binding protein 6		↑				↑	12
Htr1a	5-Hydroxy tryptamine (serotonin) receptor 1A	↓	↓			↑		14
Htr2b	5-Hydroxy tryptamine (serotonin) receptor 2B	↑			↑		↑	15
Kcnv2	Potassium channel, subfamily V, member 2, Kv8.2			↑	↑		↓	5
Grm3	Glutamate receptor, metabotropic 3	↑	↑				↑	16
Mtap2	Microtubule associated protein 2			↑			↓	5
Mutyh	MutY homolog (E. coli)	↑				↓	↑	5
Nfkb1	Nuclear factor kappa-B p50		↑				↑	17
Nrgn	Neurogranin	↑	↑		↑	↑	↑	1
Oprs1	Opioid receptor, sigma 1		↑				↑	18
Pdyn	Prodynorphin		↑				↑	3,7
Per1	Period 1		↑		↑		↑	1,3
Per2	Period 2	↑					↑	1,3
Prkg2	Protein kinase, cGMP dependent			↑		↓	↓	3
Rbmx	RNA binding motif protein, X chromosome	↑	↑			↑	↑	5
Rgs9	Regulator of G-protein signaling 9	↑					↑	19
Sema5b	Semaphorin 5B		↓		↑		↓	20
Sirt2	Sirtuin 2	↑			↑		↑	Fig 5
Sstr2	Somatostatin receptor 2		↑				↑	3
Syt1	Synaptotagmin I	↑				↑	↑	21
Tbc1d12	TBC1 domain family member 12			↑		↓	↓	5
Wnt10b	Wnt 10B protein		↓				↓	5
Zipro1	Zinc finger protein 38 (KOX25)	↑				↑	↑	5

[a]Effect of chronic cocaine on mRNA or protein expression in the NAc; see Supplementary Information for references.
[b]Unpublished data.
↑ increase; ↓ decrease ($p<0.001$).
acH3, acetylated histone H3; acH4, acetylated histone H4; meH3, dimethylated-K9/K27 histone H3; pCREB, phospho-CREB.

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CHAPTER FOUR

ΔFosB mediates epigenetic desensitization of the *c-fos* gene after chronic amphetamine exposure.

SUMMARY

The molecular mechanisms underlying the transition from recreational drug use to chronic addiction remain poorly understood. One molecule implicated in this process is ΔFosB, a transcription factor that accumulates in striatum after repeated drug exposure and mediates sensitized behavioral responses to psychostimulants and other drugs of abuse. The downstream transcriptional mechanisms by which ΔFosB regulates drug-induced behaviors are incompletely understood. We previously reported the chromatin remodeling mechanisms by which ΔFosB activates the expression of certain genes, however, the mechanisms underlying ΔFosB-mediated gene repression remain unknown. Here, we identify *c-fos*, an immediate early gene rapidly induced in striatum after psychostimulant exposure, as a novel downstream target that is repressed by ΔFosB. We show that accumulation of ΔFosB in striatum after chronic amphetamine treatment desensitizes *c-fos* mRNA induction to a subsequent drug dose. ΔFosB desensitizes *c-fos* expression by recruiting histone deacetylase 1 (HDAC1) to the *c-fos* gene promoter, which in turns deacetylates surrounding histones and attenuates gene activity. Accordingly, local knockout of HDAC1 in striatum abolishes amphetamine-induced desensitization of the *c-fos* gene. In concert, chronic amphetamine increases histone H3 methylation on the *c-fos* promoter, a chromatin modification also known to repress gene activity,

as well as expression levels of the H3 histone methyltransferase, KMT1A/SUV39H1. This study reveals a novel epigenetic pathway through which ΔFosB mediates distinct transcriptional programs and ultimately behavioral plasticity to chronic amphetamine exposure.

INTRODUCTION

Repeated use of psychostimulants such as amphetamine and cocaine often results in a transition from recreational drug use to a chronically addicted state (Hyman et al., 2006). One mechanism implicated in this process involves the transcription factor ΔFosB, a highly stable splice product of the immediate early gene *fosB*, which dimerizes with Jun family proteins to form functional AP-1 transcriptional complexes (McClung et al., 2004). ΔFosB accumulates several-fold in striatum after repeated exposure to drugs of abuse, and this accumulation has been linked to increased cocaine reward, locomotor sensitization, and self-administration (Kelz et al., 1999; Colby et al., 2003; McClung et al., 2004), which together suggest a role in the neural mechanisms involved in transitioning between recreational and addicted drug use. According to this hypothesis, ΔFosB functions in a positive feedback loop by increasing drug-seeking behaviors, which in turn induce more ΔFosB. One key outstanding question is how ΔFosB mediates its effects on drug-related behaviors. Genome-wide microarray studies in mice that overexpress ΔFosB in striatum provided the first insight into potential downstream targets (McClung and Nestler, 2003). This study suggested that ΔFosB can serve as a transcriptional activator or repressor, depending on the target gene. However, the study examined transcripts regulated in an overexpression setting, so it is not clear which of these genes are direct, physiological ΔFosB targets.

We recently identified the cyclin-dependent kinase 5 (*cdk5*) gene as a direct target for endogenous ΔFosB, which promotes *Cdk5* transcription in striatum (Kumar et al., 2005). However, the mechanisms involved in ΔFosB's repression of target genes have remained elusive. One attractive candidate is *c-fos*, a gene which is induced dramatically by acute psychostimulants

but only weakly after repeated exposure (Hope et al., 1992; Persico et al., 1993; Steiner and Gerfen, 1993), when levels of ΔFosB and ΔFosB-containing AP-1 complexes are high (Hope et al., 1992, 1994). Since the *c-fos* gene contains an AP-1-like site in its proximal promoter (Morgan and Curran, 1989), it is a plausible candidate for ΔFosB-mediated repression. Induction of *c-fos* is traditionally viewed as an early marker of neural activation, since it is rapidly and transiently induced in response to a variety of stimuli (Morgan and Curran, 1989). The *c-fos* gene is also important for behavioral responses to cocaine, as mice lacking *c-fos* in dopamine D1 receptor-containing neurons, the neuronal cell type where ΔFosB is induced by psychostimulants (McClung et al., 2004), have reduced behavioral sensitization to cocaine (Zhang et al., 2006). These findings led us to investigate whether ΔFosB controls *c-fos* gene activity after chronic amphetamine exposure. We describe here a novel epigenetic mechanism by which ΔFosB accumulation in response to chronic amphetamine feeds back to desensitize *c-fos* induction to subsequent drug doses. This novel interplay between ΔFosB and chromatin remodeling events on the *c-fos* promoter may be an important homeostatic mechanism to regulate an animal's sensitivity to repeated drug exposure.

RESULTS

ΔFosB desensitizes *c-fos* mRNA induction in striatum after chronic amphetamine exposure.

To explore whether the desensitization of *c-fos* mRNA expression is a cellular adaptation controlled by ΔFosB, we treated rats with saline or acute or chronic amphetamine and let them withdraw in their home cage for 1 to 10 days. The rats were then analyzed 1 hr after a saline or amphetamine challenge dose. As demonstrated previously (see Introduction), *c-fos* mRNA was induced 4-fold in striatum by acute amphetamine administration. In rats previously exposed to chronic amphetamine, however, the expression of *c-fos* in response to drug challenge was significantly attenuated for up to 5 days of drug withdrawal (Figure 4-1A), a point at which ΔFosB remains elevated in this brain region (Hope et al., 1994). Additionally, in rats that were withdrawn from chronic amphetamine for 5 days, we found that basal *c-fos* mRNA expression was reduced below levels found in saline-treated controls (Figure 4-1A). Importantly, the magnitude of *c-fos* induction to an amphetamine challenge was significantly attenuated at day 1 of withdrawal compared to saline-treated animals. Together, these findings demonstrate an effect of chronic amphetamine on both basal and induced *c-fos* mRNA levels, although with the two effects occurring with a complex time course.

To determine whether ΔFosB accumulation after chronic amphetamine directly contributes to the desensitization of *c-fos* expression, we first performed ChIP for ΔFosB on the *c-fos* gene promoter in striatum. As shown in Figure 4-1B, the *c-fos* promoter has significantly more ΔFosB bound after chronic amphetamine exposure, an effect seen for at least 5 days of drug withdrawal. These data correlate ΔFosB occupancy on the *c-fos* promoter with the kinetics of reduced *c-fos* gene activity. Next, to directly test whether ΔFosB causes reduced *c-fos*

induction in response to amphetamine challenge, we used an AAV vector to overexpress either ΔFosB, or GFP as a control, in striatum. We then isolated the infected striatum by laser microdissection (Figure 4-1C) and performed qRT-PCR for *c-fos* mRNA. We observed significantly less *c-fos* mRNA induced after an acute dose of amphetamine in the striatal tissue infected with AAV-ΔFosB compared to the contralateral side infected with AAV-GFP, while levels of *β-tubulin* mRNA remained unchanged (Figure 4-1D). These data suggest that *c-fos* desensitization is mediated by accumulation of ΔFosB on its promoter after chronic amphetamine exposure.

ΔFosB recruits HDAC1 to the *c-fos* promoter to mediate *c-fos* gene repression.

To explore the mechanisms by which ΔFosB mediates *c-fos* desensitization, we focused on the time point at which *c-fos* was most significantly repressed: 5 days of withdrawal from chronic amphetamine. A key mechanism involved in *c-fos* activation in response to a variety of stimuli, including cocaine (Kumar et al., 2005), is histone acetylation. We were therefore interested to determine whether histone acetylation on the *c-fos* gene promoter was also induced by acute amphetamine and whether repeated drug exposure attenuated this response. Indeed, acute amphetamine increased histone H4 acetylation on the *c-fos* promoter and, after chronic amphetamine treatment, this induction was no longer observed (Figure 4-2A). Acetylation of H4 was specific, as no effect was observed for H3 (not shown). These data suggest that reduced histone acetylation, associated with a more compact and inactive chromatin structure (Kouzarides, 2007), contributes to the desensitization of the *c-fos* gene after chronic amphetamine exposure. To directly test this hypothesis, we treated rats with chronic amphetamine and, after 5 days of withdrawal, administered the HDAC inhibitor, sodium butyrate

or its vehicle. We found that sodium butyrate reversed the amphetamine-induced repression of *c-fos* expression (Figure 4-2B), directly supporting the idea that hypoacetylation on the *c-fos* promoter is a key mechanism underlying desensitization of the gene.

To understand how ΔFosB inhibits histone acetylation on the *c-fos* promoter, we investigated whether ΔFosB interacts with enzymes that reduce histone acetylation, namely, HDACs. We first explored HDAC1 and HDAC2 because these enzymes form complexes with a variety of transcription factors to repress gene expression (Grozinger and Schreiber, 2002). Since preliminary ChIP studies identified significant HDAC1 binding on the *c-fos* promoter (see below), but no detectable HDAC2 (not shown), we performed co-immunoprecipitation experiments to determine whether ΔFosB physically interacts with HDAC1. Indeed, we found that immunoprecipitation of ΔFosB also pulled down HDAC1 in PC12 cells (Figure 4-2D). Importantly, this interaction is specific for ΔFosB, as full-length FosB, which does not accumulate after chronic psychostimulant administration (Hope et al., 1994), did not interact with HDAC1. We performed the reverse experiment *in vivo* by inducing large amounts of ΔFosB with electroconvulsive seizures. Consistent with our cell culture data, immunoprecipitation with an antibody against HDAC1 pulled down ΔFosB from brain tissue (Figure 4-2E).

Based on these findings that ΔFosB and HDAC1 physically interact *in vitro* and *in vivo*, we hypothesized that, after chronic amphetamine, ΔFosB recruits HDAC1 to the *c-fos* gene promoter. Indeed, ChIP of striatal lysates found significantly higher levels of HDAC1 on the *c-fos* promoter after chronic amphetamine exposure (Figure 4-2C), whereas amphetamine did not alter HDAC1 binding to the *β-actin* gene promoter. To directly determine whether HDAC1 was sufficient to attenuate *c-fos* induction, we transfected HEK293T cells with HDAC1 or GFP and

stimulated them with 5% serum (see Supplemental Methods). We found that serum-induced *c-fos* expression was significantly blunted in cells overexpressing HDAC1 (Figure 4-2F). These studies were extended *in vivo* by using floxed HDAC1 mice infected with AAV-GFP on one side of their striatum and AAV-CreGFP to induce local knockout of the *hdac1* gene in the contralateral striatum. AAV-CreGFP reduced *Hdac1* mRNA expression in the infected tissue (isolated by laser microdissection) by >75% compared to AAV-GFP injected controls while *Hdac2* expression remained unchanged (Figure 4-2G). Mice were then treated with chronic amphetamine followed by drug withdrawal for 5 days. The mice were analyzed 30 minutes after amphetamine challenge and the infected striatal regions were microdissected. We found that amphetamine induced significantly more *c-fos* mRNA in striatal tissue infected with AAV-CreGFP compared to AAV-GFP (Figure 4-2G), demonstrating that HDAC1 is necessary for chronic amphetamine-induced repression of *c-fos* expression. These data suggest that ΔFosB accumulation in rats after chronic amphetamine treatment results in more ΔFosB binding to the *c-fos* promoter, recruitment of HDAC1, less histone acetylation, and ultimately less activity of the gene.

Histone methylation is elevated on the *c-fos* promoter after chronic amphetamine exposure.

Repression of gene activity often involves several epigenetic modifications that occur in parallel (Kouzarides, 2007; Tsankova et al., 2007). One of the best characterized histone modifications associated with reduced gene activity is methylation of histone H3 at lysine 9 (H3K9). This histone modification, when found on promoter regions, is associated with transcriptional repression by recruiting co-repressors such as HP1 (heterochromatin protein 1) (Kouzarides, 2007). We therefore analyzed whether hypoacetylation of the *c-fos* gene, seen after

chronic amphetamine administration, is also associated with alterations in H3K9 methylation. Consistent with this hypothesis, ChIP carried out on striatal tissue from rats treated with chronic amphetamine revealed that di-methylated H3K9 (H3K9me2) was significantly increased on the *c-fos* promoter (Figure 4-3A), an effect not observed on the *β-actin* gene promoter. One of the key enzymes which mediates H3K9 methylation is KMT1A/SUV39H1, which raised the question of whether the expression of this enzyme was regulated by chronic amphetamine exposure. We performed qRT-PCR on the striatum of rats treated with chronic amphetamine and observed a significant upregulation of *Kmt1a/Suv39h1* mRNA, while the distinct chromatin modifying enzyme, *Hdac5*, remained unaffected (Figure 4-3B). Unlike HDAC1, however, co-immunoprecipitation experiments did not reveal any detectible interaction between ΔFosB and KMT1A/SUV39H1, nor were we able to identify significant enrichment of the methyltransferase on the *c-fos* promoter by ChIP (not shown). Regardless, these findings suggest that upregulation of KMT1A/SUV39H1 may hypermethylate H3 at *c-fos* and contribute to the mechanisms reducing *c-fos* gene activity after chronic amphetamine exposure.

DISCUSSION

This study identified *c-fos* as a novel downstream target gene of ΔFosB in the striatum after chronic amphetamine administration. We provide direct evidence that endogenous ΔFosB binds to the *c-fos* promoter *in vivo*, where ΔFosB recruits HDAC1 to deacetylate surrounding histones and reduce the transcriptional activity of the *c-fos* gene. Both pharmacological inhibition of HDACs and the inducible knockout of HDAC1 were sufficient to alleviate *c-fos* desensitization and elevate *c-fos* expression in the striatum of chronic amphetamine-treated

animals. We also found concurrent increases in repressive histone methylation at H3K9 on the *c-fos* promoter, an adaptation associated with amphetamine-induced upregulation of the histone methyltransferase, KMT1A/SUV39H1. Together, these findings provide fundamentally new insight into the mechanisms by which ΔFosB represses the activity of certain genes and illustrates a novel interplay between two key pathways that control behavioral responses to psychostimulants: ΔFosB induction (McClung et al., 2004) and chromatin remodeling (Tsankova et al., 2007). Our findings show how these two pathways converge on the *c-fos* promoter after chronic amphetamine exposure to alter activity of the gene.

We first observed desensitization of *c-fos* mRNA expression after chronic cocaine treatment over 15 years ago (Hope et al., 1992), but no mechanistic insight has been available into how such profoundly different transcriptional responses could occur between acute versus chronic drug exposure. In our effort to understand downstream actions of ΔFosB, we revisited control of *c-fos* expression because of this differential regulation between acute and chronic psychostimulants exposure. Since ΔFosB is elevated several-fold after chronic drug exposure, this differential induction of *c-fos* mRNA, as well as an AP-1-like site in the *c-fos* proximal promoter, suggested a potential regulatory role for ΔFosB. This also made the *c-fos* gene an attractive candidate with which to study the repressive effects of ΔFosB on gene expression (McClung and Nestler, 2003).

Chronic amphetamine attenuated *c-fos* mRNA induction or its baseline levels in striatum for approximately 5 days of drug withdrawal, a time course that is consistent with the stability of ΔFosB (Hope et al., 1994) and its occupancy on the *c-fos* promoter. Although ΔFosB can be detected after even longer periods of withdrawal, it gradually declines over time (Hope et al., 1994; Nye et al., 1995) and may be insufficient to maintain repression of the *c-fos* gene much

beyond the 5 day time point. Nevertheless the time course of *c-fos* desensitization is complex, with suppression of its fold-induction by an amphetamine challenge maximal at 1 day of withdrawal, but suppression of its basal levels maximal at 5 days of withdrawal. Our ChIP data show that ΔFosB is bound to the *c-fos* promoter at both time points, suggesting that the differential activity of the *c-fos* gene observed between 1 and 5 days of withdrawal may be due to additional transcriptional regulators recruited to the gene with a very complicated time course. Further studies are needed to understand the detailed mechanisms involved.

The behavioral significance of ΔFosB-mediated *c-fos* desensitization may be homeostatic, as mice that lack the *c-fos* gene in dopamine D1 receptor-containing neurons show reduced behavioral responses to cocaine (Zhang et al., 2006). Moreover, HDAC inhibitors, which block ΔFosB-mediated desensitization of *c-fos*, increase an animal's sensitivity to the behavioral effects of cocaine (Kumar et al., 2005; Renthal et al., 2007). These findings suggest that while ΔFosB's net effect is to promote sensitized behavioral responses to psychostimulants (Kelz et al., 1999; Colby et al., 2003), it also initiates a novel transcriptional program through *c-fos* desensitization to limit the magnitude of these same behaviors. ΔFosB would, in effect, titrate behavioral responses to psychostimulants through a complex series of downstream transcriptional events, involving the induction or repression of numerous target genes (McClung and Nestler, 2003), which, in addition to the gene encoding c-Fos as shown here, also include the AMPA glutamate receptor subunit GluR2 (Kelz et al., 1999), the serine-threonine kinase Cdk5 (Bibb et al., 2001), and the opioid peptide dynorphin (Zachariou et al., 2006), among others (McClung and Nestler, 2003). Some of these genes are activated by ΔFosB (where ΔFosB recruits transcriptional co-activators) (Kumar et al., 2005), whereas others are repressed by ΔFosB (where ΔFosB, as shown here, recruits transcriptional co-repressors). A major effort of

future research is to identify the factors that determine whether ΔFosB activates or represses a target gene when it binds to the gene promoter.

Taken together, our findings identify a novel epigenetic mechanism through which ΔFosB mediates part of its transcriptional effects in the striatum after chronic amphetamine exposure. This study also provides important new insight into the basic transcriptional and epigenetic mechanisms *in vivo* involved in the desensitization (i.e., tolerance) of a crucial gene for psychostimulant-induced behavioral responses.

EXPERIMENTAL PROCEDURES

RNA isolation and quantification

Frozen brain tissue was thawed in TriZol (Invitrogen, Carlsbad, CA) and processed according to the manufacturer's protocol. RNA was purified with RNAesy Micro columns (Qiagen, Valencia, CA). Total RNA was reverse-transcribed using Superscript III (Invitrogen). Real-time PCR was then run using SYBR Green (ABI, Foster City, CA) and quantified using the ΔΔCt method.

Chromatin immunoprecipitation (ChIP)

Chromatin was sonicated and then immunoprecipitated (see Supplemental Methods) using acetylated histone antibodies (Millipore, Billerica, MA), anti-HDAC1, or anti-H3K9me2 from Abcam (Cambridge, UK), anti-FosB(C-terminus) (Kumar et al., 2005), anti-FosB(N-terminus) (Santa Cruz Biotechnology, Santa Cruz, CA, State), or a rabbit IgG control (Millipore). The IP was collected using Protein A beads from Millipore. After washing,

chromatin was eluted from the beads and reverse cross-linked in the presence of proteinase K. DNA was then purified and quantified using real-time PCR.

Immunoprecipitation

PC12 cells were transfected with V5-tagged HDAC1 (Montgomery et al., 2007), FosB, or ΔFosB as described previously (Carle et al., 2007). Cell lysates were split and incubated with either non-immune IgG (Sigma) or anti-FosB antibodies (sc-48, Santa Cruz) overnight at 4°C. Immunoprecipitation was performed with Protein G beads (Sigma). The immunoprecipitated proteins were run with SDS-PAGE and analyzed by Western blotting using a custom polyclonal anti-FosB(N-terminus) antibody (Carle et al., 2007) and anti-V5 antibody (Abcam). To determine if HDAC1 and ΔFosB are binding partners *in vivo*, we used repeated electroconvulsive seizures to induce high levels of ΔFosB protein (Hope et al., 1994). Cortical tissue was dissected from chronic (7 daily) seizure or sham-treated rats, lysed, and immunoprecipitated as described above with anti-HDAC1 antibodies (Abcam).

Laser capture microdissection

Using stereotactic surgery, the ventral striata of mice were infected with an adeno-associated virus (AAV) expressing the indicated gene or GFP on opposite sides of the brain. After amphetamine treatment, frozen brains were processed into 8 μm-thick coronal sections and mounted onto membrane slides (Lieca, Wetzlar, Germany). AAV-infected regions were laser-dissected (Leica) to exclude non-infected cells and processed with PicoPure RNA extraction kit (MDS, Sunnyvale, CA). RNA was amplified with the RiboAmp HS kit (MDS) and reverse transcribed as described above. See Supplemental Methods for complete details.

SUPPLEMENTAL METHODS

Animals

Animals used in this study were 8 - 12 weeks old and housed on a 12-hour light-dark cycle with access to food and water *ad labitium*. Male Sprague-Dawley rats were obtained from Harlan. C57BL/6 mice were obtained from Jackson Laboratory. Floxed HDAC1 (histone deacetylase) mice were generated as described (Montgomery et al., 2007). All procedures were in accordance with UT Southwestern's Animal Care and Use guidelines.

Drugs

D-Amphetamine and sodium butyrate were purchased from Sigma (St. Louis, MO).

Treatments

Rats were treated with saline or 4 mg/kg amphetamine (i.p.) once daily for 6 days. On the 7^{th} day, saline control rats received a final dose of saline, acute amphetamine rats received their first dose of amphetamine, and chronic amphetamine rats received a 7^{th} dose of amphetamine. Rats were killed 1 hour after the last injection unless otherwise specified. Some chronically treated rats were allowed to withdrawal in their home cages for 1 - 10 days before receiving a final saline, amphetamine, and/or sodium butyrate injection.

Floxed HDAC1 mice received a similar injection protocol: chronic amphetamine (4 mg/kg) for 7 days followed by 5 days of withdrawal. They were then given a challenge dose of 2 mg/kg amphetamine and sacrificed 30 min later. Mice which received viral delivery of AAV-

ΔFosB in their striatum were given an acute injection of 2 mg/kg amphetamine and sacrificed after 30 min to assess the effect of high levels of ΔFosB on the induction of *c-fos* mRNA *in vivo*.

All animals were sacrificed by decapitation and their brains were rapidly dissected and placed into ice-cold 1X PBS. Rat brains were then coronally sectioned (1 mm) using a brain matrix and whole striatum was collected. Striatal tissue was then either fixed in formaldehyde for ChIP studies (see below) or directly frozen on dry ice for mRNA studies (see main Methods). For downstream use in laser capture microdissection, all mouse brains were washed several times in ice-cold 1X PBS, flash-frozen directly on dry ice, and stored at -80ºC until sectioning.

Chromatin immunoprecipitation (ChIP)

Rat striatum was dissected as described above and incubated in 1% formaldehyde for 12 minutes. Glycine (0.125M final concentration) was added and allowed to incubate for an additional 5 minutes to stop the cross-linking reaction. Tissue was then washed 4-5 times in ice-cold 1X PBS and stored at -80ºC until processing. Tissue was then lysed in an SDS lysis buffer (Millipore, Billercia, MA) and sonicated to an average length of 500 bp. Sonicated chromatin was diluted in ChIP dilution buffer (Millipore) and then immunoprecipitated using specific antibodies (described in main Methods) overnight at 4ºC. The antibody-chromatin complex was immunoprecipitated by incubation with Protein A beads from Millipore (06-157) for 1.5hrs. The beads were washed with ChIP dilution buffer, low salt buffer, high salt buffer, LiCl buffer, and twice with 1X TE (all from Millipore). Chromatin was eluted from the beads in 500µL of elution buffer (0.42mg $NaHCO_3$, 5mL 10% SDS, in 50mL H_2O). Chromatin was reverse cross-linked overnight at 65°C in the presence of proteinase K. DNA was then purified with phenol/cholorophorm/isoamyl alcohol, and precipitated with ethanol. DNA was eluted in water

and directly quantified using real-time PCR and promoter-specific primers. Selective ChIPs for ΔFosB were obtained by sequential immunoprecipitations as described and validated previously (Kumar et al., 2005), first using C-terminal antibody which immunodepletes full length FosB but not ΔFosB, and secondly with an N-terminal antibody to immunprecipitate the remaining ΔFosB

.

Viral mediated gene transfer

Mice were used for local injection studies since a single injection covers a much larger portion of mouse striatum than rat, and within striatum differences have been observed in ΔFosB induction by psychostimulants (Hiroi et al., 1997). Expression plasmids for ΔFosB, GFP, or Cre recombinase were subcloned into an adeno-associated viral vector (Stratagene, La Jolla, CA) and packaged into high-titer viral particles as described previously (Hommel et al., 2003). Briefly, HEK293 cells were transfected with AAV-ΔFosB, pHelper, and pAAV-RC plasmids (Stratagene) with calcium phosphate. Approximately 3 days after transfection, cells were lysed and the lysate was separated on an iodixanol step gradient. The viral fraction was purified on a heparin column and concentrated. We confirmed *in vivo* that the neurons infected with AAV-ΔFosB generate high levels of ΔFosB mRNA (data not shown) and protein (Winstanley et al., 2007). To deliver the virus into the ventral striatum, stereotactic surgery was performed on mice under general anesthesia with a ketamine/xylazine cocktail. We targeted the nucleus accumbens shell and core, the ventral portion of the striatum which is closely associated with amphetamine's behavioral effects. The following coordinates were used: +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

fluorescent microscopy to visualize GFP expression in cryosections through the nucleus accumbens.

Cell culture

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics at 37ºC and 5% CO_2. Cells were transfected with AAV plasmids (Stratagene,) expressing GFP alone or human HDAC1 + GFP using FuGene (Roche, Nutley, NJ) according to the manufacturer's instructions. Transfection efficiency was >70% and determined by visualizing GFP. Twenty-four hours after transfection, cells were starved in serum-free media for 24 hrs. Cells were then stimulated with 5% FBS in DMEM for 1 hr and processed using the RNAeasy Mico Kit (Qiagen). RNA was reverse-transcribed using Superscript III (Invitrogen), which was then quantified by SYBR-green based quantitative PCR (Applied Biosystems, Foster City, CA).

Laser capture microdissection

C57Bl/6 mice were infected with AAV-GFP on one side of the nucleus accumbens or AAV-ΔFosB on the other. 10 weeks later [2 weeks for viral expression to initiate, and roughly 8 weeks of ΔFosB overexpression required for its maximal behavioral effects (McClung and Nestler, 2003)] mice were given an acute dose of amphetamine (2 mg/kg) and analyzed 30 minutes later. Floxed HDAC1 mouse were infected with AAV-GFP on one side of the nucleus accumbens and AAV-CreGFP on the other. After 18 days for viral expression to induce recombination, which was sufficient to knock down *Hdac1* mRNA levels > 75% (Figure 4-2G), mice were treated for 7 days with 4 mg/kg amphetamine. After 5 days of withdrawal, mice

received a challenge dose of amphetamine (2 mg/kg) and were analyzed 30 minutes later. Brains were rapidly dissected, washed in 1X PBS, and flash frozen on dry ice. Frozen brains were then sliced on cryostat into 8uM sections and carefully placed on membrane slides (Lieca, Bannockburn, IL). Slides were kept at -80ºC until further processing on the laser capture microscope (Leica). Slides were then removed from dry ice and dehydrated in 70% ethanol for 20 seconds followed by 95% and 100% ethanol each for 20 seconds. Slides were then air dried and mounted in the laser capture microscope. The infected regions could be visualized by fluorescent microscopy since all viruses used co-expressed GFP. Equal amounts of infected tissue were laser-dissected and collected in a thin-walled sterile 0.2mL tube containing the RNA extraction buffer included in the PicoPure RNA extraction kit (MDS, Sunnyvale, CA). Following tissue collection, tubes were incubated at 45ºC for 30 min and frozen on dry ice until RNA purification and amplification. RNA was purified using the PicoPure RNA extraction kit (MDS) exactly as described by the manufacturer and immediately processed with the RiboAmp HS kit (MDS). One round of RNA amplification was performed before the amplified RNA was reverse-transcribed using Superscript III (Invitrogen) and quantified by qPCR (Applied Biosystems).

CHAPTER FOUR FIGURES

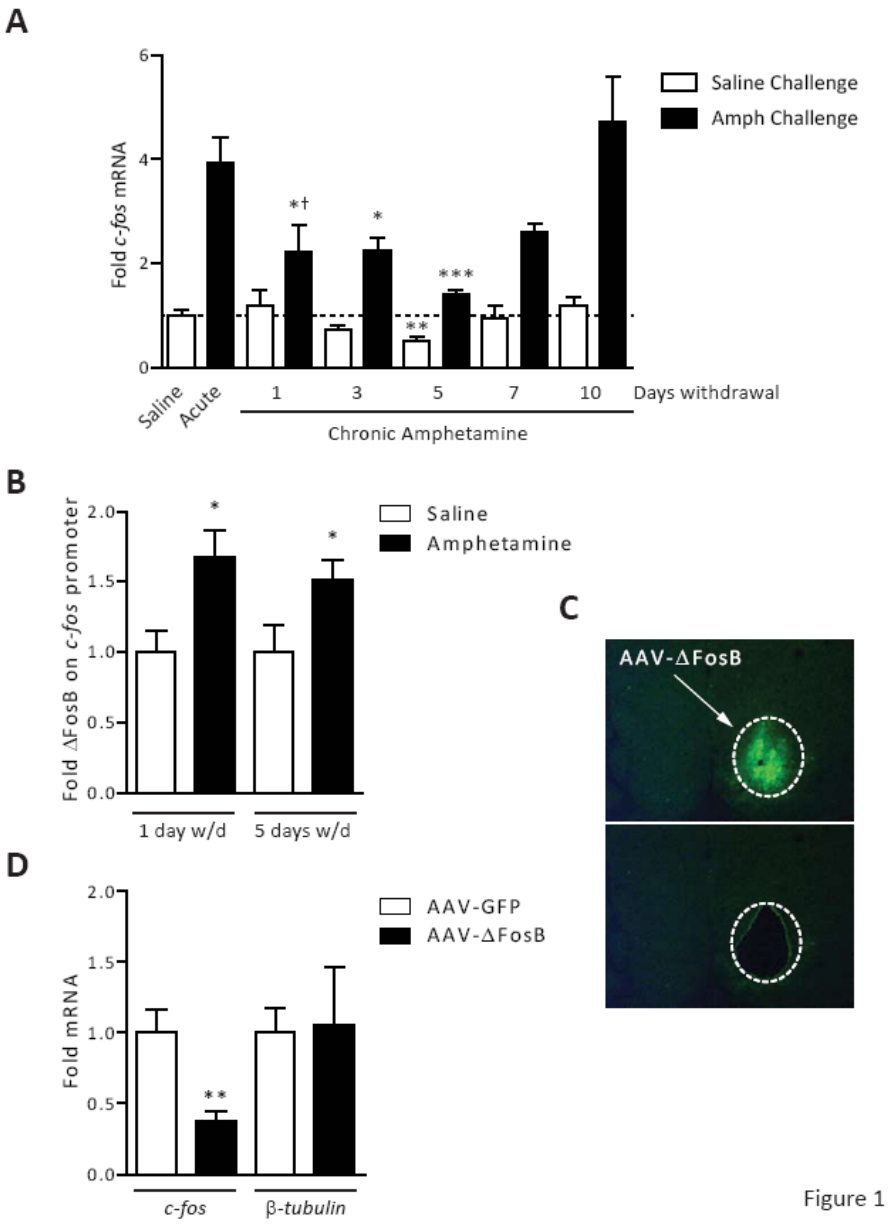


Figure 4-1. ΔFosB desensitizes *c-fos* mRNA induction in striatum after chronic amphetamine exposure.

A. Rats were treated with saline, acute amphetamine (4 mg/kg), or chronic amphetamine (7 days), and allowed to withdraw for 1 to 10 days. Rats that received chronic amphetamine received a challenge dose of either saline or amphetamine (4 mg/kg) and were analyzed 1 hr

later. The levels of *c-fos* mRNA observed after an acute dose of amphetamine were significantly reduced in rats which were previously exposed to chronic amphetamine and withdrawn from the drug for 1 through 5 days (ANOVA: significant effect of drug challenge, $F(1,35) = 132.57$, $P < 0.0001$, significant effect of withdrawal, $F(5,35) = 9.24$, $P < 0.0001$; Bonferroni post-hoc: 1 day withdrawal vs. acute; $^{*}P < 0.05$, 3 days withdrawal vs. acute $^{**}P < 0.01$, 5 day withdrawal vs. acute, $^{***}P < 0.001$, $n = 3$-5). Moreover, at 1 day of withdrawal, the fold induction of *c-fos* vs. acute was significantly blunted ($^{\dagger}P < 0.05$), with a strong trend at 3 days a 5 days of withdrawal. In rats treated with chronic amphetamine, after 5 days of withdrawal, there was a significant reduction in *c-fos* mRNA compared to naïve controls ($^{**}P < 0.01$). **B.** ChIP of striatal lysates found significantly more ΔFosB bound to the *c-fos* promoter after 1 and 5 days of withdrawal from chronic amphetamine ($^{*}P < 0.05$, n = 4-5, Student's t-test). **C**. Representative image of mouse striatum from the laser capture microscope before (top) or after (bottom) dissection of the infected region. **D.** Mice were given an acute dose of amphetamine and processed for laser capture microdissection. In striatum infected with AAV-ΔFosB compared to that infected with AAV-GFP, there was a significant decrease in *c-fos* mRNA ($^{**}P = 0.01$, $n = 3$, Student's t-test). No change was observed in *β-tubulin* mRNA ($P > 0.05$).

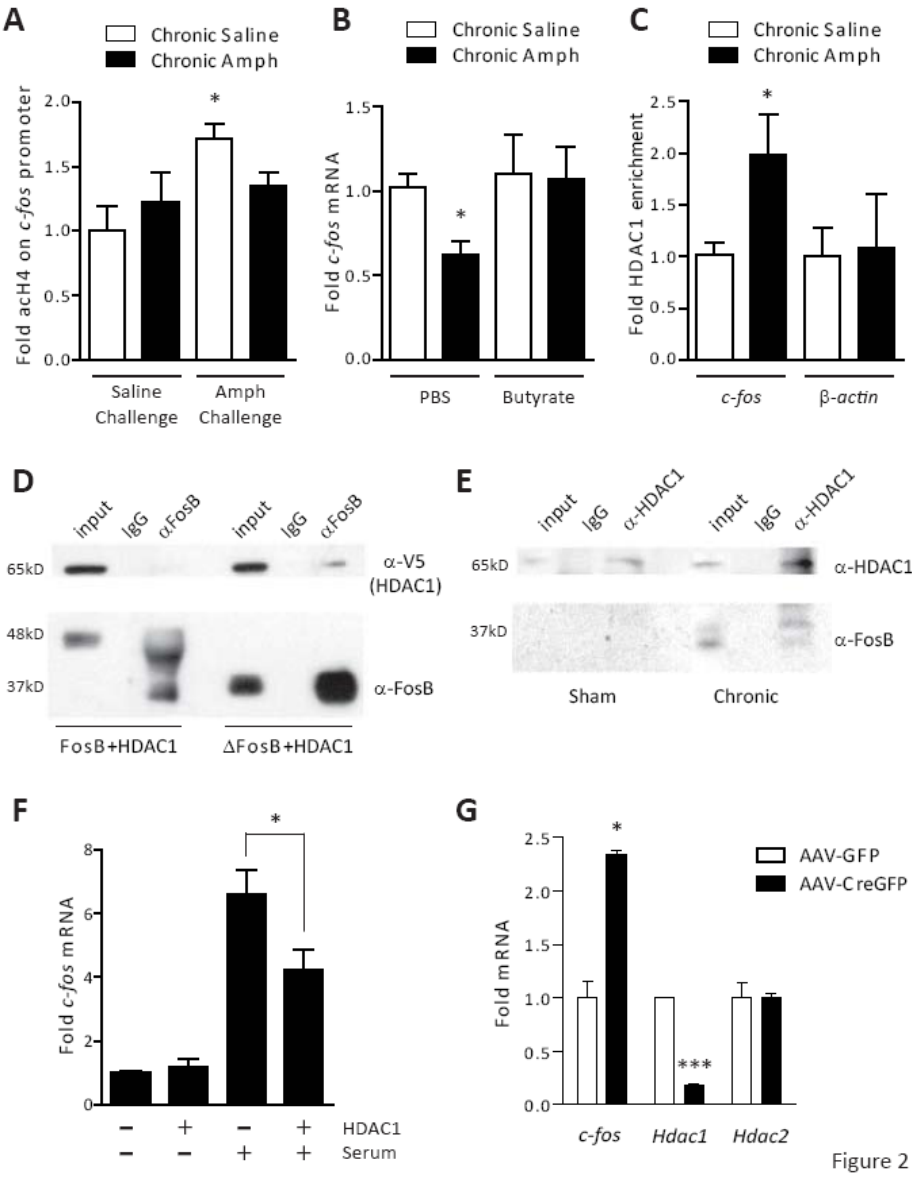


Figure 2

Figure 4-2. Recruitment of HDAC1 mediates ΔFosB action on *c-fos*.

A. ChIP of striatal lysates revealed a significant increase in acetylated histone H4 on the promoter of *c-fos* 1hr after a challenge dose of amphetamine in drug naïve rats (ANOVA, significant effect of drug, $F(1,12) = 6.26$, $P < 0.05$, Bonferroni post-hoc: $^*P < 0.05$, $n = 5$). This increase was not observed in rats previously exposed to chronic amphetamine ($P > 0.05$). **B.** The HDAC inhibitor, sodium butyrate (400 mg/kg) reversed the amphetamine-induced reduction in

c-fos mRNA observed after 5 days of withdrawal (ANOVA, significant effect of butyrate $F(1,28) = 5.29$, $P < 0.05$, Bonferroni post-hoc: $^{*}P < 0.05$, $n = 4$-9). **C.** ChIP of striatal lysates revealed significantly more HDAC1 bound to the *c-fos* promoter ($^{*}P < 0.05$, n = 5-6, Student's t-test), but not the promoter of *β-actin* ($P > 0.05$), after 5 days of withdrawal from chronic amphetamine. **D.** HDAC1 was transfected into PC12 cells with either full-length FosB or ΔFosB. HDAC1 selectively immunoprecipitated with ΔFosB, not full-length FosB. **E.** In rats that received chronic electroconvulsive seizures (7 daily seizures), a condition known to increase ΔFosB several-fold, immunoprecipitation of HDAC1 pulled down significant levels of ΔFosB. This interaction was not observed in sham-treated animals. Blots are representative of 2-3 experiments. **F.** Serum stimulation increased *c-fos* mRNA significantly less in cells transfected with HDAC1 than with GFP ($^{*}P < 0.05$, $n = 3$ independent experiments). **G.** Floxed HDAC1 mice whose striata were infected with either AAV-GFP or AAV-CreGFP on opposite sides of the brain, were treated with chronic amphetamine (7 days, 4 mg/kg) and 5 days of withdrawal. We found significantly higher *c-fos* expression in cells infected with AAV-CreGFP, where HDAC1 had been floxed out, than in cells expressing AAV-GFP after a 2 mg/kg amphetamine challenge ($^{*}P < 0.05$, $n = 2$-3). Significantly less *Hdac1* mRNA was observed in AAV-Cre infected neurons ($^{***}P < 0.001$), while *Hdac2* expression was unaffected ($P > 0.05$).

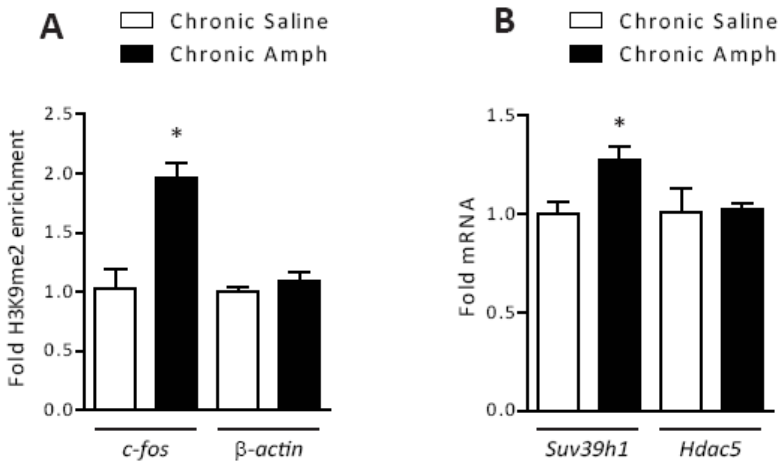


Figure 4-3. Histone methylation after chronic amphetamine exposure.

A. ChIP of striatal lysates found significantly more di-methylated histone H3 at lysine 9 (H3K9me2) bound to the *c-fos* promoter after 5 days of withdrawal from chronic amphetamine (*$P < 0.05$, $n = 3$, Student's t-test), while no change occurred on the *β-actin* promoter. **B.** Rats treated with chronic amphetamine and analyzed after 5 days of withdrawal have significantly higher *Kmt1a/Suv39h1* mRNA levels compared to saline treated rats (*$P < 0.05$, $n = 3$), while *Hdac5* levels remain unchanged ($P > 0.05$).

CHAPTER FIVE
Conclusion

SUMMARY

Through the work presented in this dissertation, I have shown that drugs of abuse broadly affect chromatin structure in a key brain reward region, the nucleus accumbens. Using genome-wide techniques, I have begun to describe the "epigenome" of a cocaine-treated mouse. Moreover, through a series of downstream statistical and bioinformatic analyses, we have begun to uncover important biological implications of this cocaine-induced chromatin regulation. Using pathway analyses we have identified many signal transduction networks that have already been implicated in addiction biology and many more pathways which have never been explored. One such pathway involves the class III histone deacetylase, the sirtuins. Pharmacological activation or inhibition of these enzymes in the nucleus accumbens potently regulates cocaine reward, suggesting that my efforts to describe and interpret the cocaine epigenome can identify novel molecules and pathways important in behavioral responses to cocaine.

In order to further understand the mechanisms by which cocaine regulates histone acetylation, I focused on enzymes which control levels of histone acetylation, histone deacetylases (HDACs). We found HDAC5 is one of the highest expressed HDACs in the NAc. Chronic, but not acute exposure to cocaine decreased HDAC5 function in the NAc, 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 cocaine. Taken together, these findings suggest that proper balance of histone acetylation in the NAc is a

crucial factor in the saliency of cocaine action, and that disruption of this balance is involved in the transition from acute adaptive responses to chronic psychiatric illness.

Finally, focusing on a specific gene highly regulated by psychostimulants, *c-fos*, I investigated the precise chromatin mechanisms occurring on its promoter and how they regulate gene activity after acute or chronic amphetamine exposure. The gene, *c-fos*, is highly activated by acute amphetamine, but after chronic exposure, the gene activity is suppressed for up to 5 days of withdrawal. At this time point, I found reduced histone acetylation on the *c-fos* promoter and higher levels of HDAC1 and the chronic-amphetamine induced transcription factor, ΔFosB. A histone deacetylase inhibitor restored *c-fos* activity to control levels, further implicating histone deacetylases in chronic amphetamine-induced *c-fos* repression. Since HDAC1 is typically recruited to promoters by interacting with sequence-specific transcription factors, I investigated whether HDAC1 and ΔFosB physically interact. Indeed both *in vitro* and *in vivo* these two proteins co-immunoprecipitate and overexpression of ΔFosB was sufficient to repress *c-fos* gene activity. In addition, the *c-fos* had significantly higher levels of histone methylation at H3K9, which is associated with gene repression, and the H3K9 histone methyltransferase, KMT1A/SUV39H1, was significantly upregulated by chronic amphetamine.

These studies provide a broad, genome-wide description of histone acetylation and methylation in the nucleus accumbens of cocaine-treated mice, investigate the regulation and behavioral significance of histone deacetylases in this brain region, and characterize the transcriptional significance of such modifications on the pscyhostimulant-induced target gene, *c-fos*. Together, this dissertation implicates the role of epigenetic mechanisms in the behavioral responses to drugs of abuse while illustrating how complex these mechanisms are.

Epigenetics and Behavior: the appeal

Epigenetics was first described by C. H. Waddington in 1942 as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being." As the molecular understanding of epigenetics has exploded in the past decade, this term has been more recently defined as "the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states" (Bird, 2007). It is relatively well accepted now that regulation of chromatin structure is a key mechanism underlying epigenetic phenomena, such as cellular differentiation, which require environmental cues to elicit a unique response. Specifically, since the genome of a liver cell is identical to a neural cell, these distinct cell types persist over time by expressing unique RNAs and proteins. The mechanisms which maintain this continuity of gene expression involve manipulations to histone proteins and DNA that either potentiate or repress the expression of a specific gene. One of the most attractive aspects of these mechanisms to neuroscience and behavior is their potential stability. Until recently, biochemical studies suggested that histone and DNA methylation were as stable as the protein or cell in which it occurred. Moreover, DNA and histone methylation are observed on the same inactivated X chromosomes for the entire life of women, illustrating the stability of these processes *in vivo*. However, the emergence of histone and DNA demethylation enzymes or processes (e.g. nucleotide excision repair) has suggested additional mechanisms must be in place. Nevertheless, behavioral neuroscientists see the stability of epigenetics as a potential mechanism by which long-lasting changes in behavior can be encoded. For example, we have observed that histone methylation at H3K27 persists over a month after chronic social defeat stress (Tsankova et al., 2006). Thus, it is interesting to speculate that such changes may contribute to the long lasting syndrome caused by chronic stress. In drug abuse, however, the

longest-lasting histone modification I have observed is 7 days following chronic drug exposure, so the search remains to find chromatin changes which persist for as long as the behavioral phenotype (months to years). Nevertheless, I did find that higher levels of histone acetylation enhance both transcriptional and behavioral responses to drugs of abuse and stress. Thus, histone acetylation may play more of a role in modulating the saliency of environmental stimuli, where higher acetylation result in stronger responses to a variety of chronic stimuli, such as cocaine, social stress, and cardiac stress.

Future Studies

Drug-induced alterations in chromatin structure have now been implicated in both the pathogenesis and maintenance of the addicted state. An important area for future research is to translate these findings from simple behavioral models such as conditioned place preference and locomotor responses to self-administration and relapse paradigms, which better model the human syndrome. Moreover, cocaine and other related stimulant addiction makes up only a small part of the substance abuse problem, making it equally important to focus on other drugs of abuse such as opiates, nicotine, alcohol, cannabinoids and inhalants. Indeed, progress is being made, as several recent findings have begun to explore other drugs of abuse. In the amygdala, for example, regulation of histone acetylation was observed in ethanol-treated rats, where acute ethanol exposure reduces HDAC activity and increases global levels of histone H3 and H4 acetylation, while withdrawal from chronic ethanol has the opposite effect (increased HDAC activity and reduced histone acetylation) (Figure 1) (Pandey et al., 2008). Changes in acetylation have also been implicated in the molecular and behavioral responses to an inhalant in *Drosophila* (Wang et al., 2007).

Another area for future research is to dissect the many types of potential changes in chromatin structure beyond histone acetylation that also occur after drug exposure. Perhaps a place to start is to explore those marks that have been shown to be regulated in brain in other behavioral models, such as the long-lasting histone H3K27 methylation seen in a mouse model of depression (Tsankova et al., 2006), or DNA methylation seen in rats with poor maternal care (Meaney and Szyf, 2005). These mechanisms, because they are generally more stable in cell culture models than histone acetylation, may contribute to persistent addictive behaviors such as relapse. In addition to numerous types of histone modifications and DNA methylation, other mechanisms of chromatin regulation such as ATP-dependent chromatin remodeling by SWI-SNF proteins and histone replacement (e.g. H3.1,3.3, H2A.Z) may also be important substrates for drugs of abuse. Regardless of the epigenetic mechanism studied, however, we must ultimately understand what is happening to the underlying expression of specific genes. In theory, any epigenetic change should be manifested by a change in gene expression or, as in the case of BDNF during cocaine withdrawal, the transcriptional potential of a gene that ultimately alters neural function (Kumar et al., 2005).

Challenges

I will discuss two key challenges I encountered studying epigenetic mechanisms in adult brain and their potential cutting-edge solutions which are about ready for use *in vivo*.

The first challenge is inherent to the structure of the brain: it's heterogeneous. There are neurons and glia, which are each made up of many subtypes. Thus, the cellular and molecular responses to cocaine can vary tremendously within a small region of the nucleus accumbens. For example, neurons which express dopamine D1 receptors will show higher levels of cAMP and its

downstream consequences, whereas neurons expressing D2 receptors will have less. There is even a small population of neurons which express both D1 and D2 receptors, even further complicating the cellular response. The end result of studying such a heterogeneous structure is that most of the observed effects will be very small since they are averaged with a variety of other, differently responding, cell types. This is particularly problematic for microarray analyses, which depend on strong effects for statistical significance. One solution that is being currently developed is fluorescence activated cell sorting (FACS) in conjunction with BAC-transgenic mice that express GFP only in specific cell types (e.g. dopamine D1 vs. D2 neurons). This will allow us to enrich for certain cell populations to study their responses to cocaine in isolation.

The second major challenge I've approached in my thesis work is determining causality from correlation. How would one determine whether histone methylation at the *c-fos* promoter *causes* transcriptional and/or behavioral responses *in vivo*? In order to do this one must induce and/or prevent histone methylation on the *c-fos* gene specifically. Overexpressing a methyltransferase may methylate numerous other target genes in addition to *c-fos* that confounds the interpretation. Just in the last few months an exciting breakthrough using zinc finger proteins may enable us to address this very challenging question. Zinc finger peptides can be designed or screened for highly sequence-specific DNA binding properties. These zinc finger peptides can then be fused to a chromatin remodeling enzyme, which would effectively target the enzyme to the promoter of a specific gene. This was for done for the first time using a DNA methyltransferase in cell culture (Smith et al., 2008), and may now permit behavioral neuroscientists to ask whether epigenetic changes at specific genes are indeed *causally* linked to the transcriptional and behavioral phenotypes observed.

Concluding Remarks

There is now considerable evidence that epigenetic mechanisms, such as histone acetylation, are involved in several behavioral models to regulate the saliency of environmental stimuli. This has important implications for the pathogenesis of drug addiction, depression, and even memory formation. However, it is still not clear whether such changes in chromatin structure are as long-lasting as the behavioral changes that define these states. Nevertheless, epigenetic mechanisms are attractive candidates for molecular substrates which mediate such long-lived changes in the brain. At the same time, such mechanisms offer fundamentally new approaches for the development of more effective treatments of drug addiction and other neuropsychiatric disorders.

APPENDIX

Cocaine Regulates MEF2 to Control Synaptic and Behavioral Plasticity

SUMMARY

Repeated exposure to cocaine causes sensitized behavioral responses and increased dendritic spines on medium spiny neurons of the nucleus accumbens (NAc). We find that cocaine regulates myocyte enhancer factor 2 (MEF2) transcription factors to control these two processes *in vivo*. Chronic cocaine exposure suppresses MEF2 activity through a novel mechanism involving the regulator of calmodulin signaling (RCS), calcineurin, and cyclin-dependent kinase-5 (Cdk5). We show that reducing MEF2 activity in NAc *in vivo* is both necessary and sufficient for cocaine-induced increases in dendritic spine density. Surprisingly, inhibiting the cocaine-induced increase in dendritic spine density (by increasing MEF2 activity) enhances behavioral sensitivity to cocaine, while increasing NAc dendritic spine density (by decreasing MEF2 activity) reduces behavioral sensitivity to cocaine. Our findings provide new insight into the mechanisms regulating cocaine-induced spine increases in NAc, and suggest that this long-lasting structural plasticity is not required for behavioral sensitization to cocaine.

INTRODUCTION

A major clinical challenge for effective treatment of drug addiction is its persistence even after long periods of drug abstinence. One of the longest-lasting neural correlates observed, across several animal models of addiction, is an increase in dendritic spine density on medium-sized spiny neurons (MSNs) in the nucleus accumbens (NAc) (Robinson and Kolb, 2004). Dendritic spines in the NAc are the primary sites of excitatory synapses from prefrontal cortex and other glutamatergic inputs. The neck of these dendritic spines receive dopaminergic inputs from the ventral tegmental area (Hyman et al., 2006). Therefore, altering the density of NAc MSN dendritic spines could have dramatic effects on the information processing from several upstream limbic structures and ultimately addiction-related behaviors. Although several groups have documented that repeated cocaine exposure increases NAc spine density (Robinson and Kolb, 1999; Robinson et al., 2001; Li et al., 2003; Norrholm et al., 2003; Lee et al., 2006), the precise molecular mechanisms that control this process have remained elusive. Moreover, the cocaine-induced increase in NAc spine density is thought to contribute to the long-lasting behavioral sensitization that occurs after repeated cocaine exposure (Robinson and Kolb, 1999), but direct evidence concerning the functional relationship between these two processes is lacking.

A recent study revealed that the cocaine-induced increase in NAc spine density is most stable in the D1 dopamine receptor-expressing neurons (Lee et al., 2006), suggesting that D1 receptor signaling plays a major role in long-lasting stabilization of altered spine density. Interestingly, the D1 receptor-expressing neurons correlated with the same population of NAc neurons that express high levels of the stable transcription factor, ΔFosB, which has been shown

to play important roles in addiction-related behaviors (Hiroi et al., 1997; Kelz et al., 1999; Colby et al., 2003). A key ΔFosB gene target in the NAc is Cdk5 (cyclin-dependent kinase 5). Chronic cocaine increases the levels and activity of Cdk5 in the NAc (Bibb et al., 2001), and chemical inhibition of Cdk5 activity in this region blocks the cocaine-induced increase in dendritic spine density (Norrholm et al., 2003). These observations led to the hypothesis that ΔFosB accumulation upregulates Cdk5, which presumably then phosphorylates key substrates to facilitate the increase in dendritic spine density.

One group of Cdk5 substrates in the brain is the MEF2 (myocyte enhancer factor 2) family of transcription factors (Gong et al., 2003). MEF2 proteins (MEF2A-D) are expressed in unique but overlapping patterns throughout the developing and adult brain (McKinsey et al., 2002; Shalizi and Bonni, 2005). They bind to DNA as hetero- and homodimers and recruit co-activators, such as p300, or co-repressors, such as class II histone deacetylases (HDACs), to regulate target gene expression. MEF2 proteins play essential roles in cardiac and skeletal muscle development, but their role in the nervous system has remained largely unknown. In addition to its initially discovered role as an activity-dependent pro-survival factor in cultured cerebellar granule neurons (Mao et al., 1999), more recent work has shown that MEF2 regulates excitatory synapses (Flavell et al., 2006; Shalizi et al., 2006; Shalizi et al., 2007). In cultured hippocampal neurons, MEF2 activity negatively regulates excitatory synapse density in part by promoting activity-dependent synapse elimination (Flavell et al., 2006). MEF2 activity in hippocampal neurons is stimulated by glutamatergic synaptic activity, which stimulates Ca^{2+} influx via L-type voltage-sensitive Ca^{2+} channels (LT-VSCCs) and activation of Ca^{2+}/calmodulin (Ca^{2+}/CaM)-dependent signaling pathways. Ca^{2+}/CaM then stimulates the protein phosphatase, calcineurin (also referred to as protein phosphatase 2B) to dephosphorylate MEF2 proteins at a

number of sites, including the inhibitory Cdk5 sites on MEF2A and MEF2D (Ser408 and Ser444, respectively), to promote MEF2 activation (Mao and Wiedmann, 1999; Gong et al., 2003; Flavell et al., 2006). Therefore, MEF2 activity appears to be regulated in part by the balance of protein kinase (Cdk5 phosphorylation of Ser408/444) and protein phosphatase (calcineurin dephosphorylation of Ser408/444) activities toward the conserved Cdk5 site. Since chronic cocaine exposure increases Cdk5 activity, glutamatergic transmission, and synapse density in the NAc , we investigated the potential involvement of MEF2 in the long-lasting cocaine-induced structural changes in dendritic spine density.

In this study, we present evidence that cocaine exposure dramatically regulates MEF2 in the NAc, a process mediated by Cdk5 and cocaine-induced inhibition of calcineurin. We show that chronic cocaine reduces NAc MEF2 activity, which is necessary and sufficient for cocaine-induced increases in dendritic spine density. We also find that manipulations of MEF2 activity in the NAc which increase dendritic spine density reduce behavioral responses to cocaine, and conversely, manipulations which reduce dendritic spine density in the NAc potentiate cocaine behaviors. Finally, using a genome-wide ChIP-chip analysis of MEF2 binding in the NAc, we identify several gene targets and signaling pathways that may mediate MEF2 regulation of structural and behavioral plasticity. Together, our findings implicate a novel transcription factor in addiction biology through which we reveal an important disconnect between dendritic spines density in the NAc and addiction-related behaviors.

RESULTS

MEF2 Transcription Factors are Regulated in the NAc by Cocaine

To test the role of MEF2-dependent transcription in cocaine-induced NAc dendritic spine plasticity, we first analyzed the expression of MEF2 proteins in the adult striatum. We observed strong, nuclear MEF2A and MEF2D immunostaining throughout the striatum, including the NAc (Fig. A-1A, and Supplemental Figs. A-S1). MEF2A and MEF2D proteins account for most of the MEF2 DNA binding activity in the NAc, since NAc extracts incubated with either anti-MEF2A- or anti–MEF2D-specific antibodies (see Supplemental Figs. A-S2A and A-S2B) show completely "supershifted" consensus MEF2 response element (MRE)-binding activity in electrophoretic mobility shift assays (EMSA) (Fig. A-1B). This also suggests that MEF2A and MEF2D bind to DNA as heterodimers in the striatum. Moreover, expression of short-hairpin RNAs (shRNAs) against both MEF2A and MEF2D in cultured striatal neurons reduces the ability of K^+ depolarization to induce endogenous MEF2-dependent transcription by ~90% (Fig. A-1C), further indicating that MEF2A and MEF2D account for most of the depolarization-dependent MEF2 activity in the NAc.

Chronic Cocaine Exposure Increases MEF2 Phosphorylation at its Inhibitory Cdk5 Site

Since chronic cocaine administration increases Cdk5 activity in the NAc (Bibb et al., 2001), and Cdk5 phosphorylates MEF2 to suppress its activity in cultured cerebellar granular neurons, we hypothesized that MEF2 activity in the NAc might be attenuated by cocaine treatment. To test this idea, we injected adult rats daily for 7 days with saline (chronic saline) or cocaine (chronic cocaine), with acute cocaine-treated animals receiving 6 days of saline followed by a single cocaine injection on day 7. The animals were then analyzed 4 hours, 24hrs, or 48hrs after the last injection, and the striatum and cerebellum were isolated for western blot analysis with a site-specific phospho-Ser408/444 antibody. At 4 hours post-injection, we observed a

robust increase in P-Ser408/444 levels in striatal lysates of rats treated with chronic cocaine compared to those treated with saline or acute cocaine (Fig. A-2A). In contrast to the striatum, neither acute nor chronic cocaine injections significantly altered MEF2 P-S408/444 levels in the cerebellum (Supplemental Fig. A-S4A). This cocaine-induced hyperphosphorylation of MEF2 in striatum persisted for 24hrs after chronic cocaine exposure (Fig. A-2B). Surprisingly, mice that received a single injection of cocaine also showed a significant increase in MEF2 phosphorylation this time point, suggesting that chronic exposure accelerates the kinetics of MEF2 phosphorylation (occurring within 4hrs) in response to the drug. MEF2 phosphorylation returned to baseline conditions 48hrs after either an acute or chronic course of cocaine (Fig. A-2B). These data indicate that chronic cocaine exposure increases MEF2 phosphorylation at the inhibitory Cdk5 sites (P-Ser408/444) in the striatum *in vivo*. Consistent with these observations, we found that Cdk5 negatively regulates MEF2 activity in cultured striatal neurons (Supplemental Fig. A-S9). Moreover, using an RNAi-based protein replacement assay (Flavell et al., 2006), we showed that phosphorylation of MEF2A/D at the Cdk5 site, Ser408/444, reduces MEF2 activity in striatal neurons. Specifically, we co-transfected plasmids expressing MEF2A and MEF2D shRNAs to reduce endogenous MEF2 proteins together with plasmids expressing RNAi-resistant MEF2A or MEF2D (Fig. A-2C). Thus, we effectively replaced endogenous MEF2 with wild-type or non-phosphorylatable forms of the protein (S408A for MEF2A; S444A for MEF2D). At similar expression levels as wild-type MEF2A or MEF2D, the non-phosphorylatable mutants of MEF2A (S408A, Fig. A-2C) or of MEF2D (S444A, Supplemental Fig. A-S3) had significantly higher basal and KCl-induced activation of MEF2 activity than their wild-type controls. Taken together, these data indicate that cocaine-induced MEF2 phosphorylation at Ser408/444 in striatum, *in vivo,* suppresses its transcriptional activity.

MEF2 Regulates Dendritic Spine Density in the NAc

Repeated exposure to cocaine stimulates long-lasting increases in dendritic spine density of MSNs in the NAc, and this structural synapse plasticity correlates with persistent behavioral sensitization to the drug. Based on the ability of MEF2 to suppress dendritic spines and excitatory synapses in cultured hippocampal neurons (Flavell et al., 2006), we hypothesized that the cocaine-induced decrease in MEF2 activity in the striatum may contribute to the induction of dendritic spines seen after chronic cocaine exposure. To test this hypothesis, we generated RNAi-expressing adeno-associated viruses (AAVs) to reduce MEF2 levels in the NAc *in vivo*. As negative controls, we generated similar AAV-shRNA viruses that expressed MEF2 shRNA with point mutations that prevented their recognition of endogenous MEF2A/2D mRNAs. All AAV-shRNA constructs co-expressed EGFP or mCherry, which allowed us to clearly visualize dendritic spines after immunostaining. We co-injected MEF2A and MEF2D shRNA viruses into the NAc on one side of the brain and co-injected the mutant RNAi control viruses into the contralateral NAc. By 2 weeks post-injection, when transgene expression is maximal, we observed a strong reduction of MEF2A and MEF2D protein in the NAc (Fig. A-3A and Supplemental Fig. A-S6) that was sustained for months after the injections. We found no detectable evidence of neuronal apoptosis in MEF2 shRNA- or mutMEF2 shRNA-infected neurons by nuclear morphology (DAPI staining), dendritic morphology (GFP staining), or TUNEL staining, suggesting that MEF2 activity is not required for NAc MSN survival *in vivo* (data not shown).

Using an established cocaine injection protocol to induce dendritic spines in the NAc (Norrholm et al., 2003), we injected mice once daily with cocaine or saline for 4 weeks before

analyzing NAc MSN dendritic spine density. Importantly, MEF2 is strongly hyperphosphorylated under these injection conditions (Supplemental Fig. A-S4C). We analyzed dendritic spine density of GFP-positive MSNs using laser scanning confocal microscopy by two blind investigators. Simplifying our analysis, MEF2 is known to specifically alter spine density without affecting dendrite morphology (Flavell et al., 2006). We found that in both saline- and cocaine-injected mice, MEF2A/D shRNA-expressing neurons had significantly higher NAc MSN dendritic spine density than the control neurons (Fig. A-3B; Supplemental Fig. A-S7). These results indicate that reducing MEF2 activity in the NAc, as chronic cocaine does, is sufficient to increase MSN dendritic spine density, and reveal an important role for MEF2 in regulating basal dendritic spine density in the NAc *in vivo*.

We next tested whether suppression of MEF2 activity by cocaine is required for the cocaine-induced increase in NAc MSN dendritic spine density. To this end, we produced AAVs that co-express EGFP together with a constitutively-active form of MEF2 (MEF2-VP16). MEF2-VP16 is a fusion between the MEF2 DNA binding and dimerization domains and the basal transcription activation domain of the viral transcription factor VP16 (Black et al., 1996). As a negative control, we produced an AAV which lacks a functional MEF2 DNA binding domain (AAV-MEF2ΔDBD-VP16). In cultured striatal neurons, we found that the AAV-MEF2-VP16 increased MEF2-dependent transcription, whereas the MEF2 DNA binding mutant (AAV-MEF2ΔDBD-VP16) had no effect compared to the vector control (Fig. A-3C). Similar to the MEF2 RNAi experiments described above, we then injected either the AAV-MEF2-VP16 or the control AAV-MEF2ΔDBD-VP16 virus into the NAc on opposite sides of the brain. After a three-week recovery and expression period, we injected the mice daily with either saline or cocaine for 4 weeks before analyzing NAc spine density of GFP-expressing neurons. Compared

to the saline-injected mice, we found that cocaine significantly increased the spine density of NAc neurons infected by the control MEF2ΔDBD-VP16 virus (Fig. A-3D). In contrast, expression of constitutively-active MEF2 in the NAc completely blocked the ability of chronic cocaine to increase NAc MSN spine density (Fig. A-4D), indicating that reduction of MEF2 activity is required for cocaine-induced changes in NAc dendritic spines. Therefore, these combined observations indicate that MEF2 activity is both necessary and sufficient to regulate cocaine-induced NAc dendritic spine density *in vivo*, and suggest that chronic cocaine exposure regulates NAc spine density in part by reducing MEF2 activity.

MEF2 Activity in the NAc Regulates Behavioral Responses to Cocaine

While several studies have documented that repeated exposure to drugs of abuse increases both NAc dendritic spine density and drug-induced behavioral responses, the functional relationship between these phenomena has remained unclear. Since MEF2 activity has a potent effect on NAc dendritic spine density, we sought to test the relationship between MEF2-dependent NAc spine plasticity and sensitized behavioral responses to cocaine *in vivo*. Since the cocaine-induced increase in NAc dendritic spines is speculated to mediate sensitized behavioral responses, we hypothesized that bilateral expression of MEF2-VP16, which blocked the cocaine-induced spine increase in the NAc, might reduce cocaine-induced behaviors. Contrary to this hypothesis, we found that NAc expression of constitutively-active MEF2 enhanced locomotor responses to cocaine (Fig. A-4C). Specifically, mice expressing MEF2-VP16 in the NAc responded normally to an initial injection of cocaine, but demonstrated significantly higher locomotor responses to cocaine on day 2 compared to control (MEF2ΔDBD-VP16 expressing) mice (Fig. A-4C), and trended higher than the control mice for the duration of the injections.

After one week of withdrawal, the MEF2-VP16-expressing mice remained significantly more sensitive to a cocaine challenge dose than the control mice (Fig. A-4D). Consistent with the sensitizing effect of MEF2-VP16 expression in the NAc on cocaine-induced locomotor behaviors, MEF2-VP16 expression also sensitizes mice to the rewarding effects of cocaine as measured by conditioned place preference. That is, mice overexpressing MEF2-VP16 in the NAc spend significantly more time in a cocaine-paired vs. a saline-paired environment (Fig. A-4E). These findings suggest that enhanced MEF2 activity in the NAc, which blocks cocaine-induced spine increases, promotes acquisition and maintenance of cocaine-induced locomotor sensitization as well as strengthens the rewarding effects of the drug.

As described above, cocaine induces the hyperphosphorylation of MEF2 in the NAc and suppresses its transcriptional activity. To explore the behavioral consequences of suppressing MEF2 activity in the NAc, we co-infected this region of adult mice bilaterally with AAV-MEF2A/2D shRNAs or mutant shRNA control viruses and tested their locomotor responses to repeated cocaine injections. Reducing MEF2 levels in the NAc, which was sufficient to increase dendritic spine density, both delayed the acquisition of cocaine-induced locomotor sensitization and attenuated behavioral responses to a cocaine challenge dose after two weeks of withdrawal (Fig. A-4A and A-4B). These findings compliment well the opposite behavioral responses observed in mice with increased MEF2 activity in their NAc (Fig. A-4D).

In sum, these data reveal that increasing MEF2 activity enhances sensitization (when spines are reduced), while decreasing MEF2 activity delays sensitization (while spines are increased). Our findings suggest that the cocaine-induced increase in dendritic spine number is not required to acquire or maintain sensitized behavioral responses to repeated cocaine exposure.

Dopamine D1 Receptor Signaling Inhibits MEF2 Activity in Striatal Neurons

In order explore the intracellular signaling mechanisms by which cocaine regulates MEF2 in the striatum and the downstream consequences it has on MEF2-dependent transcription, we transfected striatal neurons with a MEF2-luciferase reporter plasmid (MEF2-luc) and measured endogenous MEF2 activity after pharmacological and genetic manipulations. Since chronic cocaine exposure has been associated with increased glutamatergic and dopaminergic transmission in striatum, we studied whether these stimuli regulate MEF2-dependent transcription in striatal neurons. We found that membrane depolarization (60 mM KCl), which, like glutamate, stimulates calcium influx, dramatically increased MEF2-dependent transcription (Fig. A-5A). This activation of MEF2 required calcium influx through L-type voltage sensitive calcium channels (LT-VSCCs) and activation of calcineurin phosphatase activity (Supplemental Figs. S5A and S5B). While the stimulation of dopamine D1 receptors (10 μM SKF81297) alone had little effect on basal MEF2 activity, dopamine D1 receptor stimulation significantly attenuated the activation of MEF2 upon membrane depolarization (KCl) (Fig. 5A). To test whether the suppression of MEF2 activity by dopamine D1 receptor signaling was due to elevation of cAMP, we treated the cultures with the adenylyl cyclase-activator, forskolin (10 μM), and observed a similar reduction of calcium-induced MEF2 activity (Fig. 5B). This suggests that activated dopamine D1 receptors increase cAMP levels, which antagonize calcium-dependent activation of MEF2 in striatal neurons.

As a point of comparison, we also analyzed endogenous CREB activity using a CRE (cAMP response element)-luciferase reporter plasmid. As demonstrated previously, CREB activity is significantly increased in our striatal cultures by the dopamine D1 receptor agonist SKF81297. Similarly, membrane depolarization (KCl) stimulated CREB-dependent transcription

(Fig. A-5C). Unlike MEF2, however, combining membrane depolarization (KCl) with dopamine D1 receptor activation (SKF81297) synergistically activated CREB-dependent transcription (Fig. A-5C). These findings suggest that calcium signaling and dopamine signaling cooperate to activate CREB activity in striatal neurons while combining to reduce activation of MEF2, which is consistent with recent findings in hippocampal neurons (Belfield et al., 2006). Therefore, CREB and MEF2 may function to provide context-specific patterns of new gene expression depending on the relative levels of dopamine and calcium in striatal neurons .

Dopamine and Calcium Signaling Regulates MEF2 Activity through Calcineurin and RCS

Since KCl-induced MEF2 activity in these cultures requires calcineurin phosphatase activity (Supplemental Fig. A-S5B), we speculated that dopamine signaling might regulate MEF2 activity by reducing calcineurin activity. To test this possibility, we transfected striatal neurons with constitutively-active calcineurin (CaNΔCT) and measured the effect of cAMP signaling on calcium-dependent MEF2 activation. We observed that expression of CaNΔCT blocked the inhibition of MEF2 activity caused by forskolin (Fig. A-3D), which indicates that cAMP signaling attenuates MEF2 by reducing calcineurin activity. These data position the cAMP pathway at or upstream of inhibiting calcineurin activation.

Ca^{2+} influx through LT-VSCCs stimulates the formation of Ca^{2+}/calmodulin (Ca^{2+}/CaM) (Dolmetsch et al., 2001), which then binds and activates calcineurin. Recently, the regulator of calmodulin signaling (RCS) was shown to negatively regulate calcineurin activity in striatal neurons (Rakhilin et al., 2004). Activation of the dopamine D1 receptor stimulates PKA-dependent phosphorylation of RCS at Ser55, which induces direct interaction of phospho-RCS with Ca^{2+}/CaM and competitive inhibition of calcineurin activity (Rakhilin et al., 2004). To test

whether RCS regulates MEF2 activity, we expressed RCS in striatal neurons and found that in the presence of forskolin, RCS further reduced MEF2 activity (Fig. A-6A, right). Interestingly, RCS had no effect on calcium-inducible MEF2 activity in the absence of forskolin (Fig. A-6A, left), suggesting that RCS phosphorylation by PKA is necessary to inhibit MEF2 activity. Indeed, the cAMP-dependent inhibition of MEF2 activity by RCS required phosphorylation at Ser55 (PKA site) since a non-phosphorylatable RCS mutant (S55A) failed to suppress MEF2 activity in the presence of forskolin (Fig. A-6B). Importantly, unlike calcineurin inhibitors, which fully eliminate MEF2 activity in striatal neurons, the inhibition of MEF2 activity by cAMP/RCS is not complete. This may be due to limiting amounts of RCS or parallel pathways that are concurrently activating calcineurin.

In order to determine if this cAMP/RCS signaling pathway is also important for regulating MEF2 activity in adult striatum, we first generated antibodies to phospho-Ser55 RCS as well as total RCS. In 293T cells transfected with RCS or the unphosphorylatable mutant, RCS(S55A), our phospho-RCS antibody detects a significant increase in forskalin-induced RCS phosphorylation that is not observed in the S55A mutant (Supplemental Fig. A-S8A). We next incubated adult striatal sections in vechicle, forskalin (50μM), or SKF81297 and measured RCS and MEF2 phosphorylation. Consistent with the mechanism we identified in striatal culture, both forskalin and SKF81297 significantly increased RCS phosphorylation in adult striatum (Fig. A-6C). We next treated rats with chronic cocaine or saline and analyzed phospho-RCS levels in striatum 4hrs and 24hrs after the last dose. Similar to the kinetics of MEF2 phosphorylation, we observed significant increases in RCS phosphorylation in striatum 4hrs after the last injection (Fig. A-6D). Twenty-four hours later, RCS phosphorylation remained elevated in cocaine-treated rats but only as strong trend ($P = 0.07$, Supplemental Fig. A-S8B). Together, these findings

suggest a novel upstream signaling pathway involving the Dopamine D_1R/cAMP-dependent phosphorylation of RCS through which cocaine acts to suppress MEF2 transcriptional activity in the striatum.

Genome-wide Analysis of MEF2 Promoter Binding Identifies Novel Targets That Regulate Structural Plasticity

The dynamic regulation of MEF2 transcriptional activity in striatum by upstream dopamine and calcium signaling pathways led us to investigate the downstream gene targets which ultimately mediate MEF2's effects on dendritic spine plasticity and behavioral responses to cocaine. To address this question *in vivo,* we treated mice with chronic cocaine and performed chromatin immunoprecipitation (ChIP) using an antibody against MEF2A. The immunoprecipitated DNA was then amplified and hybridized to genome-wide promoter arrays to identify MEF2 target genes in the NAc of cocaine-treated mice. An example of the genome-wide MEF2-binding data is shown for chromosome 17 (Fig. A-7A). We found that in cocaine-treated mice, MEF2 significantly binds to the promoters of approximately 900 genes in the NAc at a significance level of $P < 0.0001$.

To explore the cellular processes that MEF2 gene targets regulate, we performed Ingenuity pathway analysis on the significant MEF2-bound genes. We identified several highly-enriched signaling pathways and cellular processes, including actin cytoskeleton signaling and cAMP signaling, which are known to have pronounced effects on dendritic structural plasticity and/or behavioral responses to cocaine (Supplemental Fig. A-S9A). For example, after chronic cocaine MEF2 is bound to the proximal promoters of the Wiskott-Aldrich syndrome proteins, N-WASP (*Wasl*), WAVE3 (*Wasf3*) and Profilin 1 (*Pfn1*), which are all known to regulate F-actin

and cytoskeletal remodeling (Supplemental Fig. A-S9B and A-S9C). Importantly, N-WASP, WAVE3 and Profilin 1 have also been directly implicated in dendritic spine morphology, suggesting that these target genes may contribute to MEF2-dependent cocaine-induced dendritic spine formation.

Since MEF2 remains hyperphosphorylated 24hrs after a chronic course of cocaine, we were curious if mRNA levels of MEF2 target genes were altered at this time point. To address this, we compared the genes on which MEF2 is significantly enriched with data from gene expression microarrays performed in the NAc of cocaine-treated versus saline-treated mice (Renthal et al. 2007). Since hyperphosphorylation of MEF2 suppresses its activity, we identified a subset of 82 MEF2-bound genes whose mRNA expression was downregulated >1.2 fold 24hrs after repeated cocaine administration (Fig. A-7B). Focusing on the genes where cocaine induced the strongest downregulation of mRNA and the highest levels of MEF2 enrichment, we identified the PI-3 kinase catalytic subunit, *pik3cg*. Using qPCR, we next confirmed that chronic cocaine downregulates *pik3cg* in the NAc in an independent cohort of mice (Fig. A-7D). Using quantitative ChIP, we also confirmed that MEF2 was significantly enriched at 3 near-consensus MEF2 response elements (MREs) in the *pik3cg* promoter after chronic cocaine exposure (Fig. A-7C). This MEF2 enrichment on the *pik3cg* promoter is likely a key regulator of transcriptional activity, as PC12 cells transfected with a 4-hydroxytamoxifin (4OHT)-inducible MEF2-VP16 construct show significant increases in *Pik3cg* mRNA after 4OHT treatment (when MEF2-VP16 is overexpressed) (Fig. A-7D).

These data suggest that cocaine-induced suppression of MEF2 would reduce *Pik3cg* transcription in striatum and lead to lower PI-3 kinase activity. Consistent with this idea, chronic cocaine exposure significantly reduced the phosphorylation of Akt, a key PI3-kinase substrate, in

the NAc (Fig. A-7E). Importantly, the PI-3K activity in the NAc potently controls behavioral sensitivity to cocaine, since direct infusion of the PI-3 kinase inhibitor, LY294002, into the NAc significantly blocks cocaine-induced locomotor responses (Izzo et al., 2002). This finding is consistent with data presented here in which knockdown of MEF2 in the NAc, which would also be expected to reduce PI3-kinase levels, significantly blunted behavioral sensitivity to cocaine.

Together, these data not only provide the first genome-wide analysis of MEF2 target genes in brain, but they also go on to describe how a novel MEF2 target gene, *Pik3cg*, is an important downstream mediator of the cocaine-induced behavioral effects controlled by MEF2 activity in the NAc.

DISCUSSION

In this study, we find that repeated cocaine exposure regulates MEF2 transcription factors to control aspects of long-lasting synaptic and behavioral plasticity. Our findings indicate that chronic cocaine exposure reduces MEF2-dependent transcription to promote increased MSN dendritic spine density in the NAc. Surprisingly, this MEF2-controlled increase in dendritic spine density is associated with reduced behavioral sensitivity to cocaine, suggesting that the strong correlation between NAc spine density and cocaine sensitivity may be functionally uncoupled. Our findings go on to implicate an upstream signaling pathway involving (1) upregulation of the inhibitory MEF2 kinase, Cdk5, and (2) inhibition of the activating MEF2 phosphatase, calcineurin, which together result in MEF2 hyperphosphorylation at P-Ser408/444 and reduced transcriptional activity. Finally, using ChIP for MEF2 and genome-wide promoter arrays, our data begin to describe the downstream gene targets in the NAc which ultimately mediate the structural and behavioral effects of MEF2 regulation by cocaine. .

Cocaine administration increases the inhibitory Ser408/444 phosphorylation of MEF2A/D in striatum with a complex time course. A single dose of cocaine is sufficient to induce MEF2 phosphorylation, but not for approximately 24hrs following exposure. A chronic course of cocaine accelerates the kinetics of MEF2 phosphorylation, which occurs within 4hrs of the final drug dose. By 48hrs, however, MEF2 phosphorylation in striatum returns to baseline, indicating that the regulation of MEF2 by cocaine does not persist for as long as the observed increases in dendritic spines. These findings suggest that suppression of MEF2 activity by phosphorylation, which declines over 48 hrs, more likely plays a role in the initiation of cocaine-induced spine plasticity than the maintenance.

The mechanisms underlying the kinetics of MEF2 phosphorylation by cocaine remain unclear, but increased levels of cAMP after chronic cocaine as well as elevated levels of the MEF2 kinase, Cdk5, may contribute (Hyman et al., 2006). Consistent with this idea, dopamine D1 receptor agonists and forskalin, both of which elevate cAMP in striatal neurons, potently reduce MEF2 activity through the PKA-dependent phosphorylation of RCS. Phosphorylated RCS then sequesters calmodulin, thus inhibiting calcineurin's ability to activate MEF2. Also contributing is Cdk5, which is upregulated after chronic cocaine and we show here inhibits MEF2 activity in striatal neurons. This is consistent with its known role in phosphorylating MEF2A/D at its inhibitory Ser408/444 site. Thus, after chronic cocaine, elevated levels of cAMP and Cdk5 likely work together to inhibit MEF2 and perhaps contribute to its accelerated phosphorylation kinetics.

As a key substrate of Cdk5, MEF2 may mediate some of the synaptic and behavioral effects observed from pharmacological and genetic manipulations of Cdk5 in the NAc. Specifically, chronic infusion of roscovitine into the NAc, which increases MEF2 activity in

striatal neurons (Supplemental Fig. A-S9), blocks the chronic cocaine-induced increase in NAc MSN dendritic spine density (Norrholm et al., 2003). Similarly, we find that increasing MEF2 activity in the NAc (via MEF2-VP16 expression) also blocks the cocaine-induced increase in dendritic spine density (Fig. A-3E), suggesting that downstream inhibition of MEF2 by Cdk5 is a key mechanism of roscovitine's effect on spine plasticity in the NAc. Another interesting parallel between Cdk5 and MEF2 are the recent findings that daily roscovitine injections into the NAc (Taylor et al., 2007) or conditional Cdk5 gene deletion in the NAc (Benavides et al., 2007) enhanced locomotor sensitization to repeated cocaine treatments. Consistent with these observations, we find that enhanced MEF2 activity in the NAc (via MEF2-VP16 expression) increased the locomotor responses to repeated cocaine injections (Fig. A-4A).

Together these studies provide evidence that experimental manipulations which block cocaine-induced increases in NAc dendritic spine density produce sensitized behavioral responses to cocaine. However, we cannot directly determine whether lower spine density actually causes increased cocaine sensitivity per se, because the proximal manipulation in our study is MEF2 – not the spines themselves. It is possible that MEF2 could function through independent mechanisms to control cocaine behavior and dendritic spine density. Nevertheless, one can conclude from this and previous reports, that cocaine-induced increases in NAc dendritic spine density are not required for behavioral sensitization to cocaine.

How does regulation of MEF2-dependent transcription control NAc synapse density? In cultured hippocampal neurons, MEF2-dependent transcription is induced by glutamatergic synaptic activity to promote elimination of existing excitatory synapses (Flavell et al., 2006). As such, it is possible that suppression of MEF2 activity by cocaine in the NAc increases dendritic spine density by reducing the elimination rate of existing synapses rather than increasing the

formation rate of new synapse. NAc neurons may therefore rely upon MEF2-dependent transcription to control homeostatic synaptic plasticity after repeated cocaine exposure (Turrigiano, 2007). Indeed, intrinsic neuronal excitability is reduced in the NAc of chronic cocaine-treated animals (Hu et al., 2005), perhaps due to the cocaine-induced decrease in mPFC function (hypofrontality), observed in both animal models of addiction and in human brain imaging studies of drug addicts (Jentsch and Taylor, 1999; Volkow et al., 2003). Therefore, it is interesting to speculate, that increased NAc dendritic spine density may function homeostatically to compensate for reduced NAc excitability (Dong et al., 2006), and ultimately limit an animal's sensitivity to the cocaine.

Cocaine regulation of MEF2-dependent transcription ultimately mediates structural and behavioral changes in the NAc through the altered expression of downstream target genes. We utilized genome-wide ChIP-chip technology to identify nearly 900 gene promoters in cocaine-treated NAc tissue where MEF2A binding is enriched. Many of the target genes we identified cluster to cellular functions that regulate structural plasticity, such as F-actin remodeling. The MEF2 target genes which encode the proteins, N-WASP, WAVE3, and profilin 1, are all known to potently regulate cytoskeletal remodeling as well as dendritic spine density. Importantly, dysregulation of actin polymerization with latrunculin A or a LIM-kinase peptide antagonist in the NAc, promoted cocaine reinstatement behaviors in self administering rats (Toda et al., 2006). These data suggest that the actin remodeling proteins bound by MEF2 after chronic cocaine may be key regulators of sensitized cocaine responses.

Another significantly enriched pathway was PI-3 kinase/Akt signaling, inhibitors of which are known to block behavioral responses to cocaine (Izzo et al., 2002). Since cocaine suppresses MEF2 activity in the NAc, it was notable that a catalytic subunit of PI-3 Kinase was

one of 82 MEF2 target genes also downregulated by chronic cocaine exposure. *Pik3cg* expression is also potently regulated by MEF2 activity in culture, suggesting that the cocaine-induced suppression of MEF2 activity directly contributes to the downregulation of this gene in the NAc. Indeed, not only is *Pik3cg* mRNA reduced, but we find significantly lower levels of phospho-Akt in the NAc after chronic cocaine, a key measure of PI-3 kinase activity. Moreover, knockdown of MEF2 in the NAc, which would be expected to reduce *Pik3cg* expression and PI3-k activity, reduces behavioral sensitivity to cocaine much like the infusion of a PI3-k antagonist in this brain region.

Approximately 10% of the genes occupied by MEF2 are downregulated 24hrs after chronic cocaine exposure, suggesting that other MEF2 genes targets may be regulated at distinct time points. This would be consistent with the complex kinetics of cocaine-induced suppression of MEF2 activity. Future research will focus on parsing the relative contribution of MEF2 gene targets in the dramatic effects MEF2 activity has on dendritic spine density and cocaine behavior. However our identification of specific genes and signaling pathways (e.g. PI-3k) that directly contribute to MEF2 action in the NAc represents an important first step in this direction. In this study, we found that cocaine administration regulates MEF2-dependent gene transcription in the NAc to control dendritic spine plasticity and behavioral responses to cocaine. We show here that chronic cocaine reduces MEF2 activity through a novel signaling mechanism involving Cdk5, calcineurin and RCS. We find that reducing MEF2 activity in NAc *in vivo* is both necessary and sufficient for cocaine-induced increases in dendritic spine density. Our findings also suggest that behavioral sensitization to cocaine is functionally uncoupled from these cocaine-induced increases in dendritic spine density. Taken together, these observations implicate a new transcription factor in the molecular mechanisms controlling cocaine-induced

structural and behavioral plasticity and could ultimately lead to the development of improved treatments for drug addiction.

EXPERIMENTAL PROCEDURES

Plasmids

3X MRE-luciferase, pcDNA3-MEF2-VP16, pSuper-MEF2A(1234), pSuper-MEF2D(479), pcDNA3-MEFA (rat; wild-type and RNAi-resistant (RiR), pcDNA3-MEF2D (rat; wild-type and RiR), pcDNA3-rMEF2A S408A (RiR), and pcDNA3-rMEF2D S444A (RiR) were described previously (Flavell et al., 2006). To generate pcDNA3-Flag-RCS, we used PCR amplification from reverse transcriptase reactions of purified rat NAc mRNAs. The degenerate PCR primers incorporated unique BamHI (5') and NotI (3') sites, and the coding sequence for the M2 Flag epitope tag in frame with the second amino acid of RCS. The PCR fragment was subcloned into pcDNA3, and the insert region was confirmed by sequencing.

Dissociated Striatal cultures

Embryonic striatal neurons (E18/19) were cultured from Long Evans rats (Charles River Labs) as described previously (Lindsay, 1998) with modifications. The striatal tissues were digested with papain (10 unit/ml; Worthington) for 4 minutes (37°C) before dissociation with a plastic 5 ml pipet. For luciferase and immunocytochemistry experiments, the dissociated neurons were plated at 100,000/well (24 well plate; Corning) on PDL (Sigma)- and laminin (Invitrogen)-coated 12 mm glass coverslips (Bellco) in DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), penicillin (50 μg/ml)-streptomycin (50 units/ml; Sigma) and L-

glutamine (4 mM, Sigma) and incubated at 37°C/5% CO_2 for 24 hours in a humidified incubator. Twenty-four hours after plating, the medium was changed to Neurobasal (Invitrogen), B27 supplement (2% (v/v); Invitrogen), penicillin-streptomycin (1X; Sigma) and L-glutamine (4 mM; Sigma). For western blotting and EMSA assays, "crude" striatal dissections were performed as described (Cowan et al., 2005), and plated at 8 x 10^6 cells per 10 cm plate (Corning).

Luciferase Assays in Primary Neurons

Dissociated striatal neurons were transfected using calcium phosphate as described previously (Flavell et al., 2006) at 8 days in culture. Approximately 42-48 hours after transfection, the cultures were stimulated in conditioned medium with isotonic depolarization solution (Flavell et al., 2006), indicated compounds or vehicle alone. All treatment conditions were administered simultaneously. Neurons were lysed 7-8 hours later and relative luciferase activity was determined using the dual luciferase assay (Promega). For the luciferase assays, CRE- or MRE-firefly luciferase activity was divided by TK-renilla luciferase activity, except for samples treated with SKF81297, where an independent effect on TK-renilla was observed. In these cases, the raw firefly luciferase data was averaged over 4-5 independent experiments to minimize effects of well-to-well transfection differences.

For RNAi-based protein replacement assays, pSuper vector or pSuper MEF2A(1234) and pSuper MEF2D (479) were co-tranfected together with pcDNA3, pcDNA3-MEF2A (RiR) or pcDNA3-MEF2D (RiR) to simultaneously reduce endogenous MEF2 expression and replace with expression of wild type or mutant RNAi-resistant forms of MEF2A or MEF2D. For these experiments, 500 ng of each MEF2 shRNA plasmid was used together with 10-50 ng of

pcDNA3-MEF2 (RiR). Initial titration experiments were performed to determine the optimal levels of plasmid that restore endogenous levels of MEF2 activity.

For experiments involving chemical inhibitors, the following reagents were diluted in conditioned medium at the indicated final concentrations: SKF81297 (10 μM; Tocris), forskolin (10 μM; Sigma), KN-62 (20 μM; Tocris), nimodipine (10 μM; Tocris), cyclosporin A (1 μM; calbiochem), roscovitine (10 μM; Tocris). For KCl depolarization, striatal neuron conditioned medium was supplemented with 0.5 volumes of isotonic depolarization medium (170 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4) as previously described (Flavell et al., 2006).

Animals

Adult male C57BL/6 mice (Jackson Laboratory) and adult male Sprague-Dawley rats (Harlen) 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.

Western Blots of *In Vivo* Samples

Sprague Dawley rats (male, 10 weeks old) were injected with 7 days of saline, acute cocaine (6 days saline followed by 1 dose of 20 mg/kg cocaine), or chronic cocaine (7 days of 20 mg/kg cocaine) and sacrificed 4 or 24 hours after the last injection. Cocaine was purchased from Sigma. Animals were sacrificed by microwave irradiation aimed at the head (5kW, 1.5s, Murimachi Kikai Co. Ltd., Tokyo, Japan). Striatum was rapidly dissected and frozen on dry ice. Tissues were sonicated on ice in a SDS lysis buffer (1% (w/v) SDS, 300 mM sucrose, 1 mM EDTA) containing 1 mM activated sodium orthovanadate, 1 mM PMSF (Phenyl Methyl sulfonyl fluoride), 1 mM NaF (Sigma), 100 nM Okadaic acid (Calbiochem), 1 μM Cyclosporin A

(Sigma), and 1X Complete Protease Inhibitor cocktail (Roche). Samples were then boiled for 5 minutes before centrifugation at 20,300 x g for 10 minutes at 4°C. Total protein concentration was determined by the DC assay kit (BioRad). For each sample, 30 μg of total protein were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane and incubated overnight with either anti-MEF2A (C-21, Santa Cruz; 1:1000), anti-MEF2D (Transduction Labs; 1:2000), anti-P-Ser408/444 ((Flavell et al., 2006); 1:500), anti-β-tubulin (Sigma; 1:25,000), or anti-β-actin (Abcam; 1:2500) antibodies. Blots were developed with enhanced Chemiluminiscence western blotting detection system (ECL-plus, Amersham Pharmacia Biotech). Quantification of the autoradiographs were performed using optical densitometry and NIH ImageJ software.

Electrophoretic Mobility Shift Assay (EMSA)

Tissue extracts from whole nucleus accumbens punches were prepared as described previously (Hope et al., 1992) with minor modifications. Specifically, NAc tissues were solubilized with lysis buffer (20 mM tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 0.5% (v/v) NP-40, 1 μM cyclosporine A, and 1X Complete Protease Inhibitor cocktail (Roche)). 10-20 μg of NAc extract was incubated with ^{32}P-labeled MRE duplex oligos (McDermott et al., 1993) for 20 min. at room temperature. Protein-DNA complexes were separated from unbound oligonucleotide on a 4% non-denaturing polyacrylamide gel containing 2.5% glycerol and 0.4X Tris-borate buffer (0.04M Tris borate, pH 7.5) and was visualized by phosphoimager (GE). Cold competitor MRE or mutant MRE was used at 50-fold excess. For antibody supershifts, 1 μl of specific antibodies to MEF2A (C-21, Santa Cruz), MEF2D (Signal

Transduction labs), MEF2C or CREB (Signal Transduction Labs) were preincubated with cell lysates for 30 minutes at 37^{o}C prior to addition of labeled MRE DNA probes.

Immunohistochemistry

Mice were terminally anesthetized with chloral hydrate and perfused transcardially with PBS followed by 4% (w/v) paraformaldehyde. Brains were post-fixed overnight and then cryoprotected in 30% sucrose. Coronal sections (30μm) were submerged in 100°C citric acid (pH 6.0) for heat-induced epitope retrieval and rinsed twice in PBS. The sections were blocked in 4% normal donkey serum for 1 hour at RT before overnight primary antibody incubation at 4°C. The signals were amplified by ABC-Elite kit (Vector Laboratories) following manufacturer's protocol. The labeling was revealed with Tyramide Amplification (TSA Perkin Elmer) followed by DAPI (VectaShield, Vector Labs) staining and dehydration. Biotinylated anti-Rabbit and anti-mouse secondary antibodies were obtained from Jackson Laboratories.

Recombinant Adeno-associated Viruses

For knockdown of endogenous MEF2A and MEF2D in the NAc, we subcloned specific MEF2A or MEF2D shRNA sequences (Flavell et al., 2006) into pAAV.shRNA (Hommel et al., 2003) under control of the U6 promoter. For MEF2A, pAAV.shRNA contained a CMV promoter driving expression of eGFP, and for MEF2D the CMV promoter drove expression of mCherry. To co-express MEF2-VP16 and EGFP *in vivo*, we modified the AAV shRNA vector (Hommel et al., 2003) such that the open reading frame of MEF2-VP16 (Black et al., 1996) was under control of the CMV promoter. The shRNA region was removed and replaced with an expression cassette with EGFP under control of the hEF2 promoter. Viruses were packaged as described (Hommel et

al., 2003). Briefly, HEK-293 cells were cultured in either 10 x 15 cm plates or roller bottles and tranfected with pAAV.shRNA, pHelper and pAAV.Rc plasmids (Stratagene) with calcium phosphate. Viral purification was then performed as described previously (Zolotukhin et al., 1999; Hommel et al., 2003).

Stereotactic Surgery

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) at a 10° angle. Virus was delivered bilaterally using Hamilton syringes at a rate of 0.1 μl/min for a total of 0.8 μl (0.4 ul AAV-MEF2A shRNA and 0.4 ul AAV-MEF2D shRNA were premixed). Viral placements were confirmed by staining for GFP, which was co-expressed in each virus.

Dendritic Spine Analysis

Mice were unilaterally infused into their NAc with AAVs expressing MEF2A and MEF2D shRNAs or control shRNA viruses. For MEF2 overexpression studies, mice were unilaterally infused with AAVs expressing MEF2-VP16 or its respective control virus. 19 days post-op, mice were given daily cocaine (30 mg/kg) or saline 5 days/week for 4 weeks (20 total injections) and sacrificed 24 hrs after the last dose. GFP-labeled neurons within the NAc core and shell regions were imaged at high resolution using a 100X oil immersion lens on a Zeiss LSM 510 confocal microscope. PMT assignment, pinhole sizes and contrast values were kept constant across different confocal sessions. Confocal stacks consisted of 31-178 sections at 0.23 μm in thickness imaged with a Z-step of 0.1 μm. Images were taken at 1024 x 1024 pixel

resolution to cover the entire Z dimension of the labeled neurons. Lengths of dendritic segments were measured using NIH ImageJ software. Spine densities were quantified by counting the number of spines along 30- to 100-μm segments of secondary dendrites (2-3 dendrite segments/neuron). Spine densities were expressed as spines per 10 μm. Only spines appearing continuous with their parent dendrite shaft in maximum-intensity z-projection were used for quantitative analysis. Mean spine densities were analyzed by pair-wise comparisons using the student's t-test.

Cocaine-induced Locomotor Sensitization

At the same time each day, mice were injected with saline or cocaine (IP) and were placed in standard plastic cages similar to their home cages for two hours. These cages were inside the Photobeam Activity System (San Diego Instruments, San Diego, CA), where five photobeams measured the mouse's locomotor activity in 5-minute bins. Mice received saline injections on day 1-3 to habituate them to the novel environment. The locomotor activity for their final saline day is displayed. On days 4-10 mice received cocaine injections (15 mg/kg). Challenge doses of cocaine (15 mg/kg) occurred on day 17 (1 week of withdrawal) and on day 24 (2 weeks of withdrawal; RNAi experiment only) of the experiment. Day 1 of the experiment was 21 days after viral delivery, a time point at which high levels of expression were verified. For each day, the sum of the first 30 minutes (MEF2-VP16) or 45 minutes (shRNA) of locomotor activity after injection is displayed.

Conditioned Place Preference

18-21 days following stereotactic delivery of AAV-MEF2VP16 or its control virus into the NAc, mice were conditioned to cocaine in a standard 3 chamber conditioned place preference box (gray side, middle, and striped side). Using an unbiased 6 day paradigm, mice were pretested on day 1 to balance pre-existing side bias. On days 2 and 4, mice received a saline injection and were confined to the appropriate chamber. On days 3 and 5, mice received a cocaine injection (8mg/kg) and were confined to the opposite chamber. On the final day, mice were placed again in the middle chamber with free access to all chambers and the time spent on each side was quantified. Data is expressed as time spent on the cocaine-paired side minus the time spent on the saline-paired side (CPP Score).

RNA Isolation and Reverse Transcription PCR

Bilateral 14 gauge punches of rat nucleus accumbens were rapidly dissected and frozen at -80°C. Punches were thawed in TriZol (Invitrogen), homogenized, and processed according to the manufacturer's protocol. Total RNA was reverse-transcribed using Superscript III (Invitrogen) and random hexamers.

ChIP-chip analysis

Mice were treated for 7 days with daily injections of cocaine (20 mg/kg) and sacrificed 24 hrs later. Chromatin from bilateral punches of NAc was immunoprecipitated with an antibody against MEF2A as described previously with minor modifications (Renthal et al., 2007). Chromatin was sonicated to an average of 500 bp and immunoprepitated with antibody against MEF2A (Santa Cruz, sc-313) or an IgG control (Upstate/Millipore). Antibody-bound chromatin was precipitated using Protein A beads from Upstate (06-157), which were washed

with low salt, high salt, and LiCl buffers to remove non-specific DNA binding. Eluted chromatin was reverse-crosslinked at 65^{o}C in the presence of proteinase K and EDTA. DNA was purified by chloroform extraction/ethanol precipitation and the enrichment of specific promoters was either measured directly via real time PCR or amplified by ligation-mediated PCR for genome-wide analysis (Sikder et al., 2006). Amplified DNA was then labeled with Cy3 (control IgG-enriched) or Cy5 (MEF2-enriched) and hybridized to Nimblegen (Madison, WI) MM8 mouse promoter arrays. Eight mice were pooled per replicate, and two biological replicates were performed. All microarray data will be deposited in the GEO database (accession # to be determined).

APPENDIX FIGURES

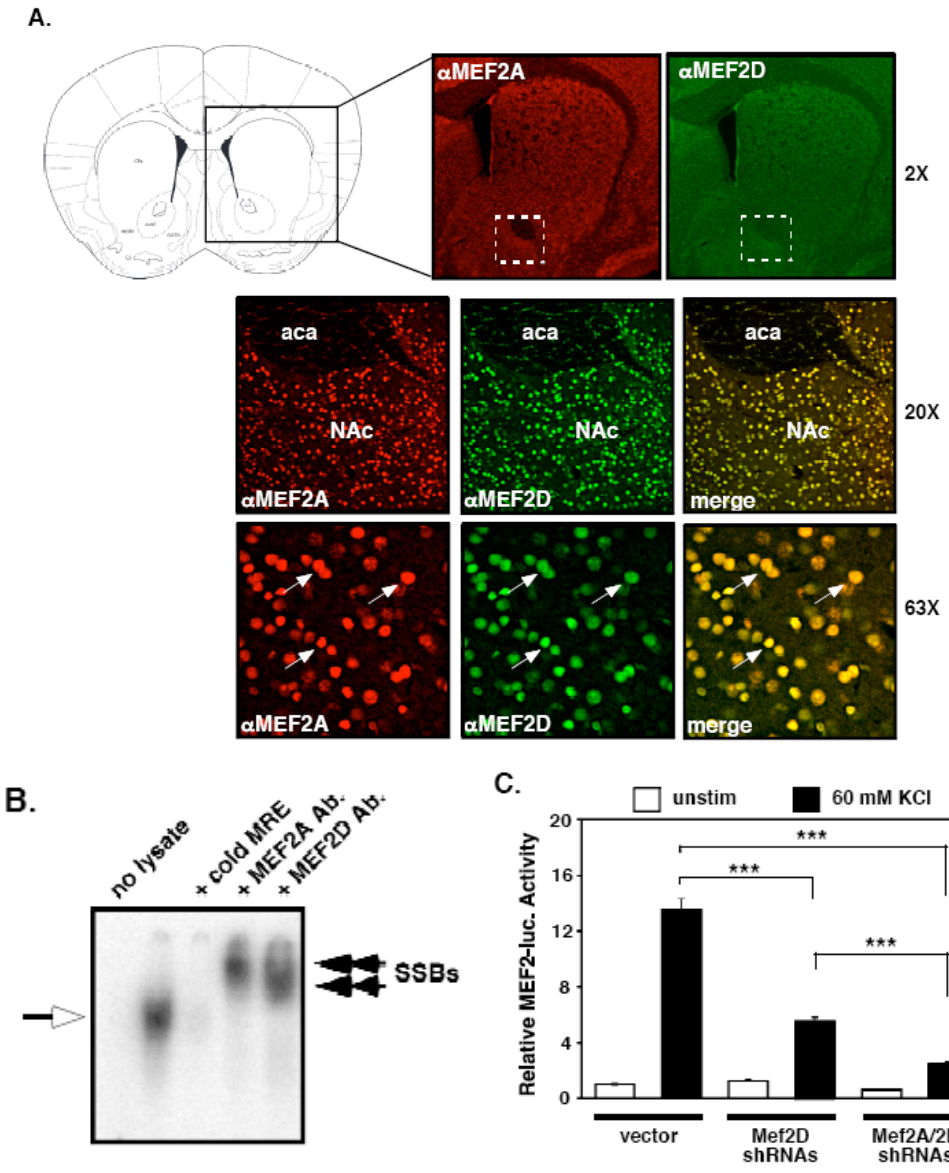


Figure A-1. MEF2A and MEF2D are highly expressed in the adult striatum.

A. Immunohistochemistry for MEF2A and MEF2D demonstrate strong nuclear staining throughout the adult striatum. MEF2A and MEF2D are highly co-localized in most of striatal neurons. **B**. Electrophoretic mobility shift assays (EMSA) were performed on 10 μg of NAc

lysate and ^{32}P-labeled MRE duplex oligos. The shifted MRE band (open arrow) was competed away with excess, unlabelled MRE. Pre-incubation with anti-MEF2A or anti-MEF2D antibodies results in supershifted MRE bands (SSBs, closed arrows) that migrate more slowly in the native gel. **C.** Cultured striatal neurons transfected with an MRE-luciferase reporter plasmid were co-transfected with either plasmids expressing MEF2A and MEF2D specific shRNAs or vector alone. Reduction of MEF2A and MEF2D significantly reduces both basal and membrane depolarization (60 mM KCl)-induced MEF2 activity (***$p<0.001$, $n=6$, two independent experiments).

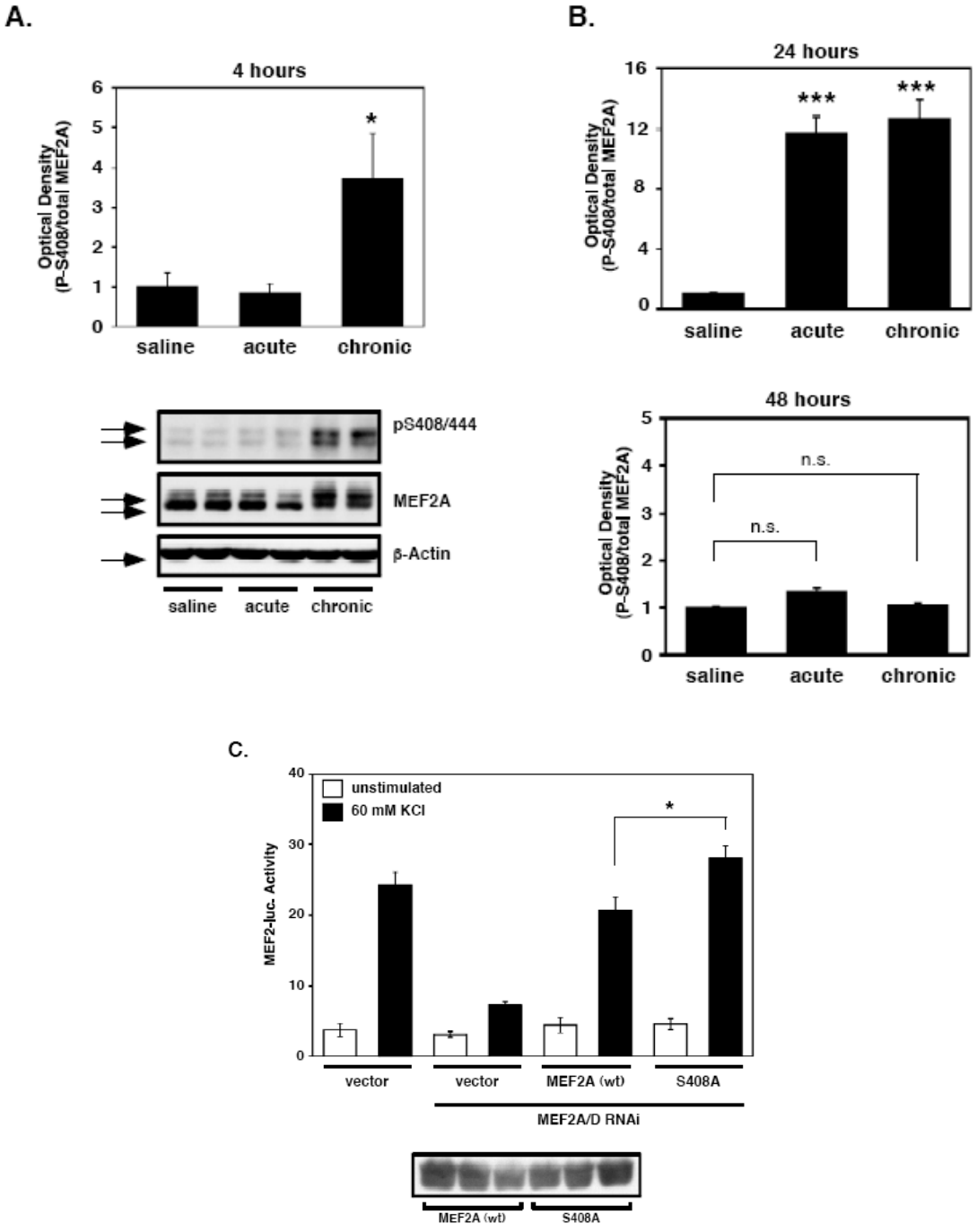


Figure A-2. Chronic cocaine regulates MEF2 activity by phosphorylation at its Cdk5 site.

A. Western blots using a MEF2A/D phospho-S408/444-specific antibody demonstrate that chronic cocaine administration significantly increases MEF2A S408/444 phosphorylation in striatum 4hrs after the last dose (*$p<0.05$, n=3). **B.** Western blots show acute and chronic cocaine administration significantly increase MEF2A/D S408/444 phosphorylation in striatum 24hrs after the last dose (***$p<0.001$, n = 4-5). This increase in phosphorylation returns to control levels by 48hrs after the final dose ($p>0.05$, n = 4-5). **C.** RNAi-based protein replacement assays comparing wild-type and S408A MEF2A proteins. MEF2A S408A has elevated KCl-induced MEF2-dependent transcription activity (representative experiment from two independent experiments, n=6 (top). Anti-MEF2A western blots of HEK-293T total cell lysates of cultures transfected with equal amounts of expression plasmid (bottom).

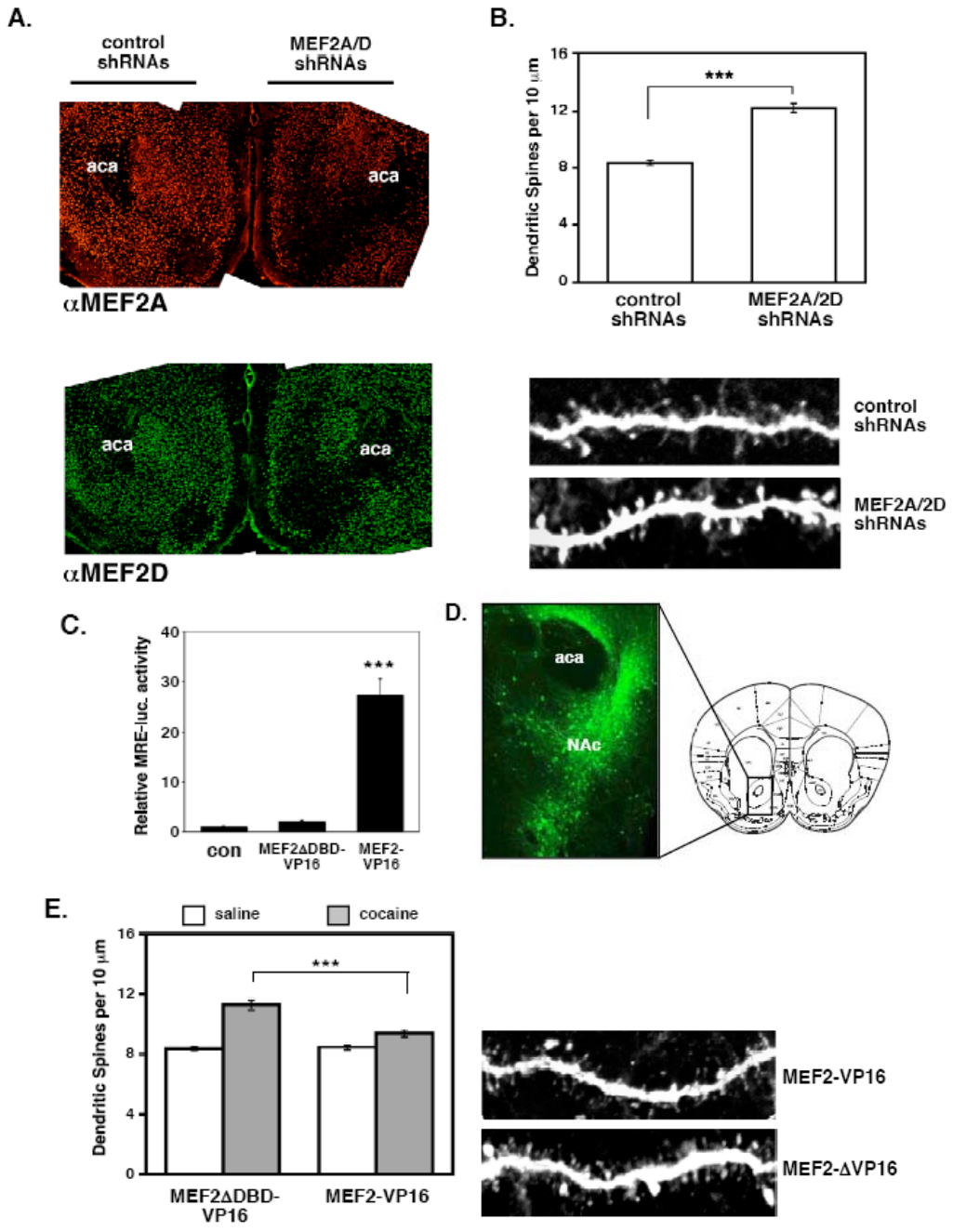


Figure A-3. MEF2 activity regulates dendritic spine density in the NAc.

A. Representative images of MEF2A (top) and MEF2D (bottom) immunostaining 28 days after stereotactic delivery of control AAV-shRNAs (left) and AAV-shRNAs against MEF2A/D into the NAc. Coronal sections through striatum reveal dramatic knockdown of both MEF2A and

MEF2D within the NAc. **B.** Knockdown of MEF2A/D in the NAc significantly increases dendritic spine density in saline-treated mice (8.35+/-0.20 vs. 12.24+/-0.33, ***p<0.001). Representative confocal scans of NAc medium spiny neurons infected with either control or MEF2A/D shRNAs (bottom). **C.** MEF2-VP16 significantly increases MEF2 transcription in cultured striatal neurons (***p<0.001, n=6, two independent experiments). MEF2-VP16 with the DNA binding domain deleted (MEF2ΔDBD-VP16) showed no significant regulation of MEF2 activity in striatal neurons. **D.** Representative image of AAV-MEF2-VP16 infection in the NAc 19 days after surgery. The virus co-expresses GFP, which was visualized by immunohistochemistry. **E.** Overexpression of MEF2-VP16 in the NAc significantly blocked cocaine-induced increases in dendritic spine density. Chronic cocaine treatment induced a significant increase in NAc dendritic spine density compared to chronic saline in mice infected with the control MEF2ΔDBD-VP16 virus in their NAc (11.31 +/-0.34 vs. 8.36 +/-0.11; cocaine vs. saline, ***p<0.001). Expression of constitutively-active MEF2 (MEF2-VP16) significantly blocked the cocaine-induced increase in dendritic spine density in the NAc by ~70% (11.31 +/-0.34 vs. 9.37 +/-0.24; control cocaine vs. MEF2-VP16 cocaine, ***p<0.001), but did not affect basal NAc dendritic spine density in saline-treated mice (8.36 +/-0.11 vs. 8.46 +/-0.17; control saline vs. MEF2-V16 saline, p>0.05). Representative confocal scans of NAc medium spiny neurons infected with either AAVs expressing control MEF2ΔDBD-VP16 or wild-type MEF2-VP16 (bottom).

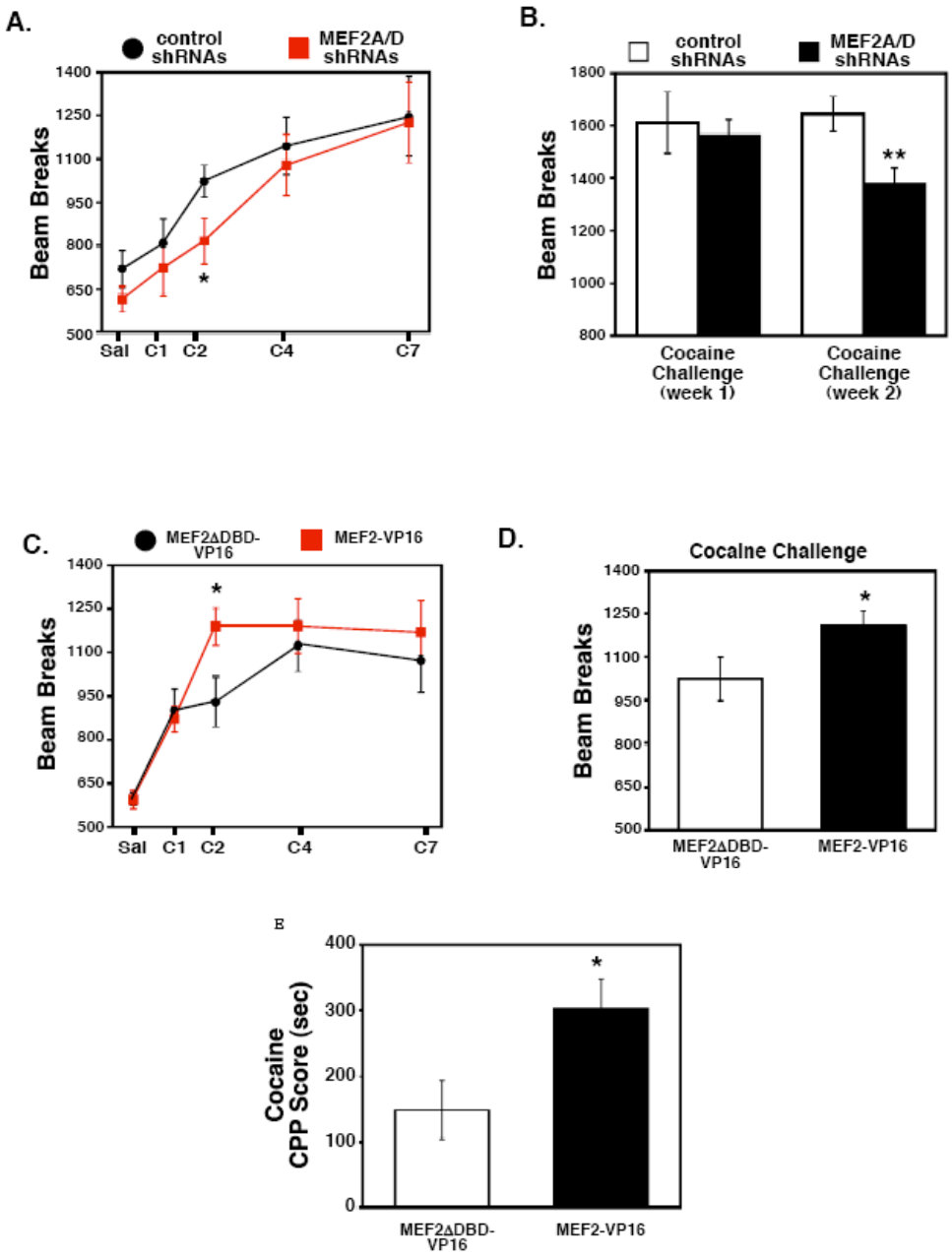


Figure A-4. MEF2 activity in the NAc controls behavioral responses to cocaine.

A. Viral-mediated knockdown of MEF2A/D in the NAc significantly reduces sensitivity to repeated cocaine administration. Mice expressing shRNAs against MEF2A/D in the NAc have normal locomotor responses to saline and the first cocaine injection (15 mg/kg) but are significantly more sensitive to the subsequent dose compared to mice expressing control shRNAs

(*p<0.05, n=9-11). **B.** Mice expressing shRNAs against MEF2A/D in the NAc show significantly less cocaine-induced locomotor activity in response to a challenge dose (15 mg/kg) given two weeks after the acquisition of cocaine sensitization (**p<0.01, n=9-11). Knockdown of MEF2A/D in the NAc has only a slight trend towards reducing locomotor responses to a challenge dose of cocaine (15 mg/kg) one week after acquisition of cocaine sensitization (p>0.05, n=9-11). **C.** Viral-mediated expression of MEF2-VP16 in the NAc significantly increases sensitivity to repeated cocaine administration. Mice expressing MEF2-VP16 in the NAc have normal locomotor response to saline and the first cocaine injection (15 mg/kg), but are significantly more sensitive to the subsequent dose compared to the MEF2-VP16 DNA binding mutant control (MEF2ΔDBD-VP16) (*p<0.05, n=9-10). **D.** Mice expressing MEF2-VP16 in their NAc remain significantly more sensitive to a challenge dose of cocaine (15 mg/kg) after one week of withdrawal (*p<0.05, n=9-10). **E.** Mice expressing MEF2-VP16 in the NAc spend more time in a cocaine-paired (8mg/kg) environment as measured by conditioned place preference (*p<0.05, n = 13).

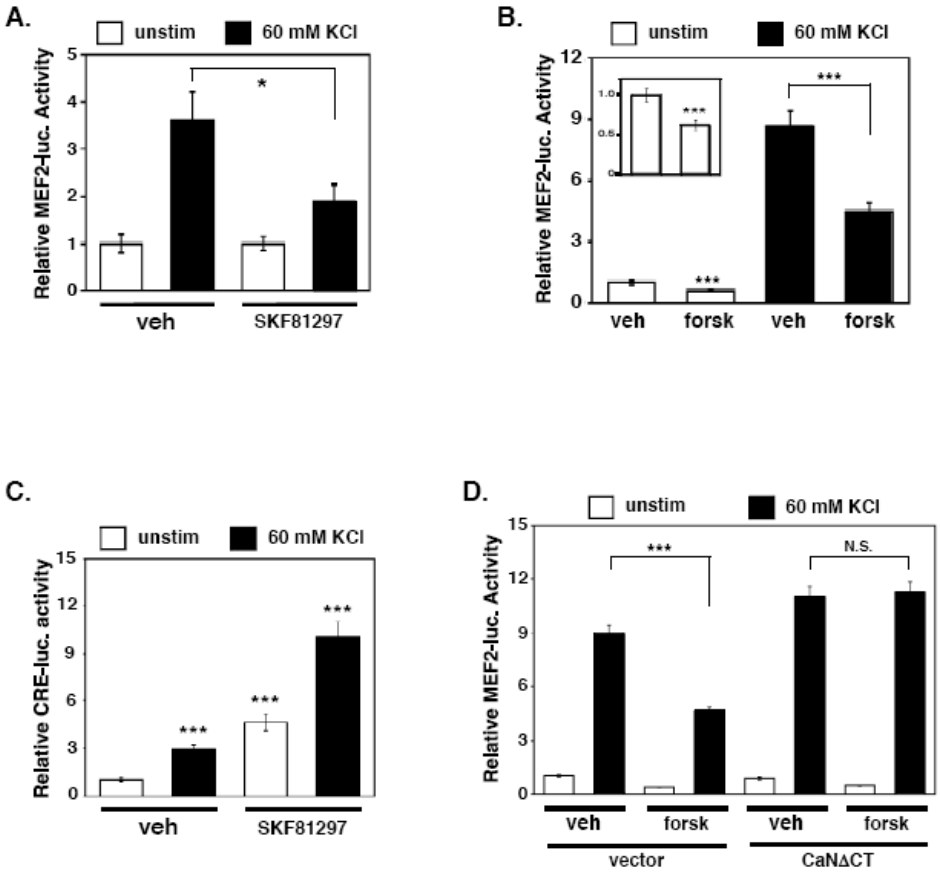


Figure A-5. Dopamine D1 receptor signaling and cAMP reduces calcium-dependent activation of MEF2 in striatal neurons.

A. Dopamine D1 receptor stimulation (SKF81297) significantly reduces calcium-dependent activation of MEF2-luciferase activity in cultured striatal neurons (*$p<0.05$; n=15, five independent experiments). **B.** Forskolin (forsk) treatment (10 μM) of cultured striatal neurons significantly attenuates basal and KCl-induced MRE-luciferase activity. The inset shows the effect of forskolin on basal MRE-luciferase activity over a smaller scale (***$p<0.001$; n=21, seven independent experiments). **C.** KCl (60 mM final) stimulation of cultured striatal neurons significantly increases CRE-luciferase activity. Treatment with dopamine D1 receptor agonist (SKF81297, 10 μM) significantly increases basal and KCl-induced CREB activity in cultured

striatal neurons (***$p<0.001$; n=9, three independent experiments). **D.** Constitutively-active calcineurin (CaNΔCT) blocks the effect of forskolin on KCl-induced MEF2 activity. Cultured striatal neurons transfected with CaNΔCT did not significantly affect basal MRE-luciferase activity. However, after depolarization with 60 mM KCl or KCl+forskolin MRE-luciferase activity was significantly higher than their respective vector controls (***$p<0.001$; n=6, two independent experiments).

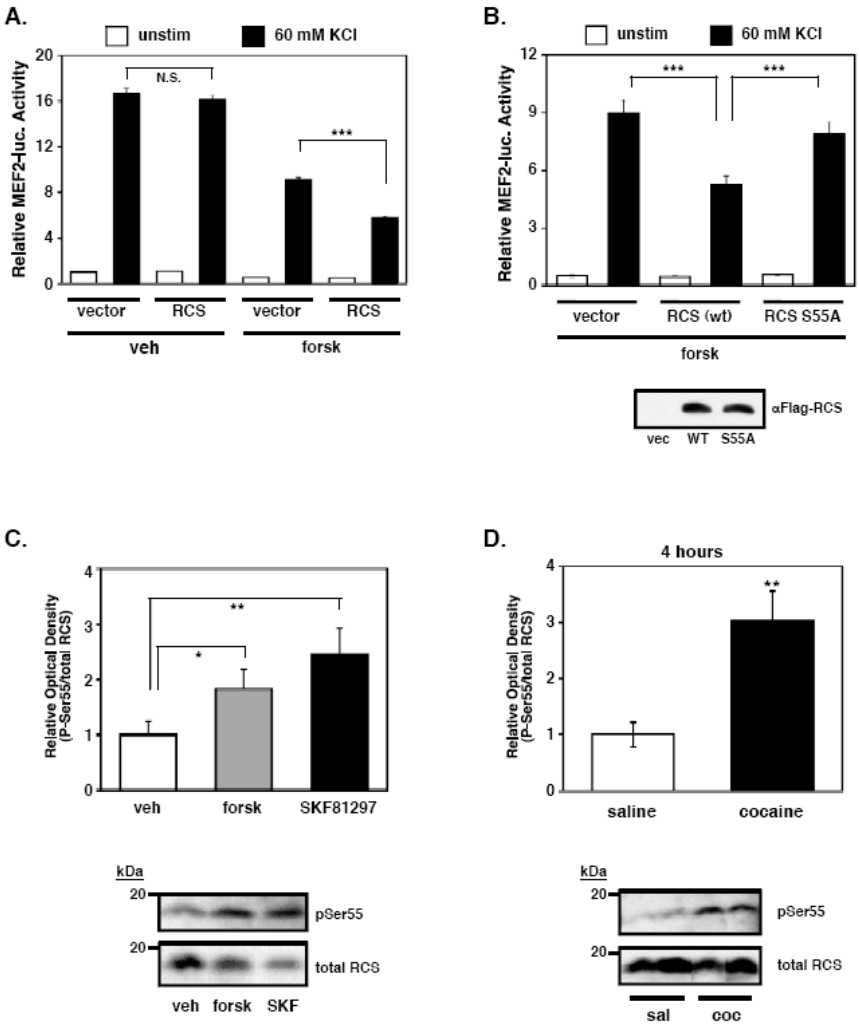


Figure A-6. RCS mediates cAMP-dependent suppression of MEF2 activity.

A. Overexpression of RCS significantly potentiates forskolin-induced inhibition of MEF2 activity. Cultured striatal neurons were transfected with an RCS expression plasmid or vector and stimulated with either vehicle or forskolin (10 μM). Overexpression of RCS did not affect KCl-induced MRE-luciferase activity, but significantly potentiated the repressive effects of forskolin on KCl-induced activity (***$p<0.001$, $n=12$, four independent experiments). **B.** Phosphorylation of RCS at its protein kinase A (PKA) site is necessary for forskolin-induced inhibition of MEF2 activity. Cultured striatal neurons were transfected with either wild-type

RCS or a mutant RCS (S55A) that cannot be activated by PKA. Neurons were then treated with forskolin alone or forskolin + 60 mM KCl. The enhanced suppression of MEF2 by wild-type RCS expression is not observed by expression of the PKA site mutant, RCS S55A. (***$p<0.001$, n=6, two independent experiments). Anti-Flag western blots showing equal expression of wild-type RCS and mutant RCS in HEK-293T cell lysates transfected with equal amounts of the respective plasmids (bottom). **C.** Sections of adult striatum were dissected in bubbled over oxygen and incubated with vehicle, forskalin, or SKF81297. Both forskalin (*$p<0.05$, n=3) and SFK81297 (**$p<0.01$, n=3) significantly increase RCS Ser55 phosphorylation. **D.** Chronic cocaine administration significantly increases RCS Ser55 phosphorylation in striatum 4hrs after the final dose ($p<0.01$, n=4).

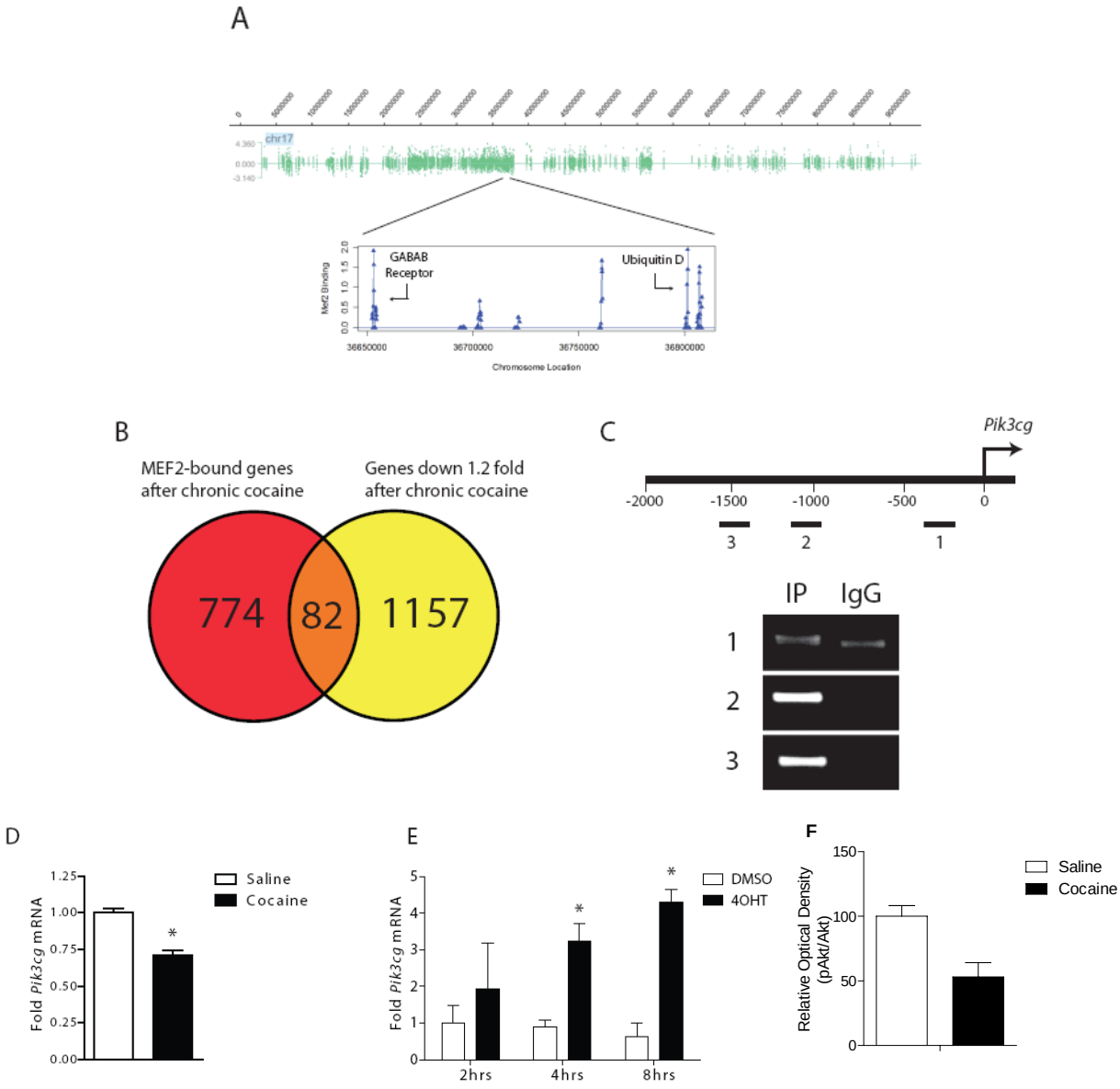


Figure A-7. ChIP-chip analysis of MEF2 in the NAc reveals target genes involved in dendritic plasticity.

A. MEF2 binding in the NAc of chronic cocaine-treated mice is displayed (log2 ratio) along chromosome 17. One region is magnified to display the MEF2 target genes, GABAB receptor and the ubiquitin protein, Ubd. **B.** Venn diagram illustrating the overlap between the genes on which MEF2 is significantly enriched and the genes whose expression is downregulated >1.2

fold by chronic cocaine in the nucleus accumbens. **C.** The location of 3 MEF2-response element (MRE)-like regions (labeled 1-3) are shown on the gene promoter of *pik3cg*, a gene both significantly bound by MEF2 and downregulated >1.2 fold by cocaine. Semi-quantitative ChIP demonstrates enrichment of MFE2 at each of these regions over the IgG control. **D.** Confirmation that *Pik3cg* mRNA is significantly downregulated by cocaine in an independent set of mice (Student's t-test, $*P < 0.05$, $n = 6$). **E.** PC12 cells transfected with MEF2-VP16-ER, which allows the inducible expression of MEF-VP16 by 4-hydroxytomaxifin (4OHT), were treated with 4OHT or DMSO for 2hrs, 4hrs, or 8hrs, and *Pik3cg* mRNA levels were quantified. MEF2-VP16 expression significantly upregulated *Pik3cg* mRNA at 4hrs and 8hrs ($*P < 0.05$, $n = 3$). **F.** Chronic cocaine (20mg/kg) significantly reduced Akt phosphorylation in the NAc 4hrs after the final dose ($p<0.01$ n = 8).

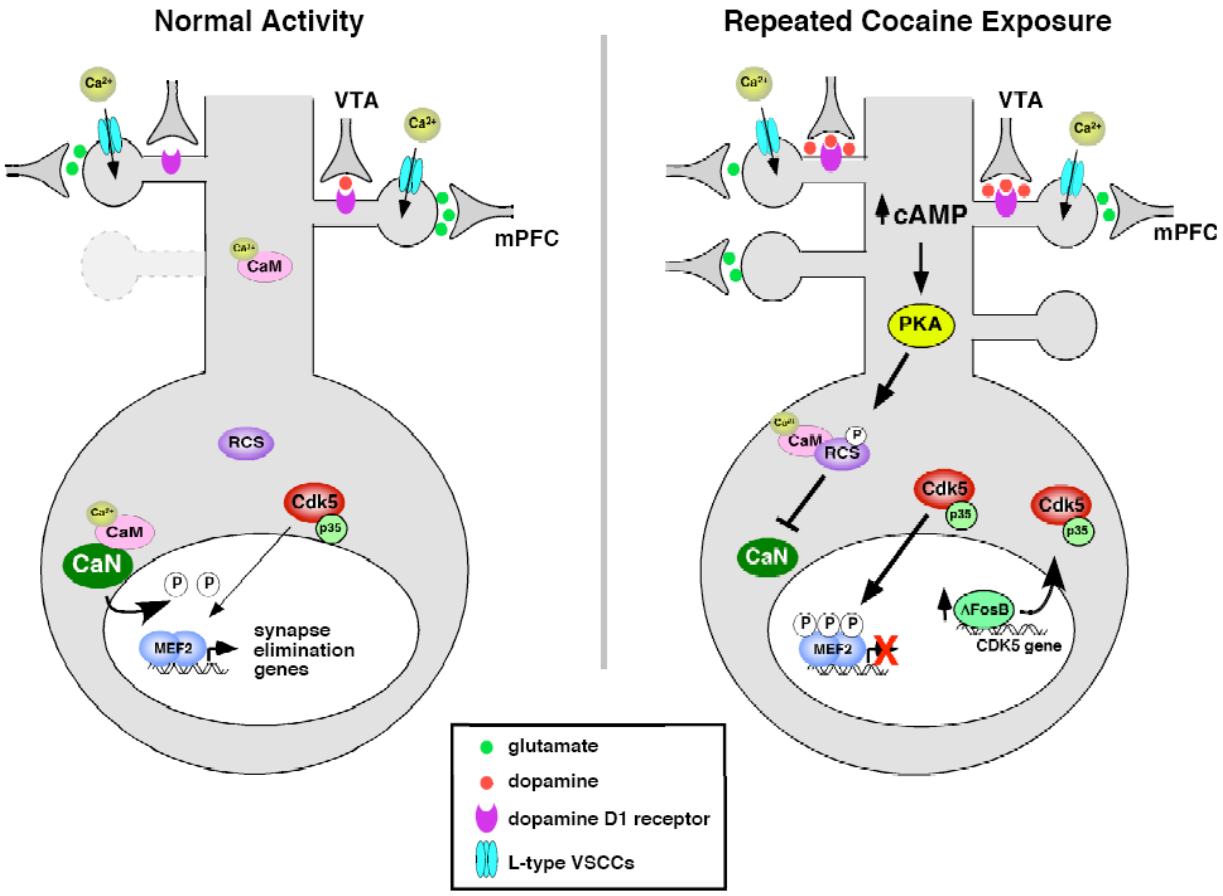
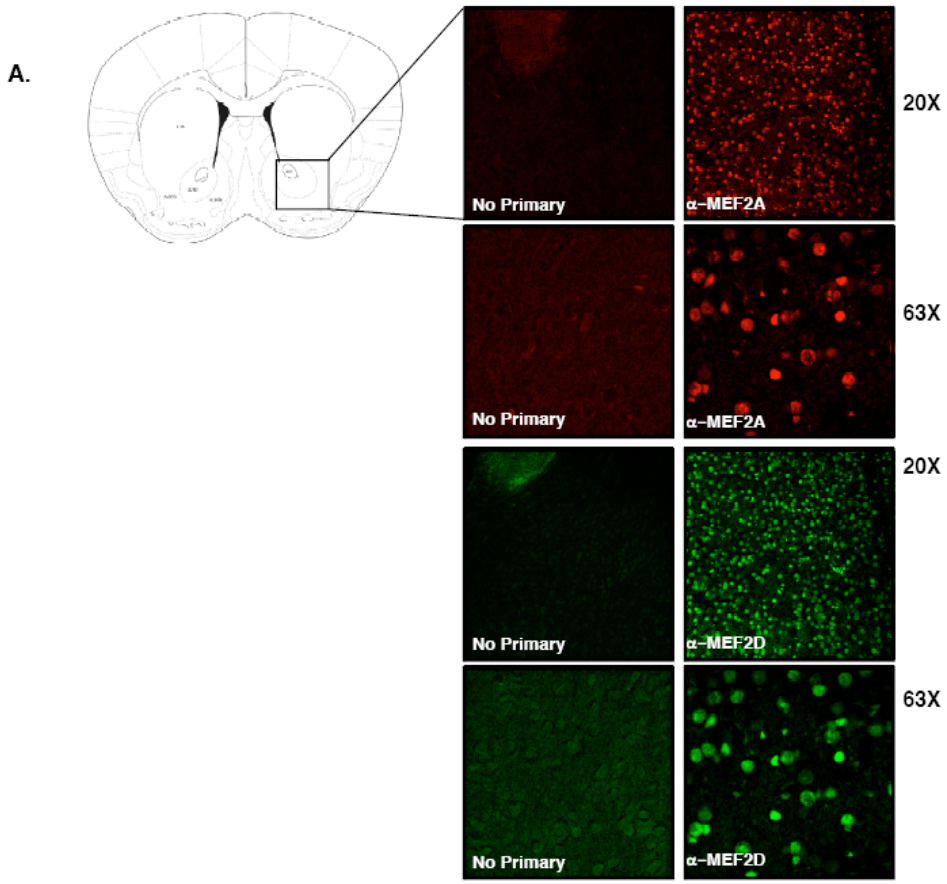
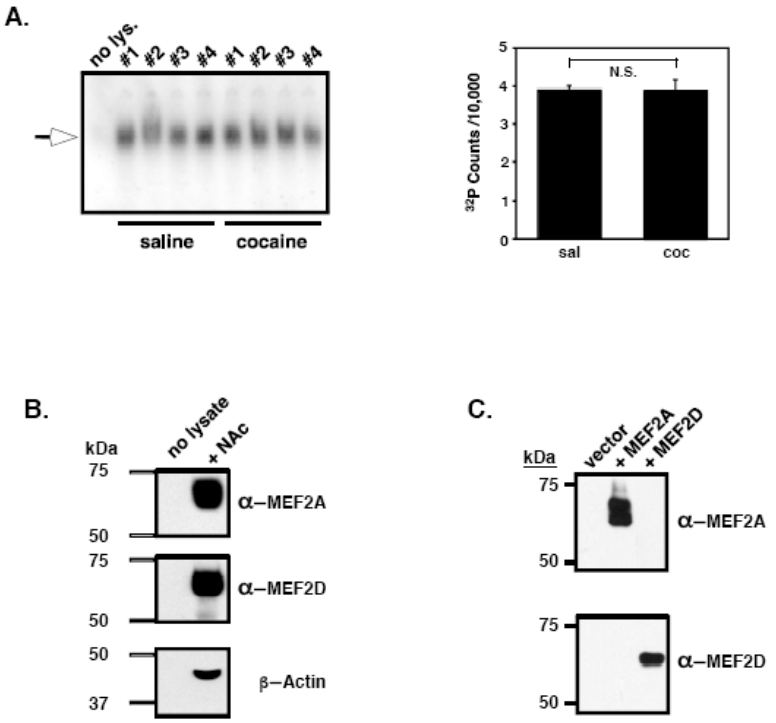


Figure A-8. Model for how cocaine regulates MEF2 in the NAc to alter dendritic spines density and behavioral responses.



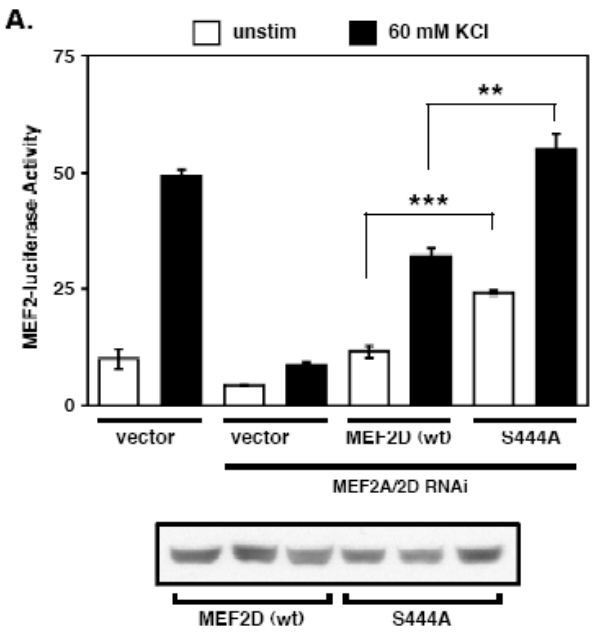
Supplemental Figure A-S1. MEF2 immunostaining in the NAc.

Immunohistochemistry was performed with MEF2A or MEF2D antibodies, or no antibody as a control. Clear MEF2A and MEF2D nuclear staining can be observed at 20X and 63X (right column), while only background fluorescence is observed with no antibody (left column).



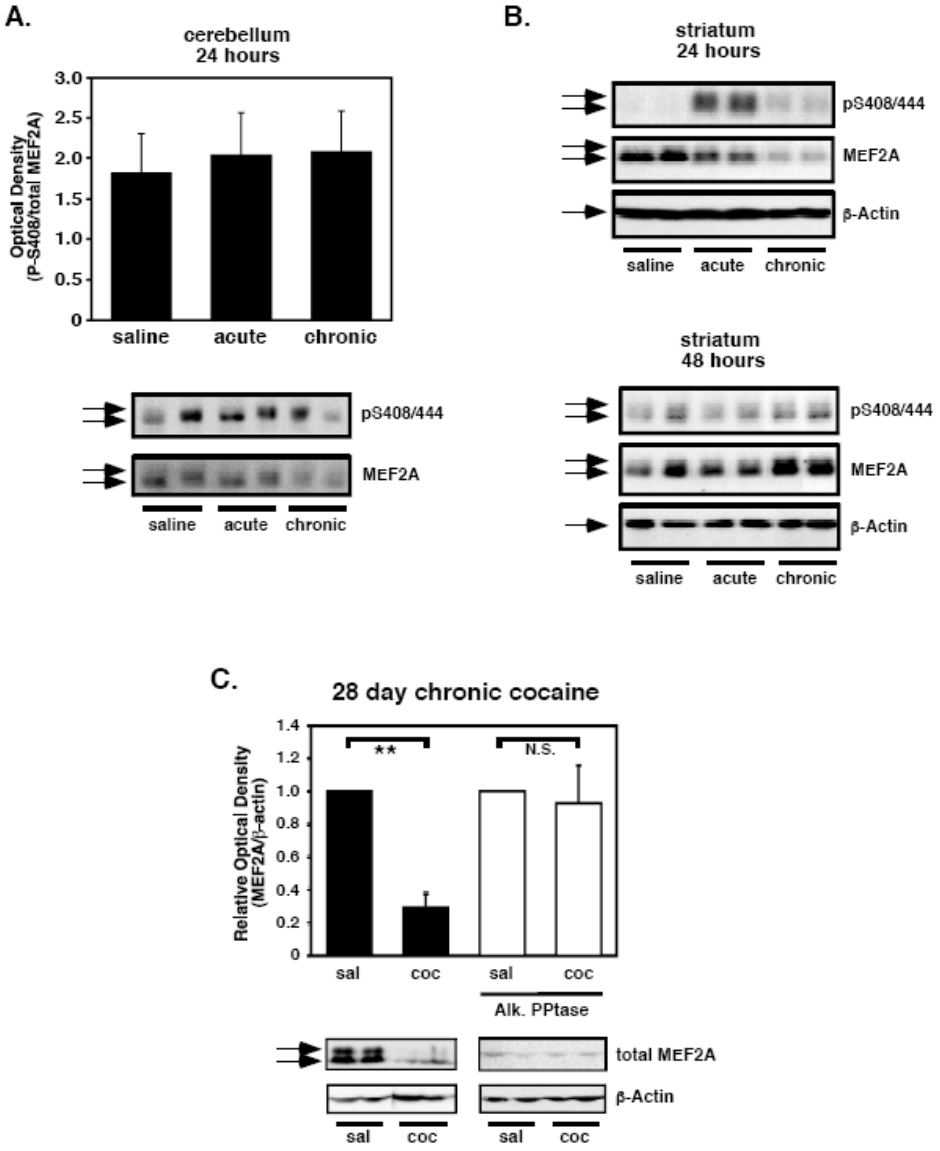
Supplemental Figure A-S2. Chronic cocaine does not alter MEF2 DNA binding activity in the NAc.

A. Chronic cocaine does not regulate DNA binding of MEF2. EMSA was performed on NAc lysates from mice injected daily for 7 days with cocaine or saline. MEF2 binding to the radioactive probe (arrow) reveals no differences in DNA binding with replicate animals (4 per conditions) (left). Quantification of the amount of radioactive MRE shifted with NAc lysates from the mice treated with saline or cocaine for 7 days (right, $P>0.05$, $n=4$). **B.** NAc lysates used for EMSA were probed with antibodies against MEF2A, MEF2D, or β-actin, to verify protein integrity. **C.** 293T cells were transfected with overexpression plasmids for MEF2A, MEF2D, or vector alone. Western blotting of these lysates confirm that the antibodies against MEF2A and MEF2D are highly specific for their respective proteins.



Supplemental Figure A-S3. RNAi-based protein replacement assays comparing wild-type and S444A MEF2D activity.

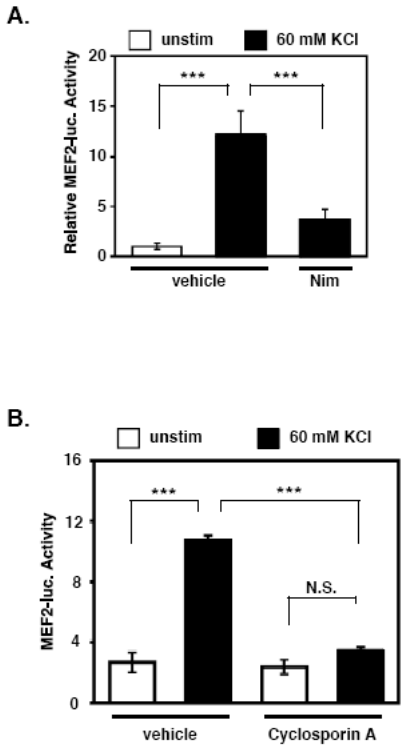
Expression of MEF2D S444A results in elevated basal and KCl-induced MEF2-dependent transcription in MEF2-luciferase assays (representative experiment (n=3) from two experiments) (top). Anti-MEF2D western blots of HEK-293T total cell lysates of cultures transfected with equal amounts of expression plasmids (bottom).



Supplemental Figure A-S4. Regulation of MEF2 phosphorylation by cocaine.

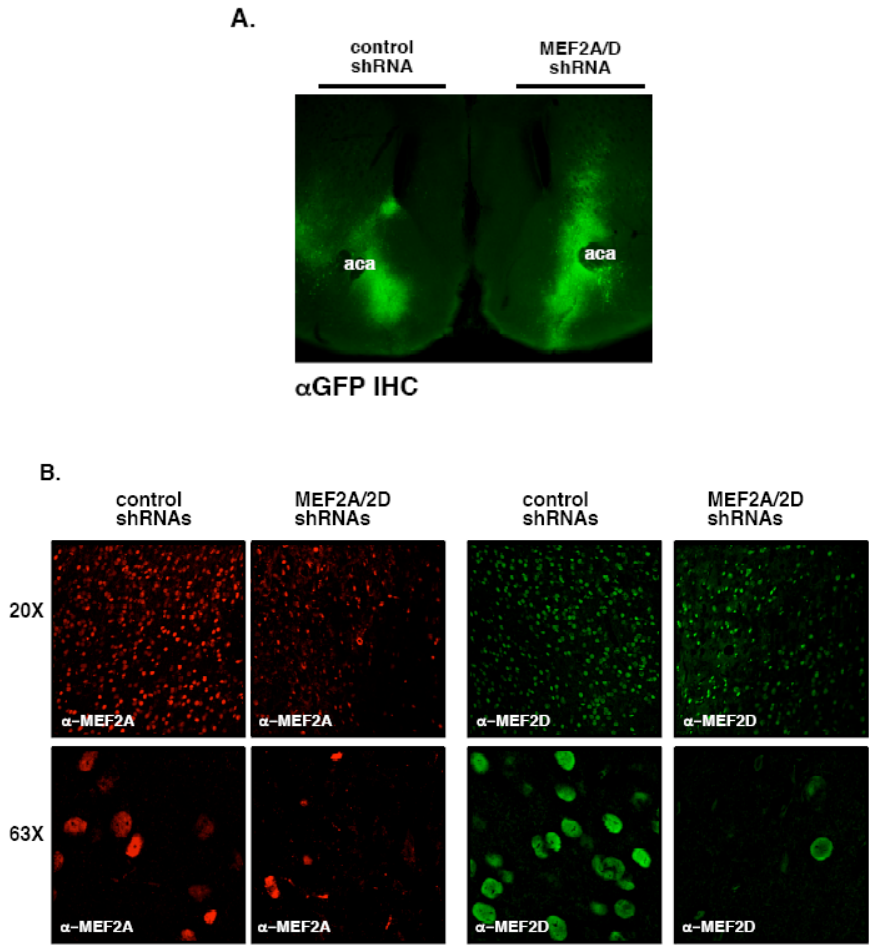
A. Effects of acute (1 day) and chronic (7 day) cocaine injections on cerebellar MEF2 phosphorylation at Ser408/444. Western blot using the MEF2A/D phospho-408/444-specific antibody revealed similar levels of phosphorylation after saline or cocaine injections ($p>0.05$, $n=3$). **B.** Representative western blots of striatal lysates from rats treated with saline, acute, or

chronic cocaine and analyzed 24hrs (top) or 48hrs (bottom) later. Quantification of these blots is displayed in Fig. 2. **C.** Rats were treated with 28 days of cocaine (20mg/kg) and NAc lysates were probed 1hr after the last dose. MEF2A was dramatically hyperphosphorylated by chronic cocaine such that the protein smeared to several molecular weights and became faint. (left). There was significantly less MEF2 staining in cocaine-treated rats compared to saline (**$p<0.01$, $n=5$). Treatment of these lysates with calf alkaline phosphatase collapsed the hyperphosphorylated MEF2 to a single band. The intensity of MEF2 staining after phosphatase treatment was indistinguishable between cocaine- and saline-treated rats ($p>0.05$, $n=5$).



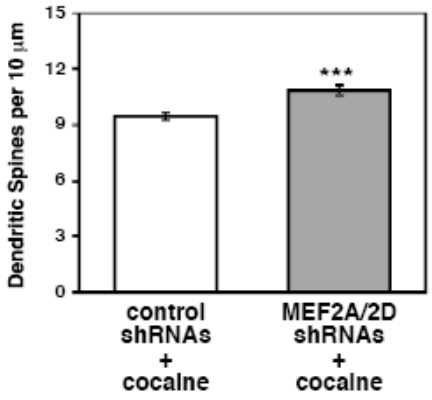
Supplemental Figure A-S5. Mechanisms of MEF2 activation.

A. Nimodipine (10 μM), an L-type calcium channel antagonist, significantly blocks the KCl-induced activation of MEF2 in cultured striatal neurons (***$p<0.001$, n=3). **B.** Cyclosporin A (1 μM), a calcineurin antagonist, significantly blocks the KCl-induced activation of MEF2 in cultured striatal neurons (***$p<0.001$, n=3).



Supplemental Figure A-S6. Knockdown of MEF2 in vivo.

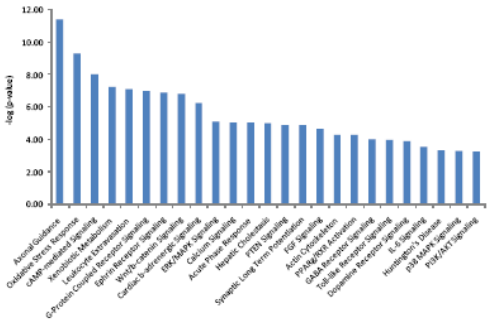
A. Representative anti-GFP immunostaining of NAc-containing coronal sections at 4 weeks after stereotactic AAV-MEF2 shRNA injections. **B.** Immunostaining of MEF2A (left) and MEF2D (right) in NAc infected with AAVs expressing control shRNAs or MEF2A/D shRNAs at 20X (top) and 63X (bottom) magnifications. AAV-infected regions show robust reduction of MEF2A (left, red) and MEF2D (right, green) protein levels in the NAc.



Supplemental Figure A-S7. **Knockdown of MEF2 increases dendritic spines in the NAc.**

Mice were infected with AAVs expressing control shRNA on one side of the NAc and AAVs expressing MEF2A/D shRNAs on the other side of the NAc. Mice were then treated with 28 days of cocaine (20mg/kg) and analyzed 24 hrs later. Knockdown of MEF2A/D in the NAc significantly increased dendritic spine density compared to the control-infected NAc (***$p<0.001$)

A

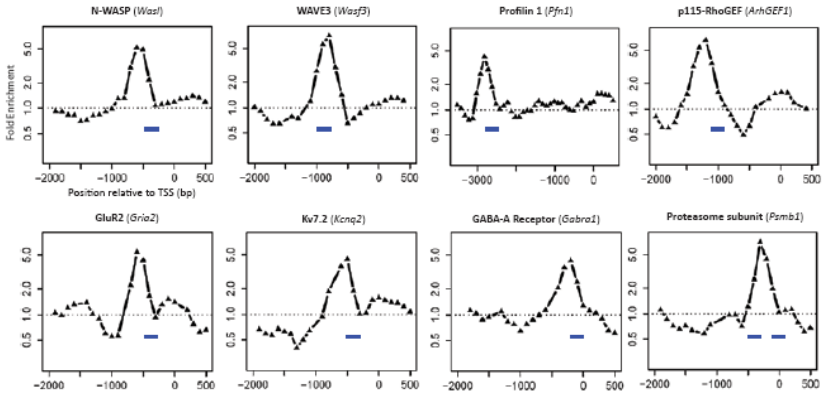


B

MEF2 target genes implicated in dendritic spine plasticity and neuronal function

F-Actin Remodeling:	cAMP Signaling:	Excitability:	Ubiquitin Signaling:
N-WASP	Adcy7	GABAAR1	Ubiquitin D
WAVE 3	ADORA2A	GABAAR2	Usp18
WASP binding protein	PDE7A	GABABR1	Usp50
Profilin 1	ATF4	Kv1.1	Ube1l
p115-RhoGEF	GRK4	Kv4.2	Ube2m
PDZ-RhoGEF	Gαi	Kv4.3	Psmb1
RhoGAP 8	κOR	Kv7.2	
RasGRF2		GluR2	
RasGRP2		5-HT3A	
RasGRP3		N-AchR	

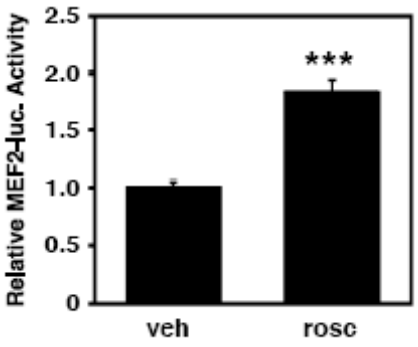
C



Supplemental Figure A-S8. Analysis of MEF2 target genes.

A. Ingenuity pathway analysis of MEF2 target genes identifies several highly enriched pathways, which MEF2 may regulate. Notably, axonal guidance, cAMP-mediated signaling, actin cytoskeleton, and dopamine receptor signaling are among the most enriched pathways and are known to be involved in cocaine responses and dendritic morphology. **B.** Examples of putative MEF2 target genes involved in F-actin remodeling, cAMP signaling, neural excitability, and ubiquitin signaling. Each of these pathways can contribute dendritic spine plasticity. **C.**

Representative genes where MEF2 is significantly bound in the NAc after chronic cocaine. The chromosomal location (x-axis) is displayed relative to the transcriptional start site (0). The blue bar underneath each plot represents a consensus or near-consensus MEF2 response element.



Supplemental Figure A-S9. **Cdk5 regulates MEF2 in striatal neurons.**

Inhibition of Cdk5 increase MEF2 activity in striatal neurons. Roscovitine (rocs, 10μM) significatntly increases MEF2-luciferase activity in cultured striatal neurons (*$p<0.001$, $n = 6$, two independent experiments).

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