THE ROLE OF DNA METHYLATION

IN ADDICTION AND DEPRESSION

APPROVED BY SUPERVISORY COMMITTEE

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

Dr. Christopher Cowan Ph.D.

Dr. Craig Powell, M.D., Ph.D.

Dr. Joel Elmquist, D.V.M., Ph.D.

DEDICATION

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Finally, I would like to dedicate this thesis to the people that have struggled from or are currently struggling with depression or addiction. I hope that this work will become a small step towards elucidating the underpinnings of these terrible diseases.

THE ROLE OF DNA METHYLATION IN ADDICTION AND DEPRESSION

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QUINCEY CHARLES LAPLANT

DISSERTATION

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THE ROLE OF DNA METHYLATION

IN ADDICTION AND DEPRESSION

QUINCEY CHARLES LAPLANT, M.D., Ph.D.

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

Despite abundant expression of DNA methyltransferases (Dnmt's) in brain, the regulation and behavioral role of DNA methylation remain poorly understood. I found that Dnmt3a expression is persistently regulated in nucleus accumbens (NAc), a key brain reward region, by chronic cocaine or chronic social defeat stress. Moreover, NAc specific manipulations that block DNA methylation potentiate cocaine reward and exert antidepressant-like effects, whereas NAc specific Dnmt3a overexpression attenuates cocaine reward and is pro-depressant. On a cellular level, I discovered that chronic cocaine selectively increases thin dendritic spines on NAc neurons and that DNA methylation is both necessary and sufficient to mediate these effects. I then used complementary genome wide techniques aimed at identifying cocaine-induced DNA methylation at gene targets. Transcriptional profiling experiments in which I virally overexpressed Dnmt3a or pharmacologically blocked DNA methylation identified an important role of DNA methylation in

regulating the mRNA expression of a number of immediate early genes—several of which are known to regulate dendritic spine morphology. Taken together, these data establish the importance of Dnmt3a in the NAc in regulating molecular, cellular, and behavioral plasticity to emotional stimuli.

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LIST OF ABBREVIATIONS

ΔFosB	Truncated form of FosB that is induced by chronic drug use
AAV	Adeno-associated virus
acH3,acH4	Acetylated histone H3 or H4
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
CamKII	Calcium/calmodulin-dependent protein kinase II
CART	Cocaine and amphetamine regulated transcript
CBP	CREB-binding protein
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
CREB	cAMP response element binding protein
DARPP-32	dopamine and cAMP regulated phosphoprotein of 32 kDa, DARPP-32
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
Dnmt1, 3a, 3b	DNA methyltransferase 1, 3a, 3b
EGR	Early growth response protein
FosB	FBJ murine osteosarcoma viral oncogene homolog B
G9a	KMT1c
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)
H3K9me2	dimethylation of histone H3 at Lys9
H3pS10	phosphorylation of histone H3 at Ser10
H3S10	Histone H3 phosphorylated at serine 10 (activating)
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDAC 1, 2, 4, 5, 7, 9	Histone deacetylase 1, 2, 4, 5, 7, 9
HMT	Histone methyltransferase
HSV	Herpes simplex virus
IEG’s	immediate early genes
LTD	long term depression
LTP	long term potentiation

MBD	Methyl-CpG-binding domain protein
mDIP	methylated DNA immunoprecipitation
meDNA	methylated DNA
MEF2	Myocyte enhancer factor 2
mEPSC	miniature excitatory post-synaptic current
MS-275	HDAC inhibitor
MSK1	mitogen- and stress-activated kinase 1
MSN	Medium spiney neurons
NAc	Nucleus accumbens
Nf-κB	Nuclear factor kappaB
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
pacH3	phosphoacetylated histone H3
PCR	Polymerase chain reaction
PP1	Protein phosphatase 1
PTMs	post translational modification
RG108	DNA methyltransferase inhibitor
RNA	Ribonucleic acid
SAHA	HDAC inhibitor (suberoylanilide hydroxamic acid)
SAM	S-adenosyl-L-methionine
SIRT1,2	Sirtuin 1,2
TSA	Trichostatin A (HDAC inhibitor)
TSS	transcription start site

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CHAPTER 1

INTRODUCTION

Preamble – The neuroplasticity paradox (why study epigenetics?)

Essentially all known mechanisms of long-lived neuronal plasticity involve transient intracellular signaling cascades. And over the past several decades neuroscientists have unraveled many mysteries of how these signaling events translate into persistent changes in synaptic function (changes which may underlie the basis of learning and memory). On a molecular scale, key examples include insertion/removal of receptor subunits from synaptic membranes, alteration of synaptic protein function via posttranslational modifications, and the regulation of the translation or degradation of proteins at the synapse. However, despite the "nobel" worthiness of these findings, a paradox exists. Despite the persistence of many neuronal adaptations, all the above mechanisms occur at the protein level and are therefore subject to eventual turnover. Neuroscientists have long ignored the fact that the only permanent structure in any given neuron is its genome. Therefore, these transient intracellular signaling cascades that initiate all forms of neuronal plasticity must in some way be recorded in the genome. In order to adapt properly (and persistently), each neuron must retain a "memory" of where it has been—what genes were expressed, and just as importantly what genes were not. Thanks to advances in sequencing and advances in our understanding of epigenetics, efforts are underway to identify the missing link in the neuroplasiticy paradox. Is it DNA methylation?

Drug addiction introduction

Drug addiction is a debilitating psychiatric disorder characterized by compulsive drug seeking and taking despite severe adverse consequences (Hyman et al., 2006; Kalivas et al., 2005; Koob and Kreek, 2007). Once a person succumbs to addiction, few effective therapies exist. Even when former addicts remain abstinent for long durations of time, they often find themselves in a lifelong struggle—always vulnerable to drug relapse. Therefore, the two major questions on which basic science research focuses relate to understanding the molecular events that occur during: 1) the transition to the addicted state and 2) the maintenance of the addicted state. A better understanding of these mechanisms would provide insight into how we can block or perhaps reverse the neuroplastic changes that define addiction.

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. A multitude of microarray studies under different experimental conditions have found drug-induced alterations in the expression levels of hundreds of mRNAs in these target regions (Freeman et al., 2010; Heiman et al., 2008; Maze et al., 2010; McClung and Nestler, 2003; McClung et al., 2005; Renthal et al., 2007; Winstanley et al., 2007; Yao et al., 2004). In response to psychostimulants, many genes, such as those encoding c-Fos, FosB, ΔFosB, ATF2 (activating transcription factor 2), ATF3, and ATF4, are

rapidly and transiently induced in response to initial drug exposures. Chronic exposure differentially affects the steady state levels of these various mRNA's as well as their degree of induction upon re-exposure to the same drug dose with some genes showing sensitized responses and others desensitized responses (Alibhai et al., 2007; Green et al., 2008; Hope et al., 1994; Renthal et al., 2008). In contrast, numerous other genes, such as those encoding CDK5 (cyclin-dependent kinase 5), several NFκB (nuclear factor κB) subunits, SIRT2 (sirtuin 2), PSD-95 (postsynaptic density protein of 95 kD), and BDNF (brain-derived neurotrophic factor), are consistently induced only by chronic drug experience, and some even increase further over several weeks of withdrawal after the last drug experience (Alibhai et al., 2007; Bibb et al., 2001; Green et al., 2008; Grimm et al., 2003; Hope et al., 1994; Renthal et al., 2008; Renthal et al., 2009; Russo et al., 2009; Yao et al., 2004).

These complex patterns of transcriptional regulation point to the need to identify the underlying mechanisms responsible for altering a gene's 'inducibility' and those capable of stably influencing transcription for prolonged periods. In focusing on psychostimulant-induced changes in the NAc, recent evidence has suggested that epigenetics—a molecular translator that interprets diverse environmental stimuli into changes in gene expression via the regulation of chromatin structure—contributes to drug-induced transcriptional and behavioral changes (Kumar et al., 2005; Levine et al., 2005; Maze et al., 2010; Renthal et al., 2007; Wang et al., 2010). I propose an integrative approach in discussing current progress being made toward understanding how epigenetic mechanisms

are regulated by cocaine and other psychostimulant drugs of abuse in the NAc to influence specific gene expression programs and how such mechanisms might contribute to addiction-related behaviors.

Epigenetics overview

Historically, the word ‘epigenetic’ refers to a heritable phenotype not coded by DNA itself, but by a cellular process ‘above the genome’. More recently, epigenetics is used to refer to the extremely complex processes of organizing the genome in a manner that allows for regulated gene expression in the appropriate cell type upon appropriate cellular stimuli. On a molecular level, the fundamental unit that accomplishes this feat is chromatin, which is composed of DNA wrapped around histone octomers made up of two copies each of H2A, H2B, H3, and H4. In the past decade, it has been appreciated that the structure of chromatin is highly regulated by post-translational modifications (PTMs) that occur on histones and DNA itself. Importantly, these modifications—via regulation of chromatin structure—profoundly influence gene expression in different ways and, since multiple PTMs can occur on a given histone octomer, it is hypothesized that the combination of these modifications summate to influence gene expression. Also known as the histone code hypothesis (Borrelli et al., 2008; Kouzarides, 2007; Lee and Mahadevan, 2009; Strahl and Allis, 2000). Highlighted in Table 1-1, are just a few well characterized examples of such PTMs, their associated effect on gene transcription, as well as the enzymes that

'write' and 'erase' such modifications (reviewed in greater detail in: Renthal and Nestler, 2008; Strahl and Allis, 2000).

DNA methylation overview

DNA methylation, or the covalent addition of methyl groups to C5-position of cytosine residues within CpG dinucleotides, forms the basis of chromatin structure and is a central component to gene regulation in all cells (ref bird). DNA methylation can impact gene transcription primarily through two mechanisms. First, methylation can physically impede the binding of transcriptional machinery to their target sequences. One well known example is how methylation of the CRE target sequence blocks the transcription factor CREB from binding to promoter elements (Chen et al., 2003; Martinowich et al., 2003). The second and probably more important way that methylation influences transcription is via recruitment of Methyl binding domain proteins (MBDs). This family of proteins is expressed at near histone octomer levels in neurons and specifically bind methylated DNA to subsequently recruit other pro-repressive histone modifying enzymes such as histone deacetylases (HDACs) (Klose and Bird, 2006; Skene et al., 2010). In addition to regulating gene expression, DNA methylation is required for gene imprinting, x-inactivation, suppression of retrotransposible elements, suppression of viral gene expression, and general chromosomal stability (Klose and Bird, 2006; Skene et al., 2010).

On a genome wide level, DNA methylation displays a curious distribution pattern which is dependent of the frequency of CpG dinucleotides and via

unknown mechanisms. Statistically, CpG sequences occur throughout the genome at a much lower frequency than expected by chance. And overall approximately 70% of CpGs in the genome are methylated. This low frequency in the CpG sequence is attributed to the high susceptibility of CpGs in undergoing spontaneous mutation (via deamination). However, there are certain hotspots in the genome called “CpG islands” that have very high frequencies of the CpG sequence. CpG islands are highly conserved in mammals and are mostly located within gene promoter regions (Gardiner-Garden and Frommer, 1987; Takai and Jones, 2002). Of all mammalian genes, approximately 40% have CpG islands and, surprisingly, most CpG islands have low levels of methylation (Klose and Bird, 2006; Takai and Jones, 2002). It is thought that the purpose of low promoter methylation levels is to render a gene subject to regulation via DNA methylation. In summary, normal cells have a mosaic pattern of DNA methylation. The genome has highly methylated individual CpG sites along with unmethylated CpG islands.

DNA methylation has been most extensively studied in the cancer field. Here it has been found that the mosaic pattern of DNA methlyation is differentially affected in cancer cells. There is a genome wide hypomethylation contrasted by hypermethylation at CpG islands. In a seminal study by Weber, a technique called mDIP-Chip (methylated DNA ImmunoPrecipitation on Chip) was pioneered in order to ultimately identify ~200 novel hypermethylated CpG islands (Weber et al., 2007). Moreover, thanks to major advances in microarray technology, recent studies have found that a much greater degree of differential

DNA methylation (10 fold more) surprisingly occurs in a region ~2 kb upstream from CpG islands. These regions have been called “CpG shores” (Irizarry et al., 2009).

The three known enzymes that methylate DNA are Dnmt1 (DNA methyltransferase 1), Dnmt3a, and Dnmt3b. Dnmt’s mediate the ezymatic transfer of methyl groups from S-adensyl-methionine to cytosine through an unusual mechanism whereby a cytosine base is flipped out of helical DNA, modified, and then replaced into the helix (Jeltsch, 2002). Aside from this mechanism, very is little is known about the upstream mechanisms that regulate Dnmt activity and genomic specificity. Functionally, Dnmt1 is often referred to as the “maintenance” methyltransferase because it appears to play a role in faithfully maintaining DNA methylation patterns in newly synthesized DNA. The evidence that Dnmt1 primarily allows for DNA methylation to be inherited after cell division comes from observations that Dnmt1 localizes with replication foci during S-phase and the catalytic efficiency of Dnmt1 is 3-10 fold higher on hemimethylated substrates compared to unmethylated DNA substrates (Bacolla et al., 1999). On the other hand, Dnmt3a and Dnmt3b are referred to as “de novo” methyltransferases because they establish methylation patterns at specific sites within the genome (Okano et al., 1999). Whether these ascribed functions (maintenance and de novo methylation) are generally applicable to all mammalian cells is a matter of debate. In all likelihood, there is functional crosstalk between Dnmt1 and the de novo methyltransferases. For example, Dnmt1 and Dnmt3a are the predominant isoforms expressed in adult, non-

dividing neurons. On a practical level, the fact that it is expressed in non-dividing cells suggests that Dnmt1 must have some de novo methylation activity as well. Consistent with this notion, a recent study found that conditional knock-out of Dnmt3a was not sufficient to reduce total brain DNA methylation levels. However, double conditional knockout of both Dnmt1 and Dnmt3a reduces DNA methylation levels in adult neurons (Feng et al., 2010).

The fact that double knock out of Dnmt1 and Dnmt3a reduces total levels of DNA methylation is also of interest because it suggests that mechanisms must exist to remove methylated DNA from the genome. DNA methylation is widely considered to be the most persistent epigenetic modification in nature. This is attributed to the theoretical difficulty in enzymatically removing methyl-cytosine's carbon-carbon bond and is also attributed to the well known role that DNA methylation plays in permanently silencing the X chromosome in females. In light of this, considerable debate exists as to whether an actual DNA demethylase exists. Several publications have sprung up over the years implicating a host of different proteins in DNA demethylation—including MBDs and even certain Dnmt's. At present, the leading candidate mechanism for demethylation involves complete removal of methylated cytosine from DNA structure via recruitment of DNA base excision repair enzymes, called GADD45's (Growth arrest and DNA damage repair proteins, Ma et al., 2009; Ooi and Bestor, 2008) (Table 1-1).

Important caveats

There are several important caveats to consider when studying epigenetics in brain. First, a close look at Table 1-1 illustrates that many enzymes each have multiple histone substrates and, in fact, although not listed in the table, it is highly likely that many have non-histone substrates as well. For example, in addition to deacetylating H4K16 (histone H4 on Lys16), SIRT2 is well known to also deacetylate tubulin as well as several major transcription factors (Renthal and Nestler, 2009). Therefore, in order to understand the cellular and behavioral role of a given enzyme and histone modification, a multifaceted approach is absolutely crucial. A second major caveat is that most of the discovered PTMs and their ascribed influence on transcription have been derived from *in vitro* work in non-neuronal cells. Therefore, it is possible that the presumed functions of known PTMs in influencing transcription in cultured cells may not necessarily be the same in brain. Moreover, it is likely that unique PTMs exist in brain. In fact, a recent proteomic study on whole brain tissue identified 196 novel histone modifications (Tweedie-Cullen et al., 2009), and a study analyzing brain DNA identified hydroxy-methylation as a novel brain-specific DNA modification (Kriaucionis and Heintz, 2009; Tweedie-Cullen et al., 2009). A final caveat is that histone PTMs, although the most widely studied, are just one of many epigenetic mechanisms. A few examples that remain virtually unexplored in brain include histone tail clipping, histone substitution, and histone

sliding/remodeling, which are reviewed elsewhere (Borrelli et al., 2008; Tsankova et al., 2007).

Epigenetic mechanisms in addiction

One of the most striking findings to date is that, despite the abundance of histone PTMs throughout the genome, drugs of abuse as well as many other types of environmental stimuli are capable of inducing changes in total levels of various histone modifications, for example, as detected by western blotting or immunohistochemistry, in specific brain regions. With respect to cocaine, most global changes observed are consistent with a state of increased gene activation (i.e., a more permissive state) within the NAc. Following a single cocaine injection, total levels of acetylated histone H4 (acH4), phosphoacetylated histone H3 (pacH3), but not acH3 alone, are increased in this brain region within 30 minutes (Kumar et al., 2005). Moreover, in a cocaine self-administration model, both acH4 and acH3 have been found to be increased in the NAc shell, not core, following chronic but not acute self-administration. Interestingly, in these animals, levels of acH3, and not acH4, were found to be positively correlated with motivation for cocaine (Wang et al., 2010). Also, after repeated cocaine injections, a recent study found a global reduction in dimethylation of H3 at Lys9 (H3K9me2), a repressive histone modification, in the NAc (Maze et al., 2010).

In concert with these global epigenetic alterations, cocaine regulates the expression of several chromatin modifying enzymes in the NAc in directions generally consistent with the global modifications (see Table 1-1). For example,

following chronic but not acute cocaine, HDAC5 is shuttled out of the nucleus of NAc neurons and HDAC activity is significantly reduced (Renthal et al., 2007; Romieu et al., 2008). At the same time, phosphorylation of histone H3 at Ser10 (H3pS10) appears to be mediated via direct phosphorylation by MSK1 (mitogen- and stress-activated kinase 1) as well as by nuclear shuttling of DARPP-32, a striatal-enriched protein which indirectly regulates phosphorylation via potent inhibition of PP1 (Brami-Cherrier et al., 2005; Stipanovich et al., 2008). Likewise, the downregulation of H3K9me2 appears to be mediated via transcriptional repression of G9a (also known as KMT1c), a histone methyltransferase that catalyzes H3K9 dimethylation (Maze et al., 2010). Thus, chronic cocaine appears to globally induce a more permissive transcriptional state in the NAc (increased acH3, acH4, pacH3; decreased H3K9me2), perhaps via differentially affecting histone modifying enzymes that activate (MSK1, DARPP-32, CBP) and repress (HDAC5, G9a) transcription.

Despite the fact that several chromatin modifying enzymes are regulated in the NAc by cocaine, it is still surprising that the absolute levels of PTMs are altered because modifications such as histone acetylation and methylation are known to occur throughout the genome. Thus, in order to have an absolute change, sweeping alterations must occur throughout vast genomic loci despite the fact that many DNA microarray studies of NAc from cocaine-treated animals have found that only a relatively small number (~100's) of specific genes are differentially expressed. Moreover, many specific gene promoters display changes in histone acetylation and methylation after cocaine that go in the

direction opposite of the global modifications (Renthal et al., 2009). Thus, further work is needed to understand the physiological significance of these global changes in epigenetic PTMs.

Insights from genome-wide regulation of gene expression and chromatin structure

Our laboratory recently utilized ChIP-chip (chromatin immunoprecipitation followed by promoter chip) technology to investigate the genome-wide binding profile of chronic cocaine-induced changes in the mouse NAc at two general modifications associated with gene activation and one modification associated with gene repression. The antibodies used in this study referred to as 'acH3', 'acH4', and 'meH3' each recognize multiple PTMs: polyacetylated histone H3 (K9, K14), polyacetylated H4 (K5, K8, K12, K16), and dimethylated H3 at two sites (K9, K27), respectively (Renthal et al., 2009).

This study reported several key findings for epigenetic regulation in brain. An obvious yet often overlooked concept is the importance and complexity of location in analyzing the epigenetic landscape of a gene's promoter. Here, analysis of the averaged genome-wide spatial binding profile revealed a distinct binding distribution pattern for each histone modification. In general, enrichment of acH3 and acH4 occurs within 500 base pairs (bp's) of a gene's transcription start site (TSS), with acH4 having a particularly sharp peak at the TSS (Renthal et al., 2009). Conversely, NAc meH3 enrichment has a broad bimodal distribution with maximal peaks at -1500 bp and +400 bp away from the TSS (Renthal et al.,

2009; Wilkinson et al., 2009). Such overt differences in spatial binding profiles have important implications. For example, a ChIP experiment that concludes robust acH3/H4 regulation and the lack of differential binding of meH3 may turn out to be a false negative simply based on the genomic region in which the IP (immunoprecipitation) and PCR reaction are focused. Moreover, based on individual gene promoter plot analyses, several examples exist, such as the CART promoter, in which, depending on the physical location analyzed, it is possible to observe an increase, decrease, or no change in meH3 binding (Fig 1-1B). This highlights the importance of genome wide studies of chromatin modifications and the need to extensively analyze different locations along the landscape of a given gene promoter.

Another major finding of the Renthal et al., 2009 study was the presence of extensive chromatin modifications found throughout the genome. With rigorous statistical criteria, roughly 1000 gene promoters for acH3, 700 for acH4, and 900 for meH3 were found to have increased promoter binding under cocaine-treated conditions relative to controls (Fig. 1-1A). These values are consistent with the aforementioned observation that absolute levels of histone PTMs are altered by cocaine. However, these findings raise a paradox: many more gene promoters, almost one order of magnitude more, appear to be cocaine-regulated at the chromatin level compared to the typical number of differentially expressed mRNA transcripts that have been identified under similar cocaine treatment paradigms (Maze et al., 2010; McClung and Nestler, 2003; Renthal et al., 2007; Yao et al., 2004). One explanation for this disparity may lie in differences in the sensitivity of

the two techniques. Alternatively, the much higher degree of chromatin regulation may suggest that each histone modification may not directly regulate steady-state mRNA levels, but rather indirectly influences transcription to subsequent stimuli.

An important related discovery is that there is relatively little overlap among the three different histone marks studied at the same gene promoters (Renthal et al., 2009). This is somewhat surprising, since coincident regulation might have been expected: for example, increased acH3, increased acH4, and decreased meH3 at a particular activated gene. However, in this study only about 20% of genes regulated by acH3 were commonly regulated by acH4. Even more striking, only ~1% of hypermethylated 'repressed' genes (9 out of 889) show hypoacetylation (Fig 1-1A). Taken together, these findings suggest that acH4, acH3, and meH3 each have distinct functions that regulate mostly unique transcriptional programs.

In support of these findings, a recent genome wide microarray study, comparing the ability of a challenge dose of cocaine to regulate gene expression in the NAc of cocaine-naïve animals to those treated previously with chronic cocaine, provides large scale evidence for at least two distinct patterns of gene regulation—"desensitized" and "primed" gene expression (Maze et al., 2010). A subset of genes, referred to as desensitized, are induced by acute cocaine and exhibit attenuated induction after chronic cocaine. This blunted induction persists for at least one week, as observed by the lack of effect of a challenge injection in chronic cocaine withdrawn animals (Fig 1-1C). In contrast, the large majority of

genes that are induced by chronic cocaine are not induced by the initial cocaine exposure, represent primed genes. Indeed, approximately 2-3 fold more genes are significantly upregulated in the NAc by chronic cocaine (~275 genes) as well as in cocaine withdrawn animals (~210 genes) compared to those upregulated by initial cocaine exposure (~90 genes) (Maze et al., 2010). From an epigenetic point of view, these findings suggest that throughout the course of acute to chronic cocaine injections, many genomic loci accumulate specific epigenetic 'hits' that render them poised for greater induction. Further, the fact that this primed state of gene inducibility remains present after a week of withdrawal from cocaine suggests a prolonged underlying epigenetic influence on transcription. In summary, this microarray study identified distinct patterns of transcriptional regulation which are consistent with our ChIP-chip data, which revealed extensive, mostly non-overlapping patterns of cocaine-induced promoter binding for acH3, acH4, and meH3.

Key concepts in psychostimulant regulation of chromatin structure

Indeed, analysis of the chromatin state and mRNA expression of many genes reveals evidence for distinct epigenetic influence in mediating both gene desensitization and gene priming (Fig. 1-2).

Epigenetic basis of gene priming. There are several well established examples of genes that are induced in the NAc by chronic, but not acute, cocaine administration (Maze et al., 2010; McClung and Nestler, 2003). Specific

examples for which the chromatin structure has also been extensively studied include the genes encoding BDNF, CDK5, NFκB (p65 subunit), and SIRT2 (Fig 1-2A). ChIP studies have found that genes which are primed to be induced develop increased acH3 and no change in acH4 in response to chronic and not acute cocaine injections (Kumar et al., 2005; Renthal et al., 2009; Russo et al., 2009; Wang et al., 2010). Interestingly, levels of H3K9me2 and its associated enzyme, G9a, exhibit increased binding at 1 hour after cocaine administration, suggesting a possible role in blockade of acute induction of primed genes (Maze et al., 2010). However, with chronic injections, both H3K9me2 and G9a binding are reduced, consistent with the notion that this loss of methylation on H3K9 may facilitate acetylation on H3K9 and other associated lysine residues and promote gene inducibility (i.e., priming). Priming is also associated with increased recruitment of SWI-SNF chromatin remodeling factors (e.g., BRG1) to the primed gene promoters (Kumar et al., 2005).

Less is known about whether steady state mRNA levels of genes primed for induction remain upregulated or return to baseline in between cocaine exposures, and about what happens to the chromatin state of these genes over this time frame. For two genes known to have prolonged regulation, CDK5 and BDNF, increased acH3 binding was found to persist for at least one week following the last drug injection (Kumar et al., 2005). Presumably, H3K9me2 remains reduced although this remains to be proven. In all likelihood, different subclasses of primed genes exist—those with a prolonged increase in steady-state levels, such as BDNF, and those whose mRNAs return to baseline levels

but remain primed for greater induction (sensitization) during re-exposure to drug, as illustrated in Fig. 1-1C.

Epigenetic basis of gene desensitization. The most well characterized example of gene desensitization is the gene encoding c-Fos, which exhibits desensitized induction in response to repeated cocaine or amphetamine administration, and shows suppressed basal expression levels over several days of withdrawal as well as enhanced desensitization to a challenge dose of the drug (Renthal et al., 2008). Unlike known primed genes, acH4 and not acH3 appears to play a key role in gene desensitization. At the c-Fos promoter, acH4 is dramatically induced by acute but not chronic drug exposure (Kumar et al., 2005). Another interesting observation lies in the temporal dynamics that occur in response to initial drug exposure, when pacH3 precedes both peak mRNA and acH4 levels, an effect that was no longer observed with chronic drug administration (Kumar et al., 2005). This suggests the possible influence of pacH3 on acH4 and subsequent gene activation. Importantly, key chromatin modifying enzymes found to be associated with the c-Fos promoter following chronic drug administration were HDAC1 and G9a, fitting with reduced acetylation (via enhanced deacetylation) and increased H3K9me2 and with gene repression (Maze et al., 2010; Renthal et al., 2008). A major goal of future research is to determine whether other genes that display desensitization exhibit similar chromatin mechanisms as observed for c-Fos.

Interplay between transcription factors and epigenetic mechanisms.

Another crucial aspect of chromatin dynamics to consider is how transcription factors and the overall transcriptional machinery interact with epigenetic modifications to ultimately influence transcription. In the addiction field, one of the most well studied examples of such interactions is the transcription factor, ΔFosB (Nestler et al., 1999; Nestler, 2008). Genome wide overexpression studies have suggested that ΔFosB plays a dual role in repressing and activating transcription of cocaine-regulated genes (McClung and Nestler, 2003; Winstanley et al., 2007). As shown in Fig. 1-2, ChIP studies of NAc from chronically treated animals find that ΔFosB is indeed bound to both primed and desensitized gene promoters and, in each case, ΔFosB is associated with a completely different entourage of modifications and chromatin modifying enzymes. For example, with primed genes, ΔFosB recruits the transcriptional activator, BRG1, whereas with desensitized genes HDAC1 is recruited to the c-Fos gene via ΔFosB (Kumar et al., 2005). Adding to this complexity, it appears that G9a and ΔFosB interact in a complex autoregulatory feedback loop. After acute cocaine, G9a is bound to, and hypermethylates H3, at the FosB promoter. This hypermethylation functionally attenuates cocaine induction of FosB gene expression, as was observed with G9a overexpression. However, due to ΔFosB's extraordinarily long half life, ΔFosB accumulates in the cell and then represses mRNA levels of G9a and reduces total levels of H3K9me2, both of which ultimately appear to reduce G9a and H3K9me2 binding at the FosB promoter during cocaine withdrawal—thereby allowing ΔFosB to potentiate its own induction (Maze et al., 2010).

Much more work is needed to understand this complex regulation, particularly within the context of the complex behavioral parameters that define an addicted state. For example, it will be critical in future investigations to delineate the role played by these numerous desensitized and primed genes (in the NAc and elsewhere) in different phases of addiction, for example, the decision to take drug initially, the transition from casual drug use to addiction, and the craving and risk for relapse that occur during withdrawal periods.

Role of epigenetic mechanisms in drug-related behaviors

As stated previously, biochemical and genome-wide evidence suggests that chronic cocaine administration causes chromatin structure to be in a generally more permissive state. This is evidenced by a reduction in total levels of the repressive histone mark, H3K9me2, as well as an increase in total levels of acH3 and acH4. On a genome-wide scale, in addition to each of the aforementioned modifications being heavily regulated by chronic cocaine at literally thousands of gene promoters, 2-3 fold more genes are activated by chronic cocaine compared to acute cocaine. Behaviorally, increasing evidence suggests that the permissive chromatin structure induced by chronic cocaine is a pathological adaptation to the rewarding effects of the drug (Table 1-2). For example, mimicking this ‘permissive state’ by systemic administration of trichostatin A (TSA) or intra-NAc delivery of suberoylanilide hydroxamic acid (SAHA), which are both selective class I and class II HDAC inhibitors, increase cocaine conditioned place preference, which provides an indirect measure of

cocaine reward (Kumar et al., 2005; Renthal et al., 2007). In addition, intra-NAc pharmacological blockade as well as intra-NAc conditional knock-out of another repressive modification, G9a (H3K9me2), also increases cocaine place conditioning (Maze et al., 2010). Conversely, experimental manipulations that promote gene repression, such as intra-NAc overexpression of HDAC4, HDAC5, or G9a, reduce cocaine place conditioning. These effects appear to depend specifically on enzymatic activity of these enzymes, since catalytically dead mutants of HDAC5 or G9a (G9a-H1093K) have no effect on cocaine reward, and since overexpression of wild type HDAC5 co-administered with TSA completely blocks HDAC5's effect on behavioral responses to cocaine. Furthermore, these effects on cocaine reward are specific to certain enzymes and are not generalizable, since, for example, NAc-specific overexpression of HDAC9—an enzyme that is not cocaine regulated, but is expressed in the NAc—does not have any effect on cocaine reward (Renthal et al., 2007).

It is important to note that this general notion that promoting a more permissive chromatin state does not always produce increased cocaine reward. For example, resvertitrol, a sirtuin (class III HDAC) agonist, and sirtintol, a sirtuin antagonist, unexpectedly increase and decrease cocaine reward, respectively (Renthal et al., 2009). This is surprising since SIRT1, a key target of these agents, is a major component of transcriptional repression complexes (Finkel et al., 2009). Also, despite both manipulations reducing H3S10 phosphorylation, MSK1 knockout increases cocaine reward whereas DARPP-32 S97A knock-in decreases cocaine reward (Brami-Cherrier et al., 2005; Stipanovich et al., 2008).

However, while sirtuins, MSK1, and nuclear DARPP-32 have effects on histones, they also have a multitude of non-histone substrates, which makes it difficult to establish a solid link between their effects on chromatin and their resultant behavioral actions—an important concept.

From a theoretical standpoint, since chromatin modifications such as histone acetylation are affected differently by acute compared to chronic cocaine, one would expect that experiments that manipulate chromatin dynamics at different times of drug administration (i.e., before receiving initial cocaine, after receiving chronic cocaine, or during cocaine extinction) may have different results. A limited number of experiments have specifically addressed this question and support this concept (Table 1-2). All of the aforementioned experiments have analyzed baseline manipulations that occur during behavioral training and testing. However, in one experiment, it was found that loss of HDAC5 and not HDAC9 results in lasting sensitization to cocaine reward: HDAC5 knock-out had no effect on cocaine place conditioning at baseline but sensitized the ability of prior cocaine exposure to enhance place conditioning (Renthal et al., 2007). Furthermore, a series of key cocaine self-administration studies have found that the timing of HDAC inhibition is likely to be crucial. For example, Romieu et al (2008) found that the daily systemic (IV) administration of TSA during the initial acquisition phase of cocaine self-administration reduced instrumental responding including reduced break point in a progressive ratio test, whereas another study found that a single systemic injection of sodium butyrate (a broadly acting and non-specific HDAC inhibitor among many other actions)

during the maintenance phase significantly enhanced administration behavior (Sun et al., 2008). Similarly, a recent study found that TSA and SAHA infusions in the NAc shell, but not core, greatly enhanced motivation for cocaine during the chronic phase of cocaine self-administration (long-term reinforcement of cocaine self-administration for more than 30 days) (Wang et al., 2010). Finally, Malvaez and colleagues found that systemic sodium butyrate injections given after CPP extinction and reinstatement training resulted in an enhancement of extinction and reduced reinstatement (Malvaez et al., 2010). Based on this range of interesting results, it is clear that, similar to ChIP and mRNA studies, acute exposure to cocaine imposes different effects compared to chronic cocaine, and presumably the environmental context also imposes unique effects—all of which are simultaneously affected by these manipulations.

Depression and epigenetic mechanisms (Brief overview)

Depression is a major cause of worldwide morbidity. It is estimated that the lifetime prevalence in the United States of developing major depression is as high as 17%. Vulnerability to depression has a strong genetic component estimated at 40-50% (Krishnan and Nestler, 2008). However, even after years of research and major advances in genome sequencing, the genetics of depression remains poorly understood. Conversely, it is clear that environmental factors such as chronic stress, emotional trauma, and several medical illnesses are major contributors to depression. Given the prevalence it is not surprising that antidepressants are consistently among the top prescribed medications in

America. What remains surprising is the paucity in understanding of how medications like fluoxetine (a commonly perscribed SSRI) exert antidepressant responses only after prolonged (weeks of) administration. Similar to addiction, the pathophysiology of depression clearly involves several brain structures. Because of its central role in influencing mood and reward, one structure of particular focus in depression is the nucleus accumbens (NAc). Also similar to drug addiction, a leading hypothesis for the basis of depression suggests that persistent changes in gene expression ultimately change neural function and behavior (Covington et al., 2009).

Such prolonged alterations in NAc gene expression are well documented in both human depression and rodent models of depression (Krishnan et al., 2007; Wallace et al., 2009). Compared to addiction research, much less is known about the basis for these changes in gene expression, however increasing evidence similarly points to epigenetic mechanisms. For example, in a ChIP-Chip study looking at the role of repressive histone methylation in two different models of depression, my colleagues and I found that gene promoters are extensively and similarly methylated in these models. Using heatmap analysis, I also found that both chronic antidepressants and resiliency to stress completely block or reverse stress-induced methylation patterns (Wilkinson et al., 2009). Ultimately, this study suggests that epigenetic mediated gene repression is a major factor in promoting depressive behavior and that mechanisms that promote gene activation should have therapeutic efficacy in treating depression. Consistent with this notion, a study by Covington found that continuous intra-NAc

infusion of HDAC inhibitors (MS-275 or SAHA, both of which promote gene activation) function as antidepressants. Next, in a microarray study comparing the expression profile of MS-275 to the standard antidepressant, fluoxetine, I found that both fluoxetine and HDAC inhibition reverse stress induced gene expression through a combination of similar and novel transcriptional mechanisms (Covington et al., 2009). One important next step will be to further identify whether other repressive modifications, such as DNA methylation, play a similar role in the NAc in promoting depressive behavior.

DNA methylation in neuroscience

In recent years, the role of DNA methylation in the CNS has gained considerable attention. As previously mentioned, this interest is largely due to the observation that the DNA methyltransferases, Dnmt1 and Dnmt3a are unexpectedly highly expressed in adult, post-mitotic neurons (Feng et al., 2005). Moreover, in the past decade, DNA methylation has been heavily implicated in several neurological disorders, particularly psychiatric diseases with developmental etiology. The most convincing data come from several published findings which have shown that various mutations in the methyl-binding domain protein, MeCP2, cause Rett syndrome (an autism spectrum disorder) (Zoghbi, 2009). These mutations do not result in neuronal cell death and, amazingly, genetic rescue with Wt MeCP2 has been shown to reverse Rett associated neurological defecits (Guy et al., 2007). Two other mental retardation disorders, Fragile X and ICF syndromes, also arise from abnormal DNA methylation. Fragile

X mental retardation is associated with expansion of a single trinucleotide gene sequence (CGG) on the X chromosome. The severity of the disorder is linked to the extent of CGG repeats and the degree of DNA methylation at these repeats which ultimately silence the expression of the FMR1 gene. ICF (Immunodeficiencey, Centromeric region instability, Facial anomalies) syndrome is a rare autosomal recessive disorder caused by mutations in the DNMT3b gene which result in genome wide hypomethylation. Finally, a series of association studies have led to the hypothesis that schizophrenia may be caused by aberrant DNA methylation. For example, high doses of the amino acid l-methionine (and Dnmt coenzyme) were found to exacerbate schizophrenic symptoms (Cohen et al., 1974; Grayson et al., 2009). Moreover, levels of SAM (s-adenosylmethionine) and Dnmt1 have been found to be elevated in the frontal cortex from schizophrenic post-mortem brain tissue (Guidotti et al., 2007; Veldic et al., 2005). Third, elevated levels of Dnmt1 correlate with increased methylation and reduced mRNA expression of GAD67 and reelin in schizophrenic tissue (Grayson et al., 2005). These studies have also been extended to mouse models. Interestingly, as originally associated in humans, it was demonstrated that chronic systemic L-methionine administration causes hypermethylation of GAD67 and reelin in frontal cortex in mice (Dong et al., 2005). In summary, aberrant DNA methylation is implicated in autism, mental retardation, and schizophrenia. Of note, these are all neurodevelopmental disorders thought to be due in part to improper neuronal plasticity.

Several studies on the role of DNA methylation in hippocampus also support the notion that methylation regulates neuronal plasticity, particularly synaptic plasticity. In hippocampal culture, Dnmt inhibition causes a marked reduction in number of functional synapses, as measured by a reduction in miniature excitatory post-synaptic current (mEPSC) frequency (Nelson et al., 2008). Other studies in hippocampal slices have found that DNMT inhibition blocks long term potentiation (LTP) (Levenson et al., 2006). More recently, it has been shown that double (and not single) conditional knock-out of Dnmt1 and Dnmt3a similarly blocks LTP (Feng et al., 2010). Behaviorally, Dnmt3a mRNA was found to be transiently upregulated immediately following fear conditioning. And both Dnmt inhibition and double cKO of Dnmt1/Dnmt3a impairs memory formation in several tested learning and memory tasks (Feng et al., 2010; Lubin et al., 2008; Miller and Sweatt, 2007).

No studies so far have explored the role of DNA methylation in the pathophysiology of addiction or depression. By focusing on the NAc, my thesis provides the first look at the role of DNA methylation in these devastating psychiatric diseases. In chapter 2, I first explore whether DNA methyltransferases are regulated by chronic cocaine or by chronic social defeat stress. I then developed and used complementary pharmacological and genetic techniques to manipulate DNA methylation in order to test how this regulation affects reward behavior and synaptic plasticity. Chapter 3 describes an extensive study, still underway, aimed at identifying the genes regulated by DNA methylation in the NAc. And the final chapter concludes my thesis with a detailed

discussion of how my findings relate to other studies on addiction and depression, the limitations of these findings, as well as future studies that will further unravel the role of epigenetics in addictive and depressive disorders.

CHAPTER 1 TABLES

Table 1-1. Histone modifications, their function, and their respective enzymes.

Modification	Effect on gene expression	"Writers" (enzymatic addition)	"Erasers" (enzymatic removal)
Phosphorylation	↑	**kinases**	**Phosphatases**
H3pS10		AURKB, **MSK1**	PP1, **DARPP32 (indirectly via PP1 regulation)**
Ubiquitination	↑	**Ub ligases** (RING2)	**Ub protease** (USP16)
Sumolyation	↓	**SUMO E2s/E3s?** (UBC9)	SUMO protease (SUSP17)
Acetylation	↑	**KATs**	**HDACs (1-11), HDAC4,5, 9**
H3K9ac		2a (GCN5), 2b, 12	"
H3K14ac		2a-b, 3a **(CBP),** 3b (p300), 6a, 6b	"
H4K16ac		5, 8	**Sirt2**
Lysine Methylation	↑↓	**KMTs**	**KDMs**
H3K4me3	↑	2a-h, 7	1, 2a, 5a-d
H3K9me1/2/3	**me1**↑, **me2/3** ↓	1a-f (1c=**G9a/EHMT2),** 8	1, 3a-b, 4a-d
H3K27me3	↓	6	6a-b
H3K36me3	↑	3a-c	2a-b, 4a-c
H3K79me3	↑	4	?
Arginine Methylation	?	**PRMTs (1-8)**	**JMJD6**
DNA methlyation	↓	DNMT1, DNMT3a, 3b	Gadd45a,b,g ?

List of histone modifications, their known effects on transcription and examples of enzymes that catalyze their "writing" (enzymatic addition) or "erasing" (enzymatic removal)
↑ = increased transcription, ↓ = decreased transcription; **Blue** = examples of enzymes in which cocaine-induced biochemal regulation has been identified and it's behavioral significance has been accessed

Table 1-2. Role of Epigenetic modifications in cocaine reward behaviors

Experimental manipulation	Route of administration	Conditioned place preference	Locomotor sensitization	Self-admin.	Reference
Histone acetylation					
Sodium Butyrate - HDACi	IP, preceeding drug injection		↑		Kumar et al., 2005
Sodium Butyrate - "	IP, after drug training	extinction			Malvaez et al., 2009
Sodium Butyrate - "	IP, preceeding drug injection			↑	Sun et al., 2008
TSA - Class I & II HDACi	IP, preceeding drug injection	↑			Kumar et al., 2005,
TSA - "	IV, preceeding drug injection		↓	↓ acquisition phase of SA	Romieu et al., 2008
TSA - "	Intra-NAc shell			↑ motivation for cocaine	Wang et al., 2010
SAHA - Class I & II HDACi	Intra-NAc shell			↑ motivation for cocaine	Wang et al., 2010
SAHA - "	Intra-NAc cont. delivery	↑			Renthal et al., 2007
resveritrol - Class III (sirtuin) HDAC ago	IP, preceeding drug injection				Renthal et al., 2009
sirtinol - Class III (sirtuin) HDACi	Intra-NAc cont. delivery	↓		↓ SA, dose response curve	Renthal et al., 2009
HDAC4 overexpression	Intra-NAc, HSV/AAV	↓		↓ motivation for cocaine	Kumar et al., 2005,
HDAC5 KO	knock out	↑ with reexpsoure			Renthal et al., 2007
HDAC5 KO + HDAC5 overexp.	knock out + intra-NAC, HSV	"- with reexpsoure			Renthal et al., 2007
HDAC9 KO	knock out	-			Renthal et al., 2007
HDAC5 ovexp.	Intra-NAc, HSV	↓			Renthal et al., 2007
HDAC5-cat ovexp.	Intra-NAc, HSV	-			Renthal et al., 2007
HDAC5-dMef ovexp.	Intra-NAc, HSV	↓			Renthal et al., 2007
HDAC5 ovexp. + TSA	Intra-NAc, HSV + IP TSA	-			Renthal et al., 2007
HDAC9 overexpression	Intra-NAc, HSV	-			Renthal et al., 2007
CBP het/KO	knock out		↓		Levine et al., 2005
Histone Phosphorylation					
MSK1 KO	knock out	↑	↓		Brami-Cherrier et

				al., 2005
DARPP-32 S97A	Knock -in	↓	↓	Stipanovich et al., 2008
Histone methylation - H3K9me2				
G9a overexpression	Intra-NAc, HSV	↓		Maze et al., 2010
G9a-H1093k overexpression	Intra-NAc, HSV	-		Maze et al., 2010
G9a inhibition (BIX)	Intra-NAc cont. delivery	↑		Maze et al., 2010
G9a cKO	Intra-NAc, AAV-CRE	↑		Maze et al., 2010
DNA methylation				
DNMT inhibition		**???**		
DNMT overexpression		**???**		

red= more permissive state
green= more repressive state

red=behavior associated w/ increased cocaine reward
green= behavior associated with decreased cocaine reward

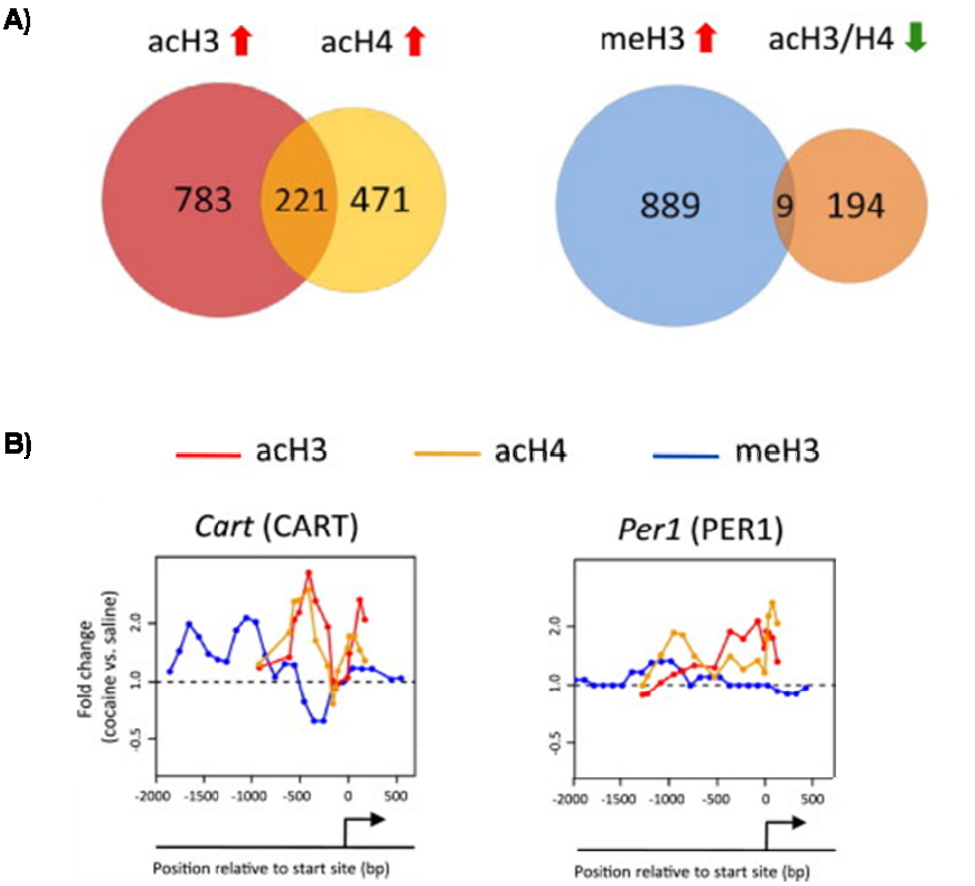

A)
acH3
acH4
783
221
471
meH3
acH3/H4
889
9
194
B)
acH3
acH4
meH3
Cart (CART)
Per1 (PER1)
Fold change (cocaine vs. saline)
Position relative to start site (bp)
C)
Significantly upregulated genes
0
50
100
150
200
250
300
Acute
Chronic
Withdrawn
Acute*
Chronic
Withdrawn
Fold expression
-2X
1X
+2X
Acute
Chronic*
Withdrawn
Acute
Chronic
Withdrawn*
Gene desensitization
Gene priming

Figure 1-1: Summary of genome wide analysis of cocaine regulation of chromatin structure and gene expression. A) Chronic cocaine causes extensive genome-wide chromatin modifications marked by little overlap. Venn diagrams from ChIP-chip studies with acetylated H3 (acH3), acetylated H4 (acH4), and methylated H3 (meH3) from nucleus accumbens (NAc) in mice that received chronic cocaine (24 hours after 7 daily cocaine injections at 20 mg/kg dose) (Renthal et al., 2009). Compared to traditional DNA microarray studies that look for genes with altered steady state mRNA levels under similar conditions, a much greater number of gene promoters are significantly regulated by each analyzed histone modification and, yet, a relatively small number of gene promoters share co-regulation of more than one modification suggesting that each analyzed modification has distinct functions. B) Example of promoter plots of two representative genes, Cart and Per1, from the Venn diagram illustrating spatial complexity in chromatin regulation. Y-axis represents fold change in binding relative to saline treated mice and the x-axis represents the physical location of binding on the indicated gene promoter relative to its transcription start site (TSS). The plots illustrate the complex patterns of acH3, acH4, and meH3 on these two representative gene promoters. C) Analysis of cocaine-induced gene expression reveals two patterns of gene induction: desensitized (black line in acute row) and primed genes (purple lines in chronic and withdrawn columns). Scaled heatmaps (*) show the number and pattern of cocaine-upregulated gene expression in NAc at 1 hour after a challenge injection of cocaine (20 mg/kg) in three groups of animals: Acute (no prior cocaine

exposure), Chronic (7 daily cocaine injections examined 1 hour after the last injection), or Withdrawn (7 daily cocaine injections followed by 1 week of withdrawal before the challenge injection). The top series of heatmaps show the genes upregulated in the Acute group and how those same genes are regulated in the other two groups. Likewise, the middle and lower series of heatmaps show the genes upregulated in the Chronic and Withdrawal groups, respectively, and how those genes are regulated in the other two groups. Notably, a small but significant number of acutely induced genes are no longer induced in animals with prior chronic cocaine exposure (desensitized genes, black bar***), whereas a larger number of genes that are induced after chronic cocaine lack induction by acute cocaine (primed genes, purple bars***). Adapted from Maze et al., 2010.

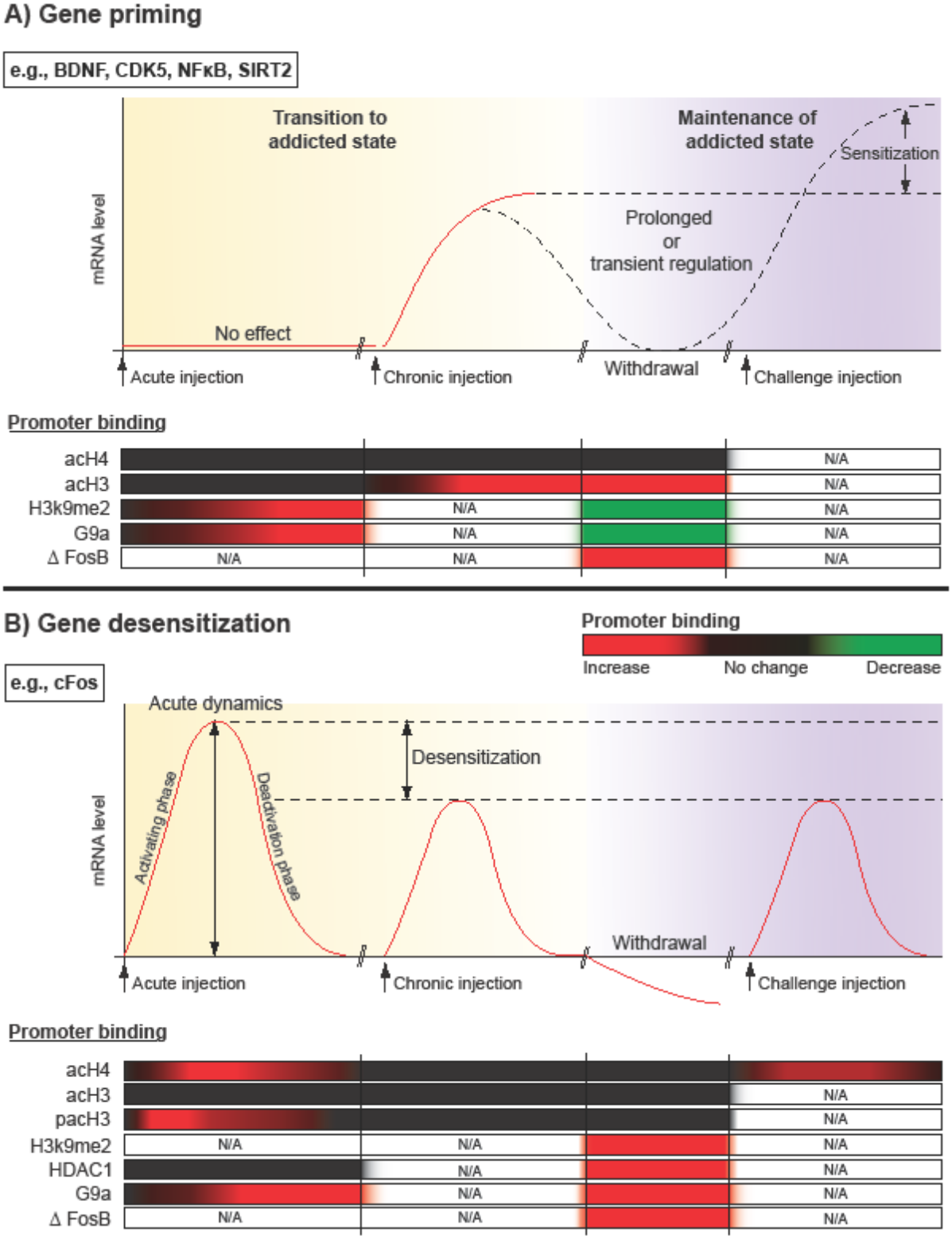

A) Gene priming
e.g., BDNF, CDK5, NFκB, SIRT2
Transition to addicted state
Maintenance of addicted state
Sensitization
mRNA level
Prolonged or transient regulation
No effect
Acute injection
Chronic injection
Withdrawal
Challenge injection
Promoter binding
acH4
acH3
H3k9me2
G9a
Δ FosB
N/A
B) Gene desensitization
Promoter binding
Increase
No change
Decrease
e.g., cFos
Acute dynamics
Desensitization
Activating phase
Deactivation phase
mRNA level
Withdrawal
Acute injection
Chronic injection
Challenge injection
Promoter binding
acH4
acH3
pacH3
H3k9me2
HDAC1
G9a
Δ FosB
N/A

Figure 1-2: Distinct patterns of cocaine-induced gene induction correspond to unique patterns of chromatin regulation. Graphs in the top of A and B display patterns of mRNA expression over time, with the underlying epigenetic binding profile depicted in the bottom tables. Promoter binding profiles are displayed in heatmap style (cocaine relative to saline, with red = increased binding, black = no change, green = reduced binding). The x-axis is divided into 4 sections that highlight mRNA and chromatin changes that occur in response to initial cocaine exposure, in response to a cocaine challenge after prior chronic drug exposure, during drug withdrawal, and in response to a subsequent challenge dose of cocaine. Of note, since chronic injections are given once daily, one can infer that chromatin changes that occur during early withdrawal (24 hours) are presumed to provide insight into events that also occur prior to the last chronic cocaine injection. A) Epigenetic basis of gene priming. Genes that are primed to be induced eventually develop increased acH3 and reduced H3K9me2 binding during a chronic course of cocaine administration. In contrast, during initial cocaine exposure—when there is no mRNA induction—the repressive histone methyltransferase, G9a, is induced and binds to and mediates H3K9me2 binding to nearby promoter regions. B) Epigenetic basis of gene desensitization. Genes that are desensitized exhibit prominent induction of acH4 binding during initial cocaine exposure, which is lost with chronic treatment. This may be due to increased binding of HDAC1. As well, G9a shows increased binding during acute and withdrawal time points. This is correlated with increased H3K9me2 during late withdrawal. Interestingly, the transcription factor, ΔFosB, is bound to both

types of genes during cocaine withdrawal, suggesting a dual role in mediating gene repression and gene activation. An important caveat is that these mechanisms of gene priming and desensitization are based on the analysis of a relatively small number of cocaine-regulated genes and must be refined as many more such genes are characterized. N/A, information not available.

CHAPTER 2

DNMT3A REGULATES EMOTIONAL BEHAVIOR AND SPINE PLASTICITY IN THE NUCLEUS ACCUMBENS.

ABSTRACT

Despite abundant expression of DNA methyltransferases (Dnmt's) in brain, the regulation and behavioral role of DNA methylation remain poorly understood. We find that Dnmt3a expression is regulated in nucleus accumbens (NAc) by chronic cocaine and chronic social defeat stress. Moreover, NAc specific manipulations that block DNA methylation potentiate cocaine reward and exert antidepressant-like effects, whereas NAc specific Dnmt3a overexpression attenuates cocaine reward and is pro-depressant. On a cellular level, we show that chronic cocaine selectively increases thin dendritic spines on NAc neurons and that DNA methylation is both necessary and sufficient to mediate these effects. These data establish the importance of Dnmt3a in the NAc in regulating cellular and behavioral plasticity to emotional stimuli.

INTRODUCTION

Chronic cocaine and chronic social defeat stress alter gene expression, neuronal plasticity, and ultimately behavior, and we and others have implicated chromatin remodeling in playing a key role in regulating these events in the nucleus accumbens (NAc), a key brain reward region (Covington et al., 2009; Kumar et al., 2005; Levine et al., 2005; Malvaez et al., 2010; Maze et al., 2010; Renthal et al., 2007; Renthal et al., 2009; Schroeder et al., 2008; Tsankova et al., 2007). However, work to date has focused primarily on more labile epigenetic modifications such as histone acetylation and methylation. Given the persistent nature of addiction, an intriguing possibility is whether more stable epigenetic modifications, such as DNA methylation, can more persistently influence gene expression in the NAc to maintain this behavior.

Despite abundant neuronal expression of DNA methyltransferases **(**Dnmt's)(Feng et al., 2005), little is known about the function of DNA methylation in brain. Behavioral studies suggest that DNA methylation is required for hippocampal-dependent memory formation (Feng et al., 2010; Miller and Sweatt, 2007). Pharmacological inhibition of DNA methylation blocks long-term potentiation in hippocampus (Levenson et al., 2006) and this effect depends on the activity of both Dnmt1 and Dnmt3a (Feng et al., 2010). Moreover, Dnmt inhibition dramatically reduces functional synapses formed by cultured hippocampal neurons as measured by a reduction in mEPSC frequency (Nelson

et al., 2008). Together, these studies point to DNA methylation playing a crucial role in hippocampus in memory formation and associated effects at the synapse.

In the present study, we focus on the NAc to test if these concepts regarding DNA methylation extend to drug addiction and depression models. By analyzing the role of DNA methylation in the context of both chronic cocaine (a rewarding stimulus with persistent effects) and chronic social defeat stress (an aversive stimulus with persistent effects), these studies complement one another by establishing the influence of this lasting epigenetic modification in the NAc across a spectrum of complex behaviors in which this brain region plays an important role. Toward this goal, we identify which specific DNA methyltransferases (Dnmt1, Dnmt3a, or Dnmt3b) are regulated in chronic cocaine and chronic stress paradigms, whether manipulating DNA methylation affects addictive- and depressive-like behavior, and whether DNA methylation affects synaptic plasticity in the NAc.

METHODS

Animals

We used adult male c57bl6j mice (Jackson) and Long-Evans rats (Charles River). Animals were habituated in our facility 1 week prior to experimentation and were housed on a 12 hr light-dark cycle with access to food and water *ad libitum*. All animal experiments were approved by IACUC's of Mount Sinai School of Medicine and UT Southwestern.

Drugs

For chronic cocaine (Sigma) experiments, we used a standard cocaine injection paradigm (Kumar et al., 2005; Maze et al., 2010; Renthal et al., 2007; Renthal et al., 2009): 7 daily intraperitoneal (IP) injections of 20 mg/kg cocaine. For acute experiments, mice were injected for 6 days with saline before a cocaine injection on day 7. Controls were injected with an equivalent volume of saline. For spine analysis, we used an established injection schedule known to increase spine density, which consists of five 20 mg/kg injections. This protocol is shortened to coincide with the duration of HSV-mediated overexpression which wanes 6 days post-op (Barrot et al., 2002; Russo et al., 2009).

For methionine (MP Biochemicals) experiments, animals were injected subcutaneously with 0.78 g/kg L-methionine 2x/day for 10 days. This was timed to ensure that the minimum dose and injection duration previously shown to increase DNA methylation in the striatum (Dong et al., 2005; Dong et al., 2008),

would coincide with the beginning of CPP training. During training, animals were injected with methionine 2-3 hrs prior to behavioral experiments.

For RG108 (Sigma) experiments, we used 7-10 days of continuous (0.25 □l/hr) intra-NAc delivery of 100□M RG108 dissolved in 5% hydroxypropyl β-cyclodextrin vehicle (Trappsol). This dose of RG108 was found to decrease DNA methylation *in vivo* and was void of evidence of neurotoxicity as assessed by activated caspase-3 staining. RG108 was chosen over other Dnmt inhibitors, such as 5-aza-cytidine, because 1) it inhibits methylation without incorporation into DNA which has been shown to be linked to cytotoxicityJuttermann et al., 1994 and 2) it is chemically stable with a mean 37°C t½ of 20 days, whereas the 37°C t½ of 5-aza-cytidine is on the order of hr (Brueckner et al., 2005). Osmotic delivery was performed as previously described (Covington et al., 2009; Maze et al., 2010; Renthal et al., 2007).

For fluoxetine (Tocris) experiments, we injected mice with 20 mg/kg fluoxetine IP for 14 days, as described previously (Berton et al., 2006; Covington et al., 2009).

Cocaine self-administration

Self-administration was performed as previously described (Noonan et al., 2008; Spano et al., 2007). Animals had 3 hr daily access to cocaine (0.75 mg/kg/infusion) under a fixed ratio-1 (FR1) reinforcement schedule. For the 24 hr withdrawal experiment, rats had 13 days of cocaine intake; for the 28 day withdrawal experiment, rats had 3 weeks of intake.

Herpes simplex and adeno-associated virus injections

We used the bi-cistronic p1005+ HSV vector, which expressed GFP alone or GFP with Dnmt3a. In this system, HSV infection occurs selectively in neurons. GFP expression is driven under the human immediate early cytomegalovirus (CMV) promoter, while the gene of interest, Dnmt3a, is driven by the IE4/5 promoter (Clark et al., 2002). We used a previously cloned mouse Dnmt3a-1 plasmid (Linhart et al., 2007). AAV-GFP or AAV-CreGFP was exactly used as describedMaze et al., 2010. Stereotaxic surgery was performed based on published methods (Maze et al., 2010; Renthal et al., 2007).

Conditioned place preference

We used a standard, unbiased CPP procedure (Renthal et al., 2007; Renthal et al., 2009; Russo et al., 2009). In brief, before experimental manipulation, animals were pretested for 20 min in a photo-beam monitored box with free access to environmentally distinct chambers. The mice were then arranged into control and experimental groups with equivalent pretest scores. After experimental manipulation, mice underwent four 30 min training sessions (alternating cocaine and saline pairing). On the test day, mice had 20 min of unrestricted access to all chambers, and a CPP score was ultimately assigned by subtracting time spent in the cocaine-paired chamber minus time spent in the saline-paired chamber.

Cocaine-induced locomotor sensitization

We used a standard locomotor sensitization procedure (Pulipparacharuvil et al., 2008). In brief, mice (saline habituated) were injected daily with cocaine (IP) 30 min after being placed in standard plastic cages similar to their home cages. Total locomotor activity was measured via photobeam breaks for 30 min following their injection (20mg/kg).

Social defeat stress

Experimental C57bl6j mice were subjected to 10 days of social defeat stress as previously described (Berton et al., 2006; Krishnan et al., 2007; Krishnan et al., 2008). In summary, mice are exposed daily to an unfamiliar aggressive CD1 mouse for 5 min. For the remaining 24 hr, the defeated mouse was housed in a 'protected' compartment of the same cage where it endured chronic stress from the CD1. On the 11^{th} day, mice underwent a social interaction test to verify defeat-induced social avoidance (Berton et al., 2006; Tsankova et al., 2006). As previously published, control mice spend more time interacting with a social "target" as compared to "no target", whereas chronically defeated mice spend significantly less time interacting with the target mouse compared to "no target" (Berton et al., 2006; Krishnan et al., 2007; Tsankova et al., 2006).

For RG108 and fluoxetine experiments, we selected mice displaying robust social avoidance—showing "Day 11" interaction times <40 sec. Then, using baseline interaction times, we divided animals into 3 similar groups as follows – Controls: intra-NAc vehicle (75.1 ± 3.1 sec), intra-NAc RG108 (75.4

±4.1 sec), or systemic fluoxetine (75.5 ± 6.1 sec); Defeats: intra-NAc vehicle (20.5 ± 3.3 sec), intra-NAc RG108 (20.4 ±3.6 sec), fluoxetine (20.1 ± 3.1 sec).

To test for a pro-depressive phenotype, we performed a submaximal defeat experiment as previously described (Krishnan et al., 2007; Krishnan et al., 2008): 3 days following HSV surgery, naïve mice are subjected to 3 consecutive 5 min defeat episodes interspersed by 15 min rest periods. Mice underwent the social interaction test the following day. In this paradigm, these stressors are not sufficient to cause social avoidance in HSV-GFP mice as indicated by a significant increase in “target” interaction time versus “no target” interaction time (Krishnan et al., 2007; Krishnan et al., 2008).

Forced swim test

Immobility measures were obtained using a 2-day forced-swim test procedure commonly used in rats (Krishnan et al., 2008; Porsolt et al., 1977). Three days following surgery, Sprague-dawley rats were forced to swim for 15 min in plastic cylinders (20 × 45 cm). The following day, rats were tested under identical conditions for 5 min. All sessions were videotaped and scored by a blinded observer. Latency to immobility was defined as the time that the rat first initiated a stationary posture that did not reflect attempts to escape.

Novel object recognition and object habituation tests

Tests were performed according to previous publications with minor modifications (Frick and Gresack, 2003). Briefly, for the novel object recognition

test, mice were placed in an open field area to habituate (15 min). After 3 min of rest, they were exposed to 3 objects for 5 min in the same arena. Each object was placed in opposite corners of the open field arena. Mice were reexposed two additional times to the same objects in the same locations and on the final trial, one object was switched with a novel object. A normal mouse with intact memory is expected to spend more time with this novel object as compared to the familiar objects. For habituation to a novel object, we exposed mice to a single object for 150 sec and the re-exposure time was extended to one hr. Here, as mice are re-exposed to this object, they are expected to become disinterested in the object, as measured by number of object investigations.

RNA isolation, reverse transcription, quantitative PCR, and primers

Bilateral NAc punches were obtained and the tissue homogenized in TriZol (Invitrogen). RNA was purified with RNeasy Micro columns (QIAGEN). Reverse transcription of RNA was carried out using iScript (Biorad). qPCR was run using approximately 5 ng of cDNA per reaction. Each reaction was run in triplicate and quantified using the ΔΔCt method as previously described (Tsankova et al., 2006). A complete list of primers is given below.

Global DNA methylation analysis

We used Epigentek's Methylamp Global DNA Methylation Quantification Ultra Kit and followed manufacturer's instructions. This method is ideally suited for our experiments on NAc tissue because of its ability to use small amounts of

input DNA and for high degree of sensitivity. Raw values were colorimetrically quantified and total methylation levels estimated by generating a standard curve from Epigentek's methylated DNA standard. Values are then represented as methylation % relative to vehicle control.

ChIP promoter analysis

Chromatin from NAc tissue punches was immunoprecipitated with an antibody against me3K4H3 (Abcam ab1012) as described previously without modifications (Kumar et al., 2005; Renthal et al., 2009; Wilkinson et al., 2009). For the 24 hr experiment, samples were amplified using GenomePlex whole genome amplification kit (Sigma), labeled with Cy3 (input) or Cy5 (me3K4H3 enriched), and hybridized to NimbleGen MM8 mouse promoter arrays with 3 biological replicates used per condition. Each replicate consisted of bilateral NAc punches pooled from 10 mice. ChIP-chip analysis was performed as described previously without modification (Renthal et al., 2009; Wilkinson et al., 2009). Based on these analyses, the Dnmt3a promoter was found to have significantly reduced binding at approximately -500 bp upstream of the transcriptional start site. ChIP's were then performed on additional sets of 4 and 24 hr cocaine-injected mice and PCRs were performed using primers designed around this region as well as -2000 bp upstream.

Dendritic spine analysis (GFP immunostaining and single-cell dye filling)

For viral-based (GFP immunostaining) spine analysis (**Fig. 2-4a**), mice were perfused with 4% PFA 4 hr following the final cocaine injection and brains sectioned at 100 μm using a Leica vibratome. Slices were immunostained with an anti-GFP antibody (Millipore). Sections were mounted, coded, and confocal imaging (Zeiss LSM 710) was performed with the rater blind to experimental conditions. We imaged secondary and tertiary dendrites of NAc medium spiny neurons under a 100x oil-immersion objective at a resolution of 0.027 μm x-axis x 0.027 μm y-axis x 0.3 μm z. Approximately 7-10 neurons were imaged per animal (average total dendritic length 400 μm/animal, n=5-6). Dendritic length was measured using NIH ImageJ software and spine numbers were counted manually by a trained observer who was also blind to experimental conditions. To account for any possible surgical or sampling bias, we annotated anterior/posterior or NAc core/shell location of all images and found no significant differences in location or region.

Because HSV-GFP (like other HSV encoded transgenes) is no longer expressed after 7 days, we performed single-cell dye filling experiments (**Fig. 2-4b,c**). As previously described (Radley et al., 2006), random cells in the NAc shell were impaled with a micropipette containing 5% Lucifer Yellow (Molecular Probes) and injected with 1-10 nA of current. Slices were washed in PBS, mounted and imaged on the confocal microscope using the same imaging parameters as the above GFP experiment. On average, for each animal 12 dendrites from 6 neurons were imaged (average total dendritic length of 500 μm/animal, n=4-8). Spine density and spine type analysis were performed using

the semi-automated software, NeuronStudio (http://research.mssm.edu/cnic/tools-ns.html) (Rodriguez et al., 2008). Of note, NeuronStudio analysis is not amenable to other dendritic images, such as GFP, Golgi, or diolistics. NeuronStudio analyzes single-cell filled deconvolved images in 3 dimensions (3D) and, as an extra advantage, this software uses an algorithm of voxel clustering and Rayburst Sampling to ultimately classify spines into the 3 major morphologic types—thin, mushroom, and stubby. After NeuronStudio processing, a human operator verifies that all spines have been appropriately identified and manually corrects any errors in spine characterization. For the operator, the manual aspect of analysis takes about 10 min per dendritic image. Due to the labor intensive nature of 3D analysis, we had a separate NeuronStudio operator for each of the 3 cell-filling experiments. For each experiment, the NeuronStudio operator randomly analyzed coded images. Upon finishing analysis, the spine density (as well as spine type) was calculated by dividing the total number of spines present by the dendritic length of the segment. Human-operated NeuronStudio was previously shown to have a range of 68% to 90% inter-operator variability in spine density counts (Rodriguez et al., 2008). Similarly, we found a high degree of inter-operator variability in our 3 datasets and, therefore, to control for such variability and to allow for comparison across experiments, we normalized the spine density and spine type data from each experiment with respect to its own control. Data are expressed as % change in spine density relative to saline controls.

Statistical analysis

Statistical significance was measured using an unpaired two-tailed Student *t*-test when comparing two groups. One-tailed Student *t*-tests were used for pharmacological verification of changes in global methylation and 7day-4hr spine density analysis since values are expected to fall in an expected sampling distribution. For mRNA analysis, HSV-GFP dendritic spine analysis, submaximal defeat susceptibility, and antidepressant tests in defeated mice, two-way ANOVA's were performed since experiments contained multiple groups. Repeated measures ANOVA was used for locomotor sensitization analysis. For ANOVAs, post-hoc comparisons were performed when appropriate.

RESULTS

Transcriptional regulation of Dnmt3a in NAc by cocaine

As a first step in determining the role of DNA methylation in cocaine action, we performed quantitative PCR (qPCR) on NAc tissue of mice treated acutely or chronically (7 injections) with cocaine for all known Dnmt's and methyl-binding domain proteins. Among these genes, we found that Dnmt3a is selectively upregulated at 4 hr and downregulated after 24 hr following both acute and chronic cocaine administration, with limited changes observed for the other genes analyzed (**Fig. 2-1a, Supplemental Fig. S2-1**). Dnmt3a's upregulation during early withdrawal time points is supported by equivalent findings from a recently published microarray study (see supplemental gene lists) performed 4 hr after 15 chronic cocaine injections (Heiman et al., 2008). Dnmt3a's downregulation at 24 hr is not persistent, since mRNA analysis at 48 hr after the last chronic injection showed no change from control values (data not shown). Together, these data support the notion that Dnmt3a expression in the NAc is biphasically regulated in response to each cocaine injection. This biphasic regulation of Dnmt3a appears to occur via transcriptional mechanisms, since chromatin immunoprecipitation (ChIP) analysis of a well known marker of gene activation, histone 3 tri-methyl-Lys4 (me3K4H3), indicated that the Dnmt3a gene, and not the Dnmt1 or Dnmt3b gene, exhibits transiently enhanced (at 4 hr) and suppressed (at 24 hr) me3K4H3 binding at its promoter (**Fig. 2-1c**). To provide further relevance to addiction, we analyzed tissue from rats that chronically (13

days) self-administered cocaine and found a significant downregulation in Dnmt3a expression in NAc at 24 hr (P<0.05) after the last cocaine infusion (**Fig. 2-1b**). Interestingly, Dnmt1 is significantly downregulated by acute cocaine (P<0.05); however this effect was no longer significant with chronic cocaine or self-administered cocaine (**Fig. 2-1a, b**). Dnmt3b was not regulated under any condition analyzed. Moreover, analysis of relative levels of each DNA methyltransferase in mouse and rat NAc revealed that Dnmt3a is by far the most predominant DNA methyltransferase expressed in the NAc (**Supplemental Fig. S2-2**). Given Dnmt3a's dynamic regulation by cocaine, and its enrichment in the NAc, we focused further on this enzyme subtype.

While the molecular events that occur during the transition to the addicted state, such as the aforementioned biphasic regulation of Dnmt3a, are an important component underlying the pathophysiology of addiction, a second major area of research focuses on the mechanisms that maintain the addicted state. As stated in the Introduction, more long-term regulation of DNA methyltransferases is of particular interest given the theoretical, persisting influence that these enzymes may have on downstream gene targets and behavior. Therefore, we injected mice with cocaine for a more prolonged time period (28 days) and analyzed Dnmt mRNA levels after an additional 28 days of withdrawal. This injection paradigm causes robust and long-lasting molecular and cellular changes, such as increased dendritic spine density on NAc neurons (Lee et al., 2006; Pulipparacharuvil et al., 2008; Robinson and Kolb, 1999). Surprisingly, under these conditions, we found that Dnmt3a mRNA expression in

the NAc is selectively increased (**Fig. 2-2a**). We also found that Dnmt3a mRNA levels are similarly increased in the NAc of rats that underwent 3 weeks of cocaine self-administration followed by 28 days of withdrawal (**Fig. 2-2b**). Together, these mRNA data indicate that Dnmt3a is induced in a sustained manner after relatively long periods of cocaine withdrawal, and may thereby play an important role not only in the transition to addiction, but also in the maintenance of the addicted state.

DNA methylation modulates behavioral responses to cocaine

To understand the behavioral significance of the dynamic regulation of Dnmt3a in the NAc by cocaine, we utilized complementary pharmacological and genetic tools to manipulate DNA methylation in this brain region in the context of cocaine conditioned place preference (CPP), which provides an indirect measure of cocaine reward. We first administered methionine (a methyl donor) systemically with an injection regimen reported to hypermethylate ~5% of gene promoters in brain (Dong et al., 2008) and found that this manipulation caused a robust decrease in cocaine CPP (**Fig. 2-3a**). A key limitation of this experiment is its lack of anatomical and biochemical specificity. Therefore, we next tested whether intra-NAc delivery of RG108, a potent, highly specific, and non-nucleoside inhibitor of DNA methylation (Brueckner et al., 2005), influences reward behavior. Indeed, at a dose that decreased global DNA methylation, the continuous intra-NAc infusion of RG108 markedly enhanced cocaine CPP as well as the induction of locomotor sensitization to the drug (**Fig. 2-3b-d**).

These data suggest that DNA methylation in the NAc, possibly via Dnmt3a, negatively regulates cocaine reward. To further test this possibility, we developed a Herpes Simplex Virus (HSV) vector to temporally and specifically overexpress Dnmt3a in the NAc (**Fig. 2-3e**). Dnmt3a overexpression increased global DNA methylation in this brain region (**Fig. 2-3f**) and, consistent with methionine administration, attenuated cocaine CPP (**Fig. 2-3g**). To obtain the converse information, we administered an Adeno-Associated Virus (AAV) vector that expresses Cre recombinase into the NAc of mice homozygous for a floxed Dnmt3a gene. Such NAc specific knock out of Dnmt3a potentiated cocaine CPP, an effect not seen for Dnmt1 (**Fig. 2-3h**). Importantly, none of these manipulations of DNA methylation in NAc altered baseline locomotor behavior nor did Dnmt3a overexpression impair general tests of learning and memory (**Supplemental Fig. S2-3**). These behavioral data, coupled with the dynamic regulation of Dnmt3a expression, suggest that increased Dnmt3a expression in the NAc negatively regulates cocaine reward, whereas decreased Dnmt3a enhances cocaine reward.

DNA methylation regulates dendritic spine density in NAc

Among the most persistent drug-induced neuroadaptations known is cocaine's ability to increase dendritic spine density of NAc neurons (Robinson and Kolb, 1999). However, the contribution of spinogenesis to the addicted state remains unclear (Russo et al., 2010) (see Discussion). To gain insight into this issue, we analyzed NAc neuron spine density in mice that received intra-NAc

HSV-Dnmt3a or a control virus after chronic cocaine or saline treatment. Due to the limited time course of HSV overexpression (which wanes within 6 days of injection), we used a “5 injection” chronic cocaine schedule which has been shown to increase spine density 4 hr after the last injection (Maze et al., 2010; Russo et al., 2009). As expected, cocaine increased NAc spine density and, interestingly, Dnmt3a overexpression alone was sufficient to increase spine density to cocaine-comparable levels (**Fig. 2-4a**).

We next performed a more detailed spine analysis under identical conditions where we found Dnmt3a expression to be cocaine regulated: at 4 and 24 hr following the last of 7 daily cocaine injections (**Fig. 2-1a**). Here, we imaged Lucifer yellow filled NAc medium spiny neurons and analyzed dendrites using NeuronStudio software; these techniques are amenable to spine density as well as spine type analysis (Rodriguez et al., 2008). We found an increased spine density at 4 hr of withdrawal from chronic cocaine ($P<0.05$) and at 24 hr ($P<0.01$) (**Fig. 2-4b**). Moreover, spine type analysis revealed that both of these effects are due to a selective increase in thin spines, with no effect seen in mushroom or stubby spines (**Fig. 2-4c**). The shared increase in thin spine density suggests a mechanistic link for these two time points, which might reflect a lasting consequence of the transient induction of Dnmt3a seen at 4 hr. To test this hypothesis, we followed the same chronic cocaine dosing regimen while continuously delivering the Dnmt inhibitor, RG108, into the NAc and analyzed spine density and spine type 24 hr after the last cocaine dose. We found that RG108 completely blocks cocaine-induced spinogenesis (**Fig. 2-4c**). Moreover,

spine type analysis revealed that this effect is due to a specific effect on thin spines (**Fig. 2-4c**).

DNA methylation in NAc regulates depression-like behavior

In addition to playing an important role in addictive behavior, the NAc is known to be critically involved in depression and, as with cocaine models, labile histone modifications such as acetylation and methylation in NAc have been implicated in rodent models of depression (Covington et al., 2009; Renthal et al., 2007; Wilkinson et al., 2009). However, the role of DNA methylation in depressive behavior remains unexplored. We therefore analyzed mRNA levels for Dnmt's and methyl-binding domain proteins in NAc at 1 and 10 days following chronic social defeat stress—an ethologically-relevant model of depression that induces several depressive-like behaviors including prolonged social avoidance (Krishnan et al., 2007). We found increased Dnmt3a levels in the NAc at both time points after chronic defeat stress (**Fig. 2-5a**). In contrast, no regulation was seen for any of the other proteins studied (**Supplemental Fig. S2-4)**. As well, no significant regulation of Dnmt3a mRNA was observed 90 min after the last defeat nor 2 and 24 hrs after an acute defeat (data not shown).

We next tested the influence of Dnmt3a on susceptibility to social defeat by subjecting mice, which received HSV-GFP or HSV-Dnmt3a injections into the NAc, to submaximal defeat stress. In this paradigm, animals undergo just one day of defeat, which is not sufficient to induce social avoidance; in fact, normal mice display increased interaction with a social target under these conditions

(Krishnan et al., 2007; Krishnan et al., 2008). As expected, HSV-GFP mice exhibit this significantly increased interaction time ($P<0.05$). In contrast, Dnmt3a overexpression in the NAc attenuates social interaction, consistent with a pro-depressive-like phenotype (**Fig. 2-5b**). To complement these data, we assessed a second model of depressive-like behavior (Porsolt et al., 1977) and found that Dnmt3a overexpression significantly reduces the latency to immobility ($P<0.001$) in the rat forced swim test (**Fig. 2-5c**), also a pro-depression-like effect. These data suggest that the prolonged induction of Dnmt3a in NAc by chronic social defeat stress promotes depressive behavior. To further test this hypothesis, we continuously infused the Dnmt inhibitor RG108 into the NAc between 1 and 10 days after defeat stress, when we observed increased Dnmt3a mRNA expression in this brain region. We found that such local RG108 infusion reversed social avoidance in defeated mice, an effect similar to that observed by the standard antidepressant fluoxetine (**Fig. 2-5d**), indicating that Dnmt inhibition in this brain region exerts antidepressant-like effects.

DISCUSSION

Results of the present study demonstrate that Dnmt3a expression is subject to dynamic regulation in NAc by two types of chronic emotional stimuli. Chronic defeat stress induces a persistent upregulation of Dnmt3a in this brain region. In contrast, cocaine biphasically regulates Dnmt3a expression on a short timescale, whereas with longer-term withdrawal persisting upregulation of Dnmt3a is observed as well. Functional experiments establish that the cocaine-induced downregulation of Dnmt3a enhances cocaine reward, whereas upregulation of Dnmt3a exerts the opposite effect. Likewise, functional experiments in the chronic social defeat stress and forced swim paradigms suggest that prolonged upregulation of Dnmt3a in NAc drives depressive-like behavior. Taken together, these data suggest that an appropriate balance of DNA methylation in NAc crucially gates behavioral responses to emotional stimuli—a hypermethylated state dampens responses to rewarding stimuli and heightens responses to aversive stimuli, conversely, a hypomethylated state heightens responses to rewarding stimuli and dampens responses to aversive stimuli.

Our analysis of dendritic spine density on NAc neurons demonstrates that DNA methylation is an essential mediator of cocaine-induced spinogenesis: Dnmt3a overexpression in NAc mimics the cocaine-induced increase in spines, while RG108 infusion into this region blocks cocaine's action. A key question is how such a role for Dnmt3a in NAc spine regulation relates to the highly dynamic

regulation seen for Dnmt3a expression. Since Dnmt3a overexpression alone is sufficient to regulate spine density, and because DNA demethylation is an enzymatically unfavorable chemical reaction and debate still exists regarding the enzymatic basis of active DNA demethylation, we speculate that the transient reduction in Dnmt3a expression, such as what we observe 24 hr after chronic cocaine, is likely not of a sufficient timescale to downregulate spines (Ooi and Bestor, 2008). Rather, we speculate that the transient increase in Dnmt3a expression seen 4 hr after each cocaine exposure leads to the progressive accumulation of DNA methylation and is thereby responsible for the overall induction of NAc dendritic spines. The fact that a highly persistent increase in Dnmt3a expression is seen at 4 weeks of withdrawal, a time point when NAc spine density is robustly induced as revealed by multiple laboratories and methods (Lee et al., 2006; Pulipparacharuvil et al., 2008; Robinson and Kolb, 1999), is consistent with our hypothesis. The mechanisms responsible for the complex time course of Dnmt3a regulation in NAc–with early induction, quickly followed by suppression, and then followed by a slowly developing but very sustained induction–is unknown and requires future exploration (see below). Likewise, the persistent induction of Dnmt3a in NAc after chronic social defeat stress raises the novel possibility that NAc spine density may be regulated under these conditions as well, something in fact observed in preliminary investigations (Christoffell et al., 2010).

Our findings that Dnmt3a induction increases NAc spine density, while it attenuates cocaine reward, highlights an important question in the addiction field:

What is the behavioral relevance of cocaine's induction of dendritic spines on NAc neurons? Several conflicting reports exist on this subject (Russo et al., 2010). Robinson and colleagues positively correlated spine induction with increased locomotor sensitization (Yao et al., 2004). Three studies that directly manipulated genes (ΔFosB, NFκB, or G9a) in the NAc, known to regulate spines, found that manipulations that block spine induction also block cocaine's behavioral responses (Maze et al., 2010; Russo et al., 2009). However, two other studies yielded conflicting results: blocking CDK5 or activating MEF2 blocks cocaine-induced spinogenesis but enhances cocaine reward (Norrholm et al., 2003; Pulipparacharuvil et al., 2008). The pattern seen for Dnmt3a matches these latter findings. The basis for these paradoxical results is unknown. One possibility is that regulation of different types of spines might exert very different functional effects on NAc neurons and consequently on behavior. A recent study reported highly complex regulation of various spine types over a course of chronic cocaine exposure and withdrawal (Shen et al., 2009), and we show a selective effect of DNA methylation on the regulation of thin spins. The observations that Dnmt3a induces thin spines, but blunts cocaine's behavioral effects, raise the possibility that the induction of thin spines actually represents a homeostatic adaptation that serves to oppose the behavioral effects of cocaine. Clearly, this question requires much further investigation.

The electrophysiological function of cocaine-induced, DNA methylation-dependent thin spines remains speculative at this point, however, interesting electrophysiological correlates have been reported under similar cocaine

injection regimens. In association with the increase in thin spines, chronic cocaine causes: 1) a reduction in firing rate in the NAc shell (Kourrich and Thomas, 2009), 2) synaptic depression (decreased AMPA/NMDA ratio) during early (24 hrs) withdrawal (Kourrich et al., 2007), and 3) synaptic potentiation (increased AMPA/NMDA ratio) during late (10-14 days) withdrawal (Kourrich et al., 2007). First, some evidence exists for spines driving neuronal firing rate, as it was recently found that the spine density reductions that are associated with dopamine depletion induce a homeostatic response of increased firing of medium spiny neurons (Azdad et al., 2009). Second, synaptic depression, indicated by decreased AMPA/NMDA ratio, may represent an increased pool of AMPA receptor-lacking silent synapses, which are also known to be regulated by cocaine (Huang et al., 2009). Since thin spines represent highly plastic, newly formed spines which can lack AMPA receptors, we speculate that cocaine-induced DNA methylation induces thin spines that form silent synapses (Kasai et al., 2010). Finally, the influence of DNA methylation on synaptic plasticity is heavily supported in the hippocampal literature. In cultured hippocampal neurons, consistent with our discovery that RG108 reduces cocaine-induced spine density of NAc neurons, Dnmt inhibition causes a marked reduction in functional synapses in an activity-dependent manner as measured by a reduction in mEPSC frequency (Nelson et al., 2008). Moreover, both Dnmt inhibition and conditional knock out of Dnmt1 and Dnmt3a block long-term potentiation, a process thought to involve the formation and/or consolidation of dendritic spines (Feng et al., 2010; Miller and Sweatt, 2007). These studies point to the

importance of understanding the electrophysiological changes brought about by DNA methylation in the NAc. Such studies will provide key insight into whether cocaine-induced, DNA methylation-dependent changes in thin spines are a cause or consequence of changes in firing rate and/or synaptic depression.

As noted earlier, the mechanisms responsible for cocaine's short-term biphasic regulation of Dnmt3a mRNA expression are unknown. In hippocampus, Dnmt3a was also found to be rapidly upregulated by fear conditioning, but the expression pattern following this upregulation over longer time points has not been assessed. Since Dnmt inhibition in hippocampus blocks memory formation, it was presumed in this study that Dnmt3a's transient increase initiates downstream methylation of target genes that ultimately influence fear memory (Miller and Sweatt, 2007). This is analogous to our proposal that the transient increases in Dnmt3a that occur with each cocaine injection cause more lasting regulation of cellular and behavioral plasticity. In our study, the fact that me3K4H3 binding to the Dnmt3a promoter is commensurately associated with Dnmt3a mRNA regulation at both the 4 and 24 hr time points suggests that a histone 3, Lys4 methyltransferase, such as KMT2A (also known as MLL1), may regulate Dnmt3a's expression. Additionally, me3K4H3 is well known to inversely correlate with DNA methylation at particular gene promoters, raising the intriguing possibility that Dnmt3a may feed back and regulate its own mRNA expression (Weber et al., 2007). On a general level, one hypothesis that may explain Dnmt3a's complex regulation could be a transcriptional response to the rapid fluctuations in levels of dopamine or BDNF that are seen with cocaine

exposures (Graham et al., 2007). This is an intriguing possibility since 1) BDNF protein, like Dnmt3a mRNA, also accumulates with prolonged cocaine withdrawal, and 2) chronic social defeat stress induces lasting enhancement of BDNF signaling in the NAc (Berton et al., 2006; Krishnan et al., 2007) and, as we show here, also causes lasting increases Dnmt3a expression in this brain region.

Finally, these data, in conjunction with studies of histone acetylation and methylation (Covington et al., 2009; Maze et al., 2010; Renthal et al., 2007; Renthal et al., 2009; Wilkinson et al., 2009), support an emerging model that epigenetic alterations in the NAc have profound effects on the regulation of emotional behavior in models of both drug addiction and depression. The regulation is complex, with different epigenetic mechanisms apparently exerting distinct effects on behavioral endpoints. A promising line of future research would be to assess the behavioral function of additional enzymes that mediate still other forms of epigenetic modifications. In parallel, the gene targets (and their degree of overlap) for these various enzymes remain largely unknown. The elaboration of these targets should help us identify the many ways in which epigenetic mechanisms, including Dnmt3a-mediated DNA methylation, regulate NAc neuronal function to mediate the complex behavioral phenotypes of addiction and depression.

In this study, we found that both cocaine and chronic stress differentially regulate the de-novo DNA methyltransferase, Dnmt3a, in the NAc to control dendritic spine plasticity and behavioral responses to cocaine and stress. We find that Dnmt3a activity in the NAc *in vivo* is necessary for cocaine-induced

increases in dendritic spine density selectively for the thin type of spines. Our findings also suggest that the rewarding responses to cocaine negatively correlate with thin spines and also raise the possibility that depressive responses to chronic stress may positively correlate with increased spine density. Taken together, these observations implicate a new epigenetic modification–DNA methylation–in the molecular mechanisms controlling cocaine- and stress-induced structural and behavioral plasticity and could ultimately lead to the development of improved treatments for drug addiction and depression.

CHAPTER 2 FIGURES

Figure 1

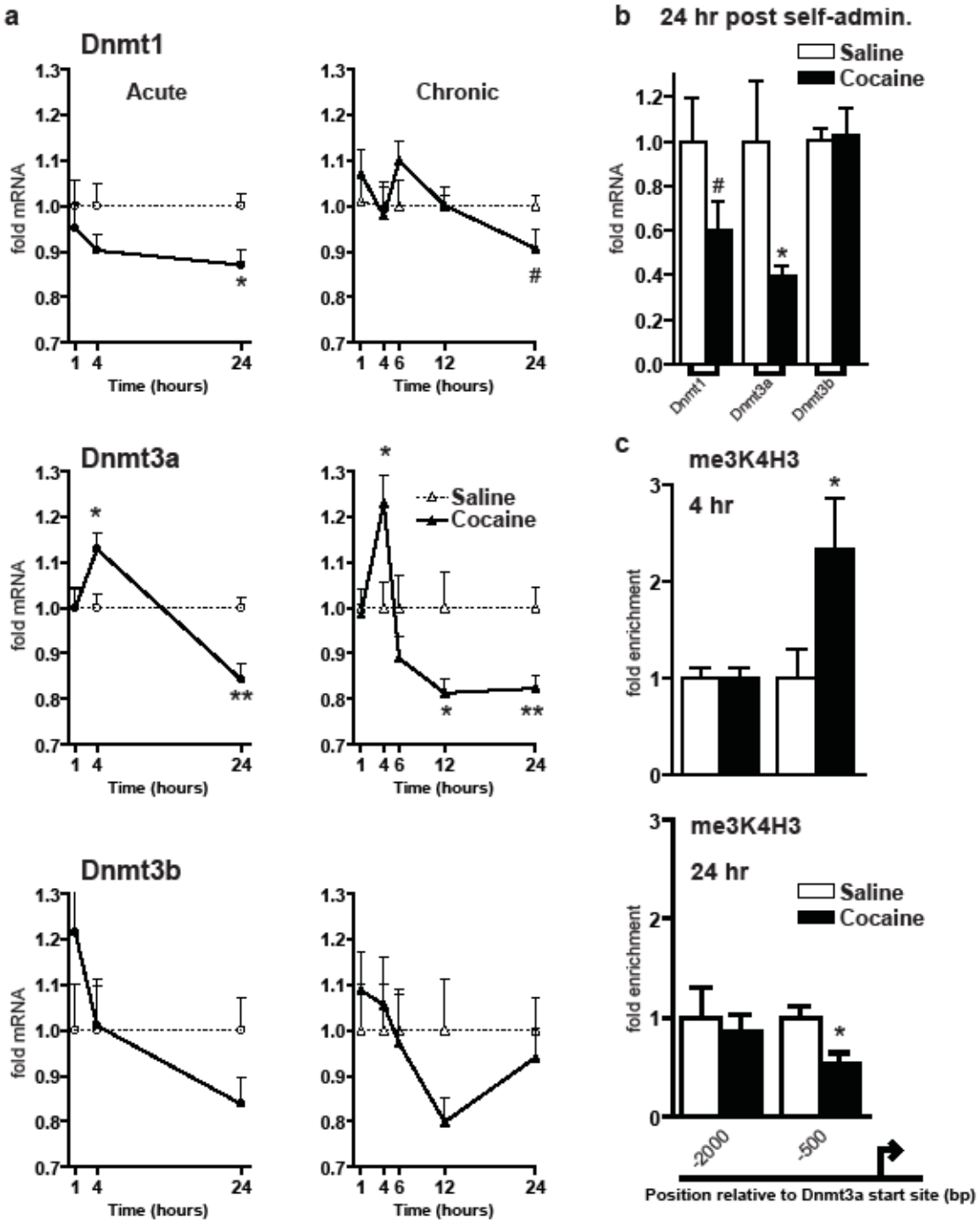


Fig. 2-1. Transcriptional regulation of Dnmt3a by chronic cocaine. **(a)** qPCR analysis of NAc of acute and chronic (7 days) cocaine treated mice (20 mg/kg/day IP) demonstrated transient increases in Dnmt3a mRNA levels 4 hr after the final injection (**P*<0.05, n=14 acute, n=7 chronic) and a decrease after 24 hr (***P*<0.005, n=6 acute, n=16 chronic). Dnmt1 (#*P*=0.08) and Dnmt3b transcripts were not altered significantly by chronic cocaine, however, acute cocaine significantly reduced Dnmt1 expression at 24 hr (**P*<0.05, n=6). **(b)** Dnmt3a mRNA was significantly reduced in the NAc of chronic (13 days) self-administering rats examined 24 hr after the last drug dose (**P*<0.05, #*P*=0.12, n=6 control, n=7 self-administration). **(c)** ChIP analysis revealed a significant cocaine-induced increase (4 hr) and decrease (24 hr) in me3K4H3 binding -500 bp upstream to the Dnmt3a promoter (**P*<0.05, n=4, 5 mice pooled/n), with no regulation seen -2000 bp upstream from the promoter.

Figure 2

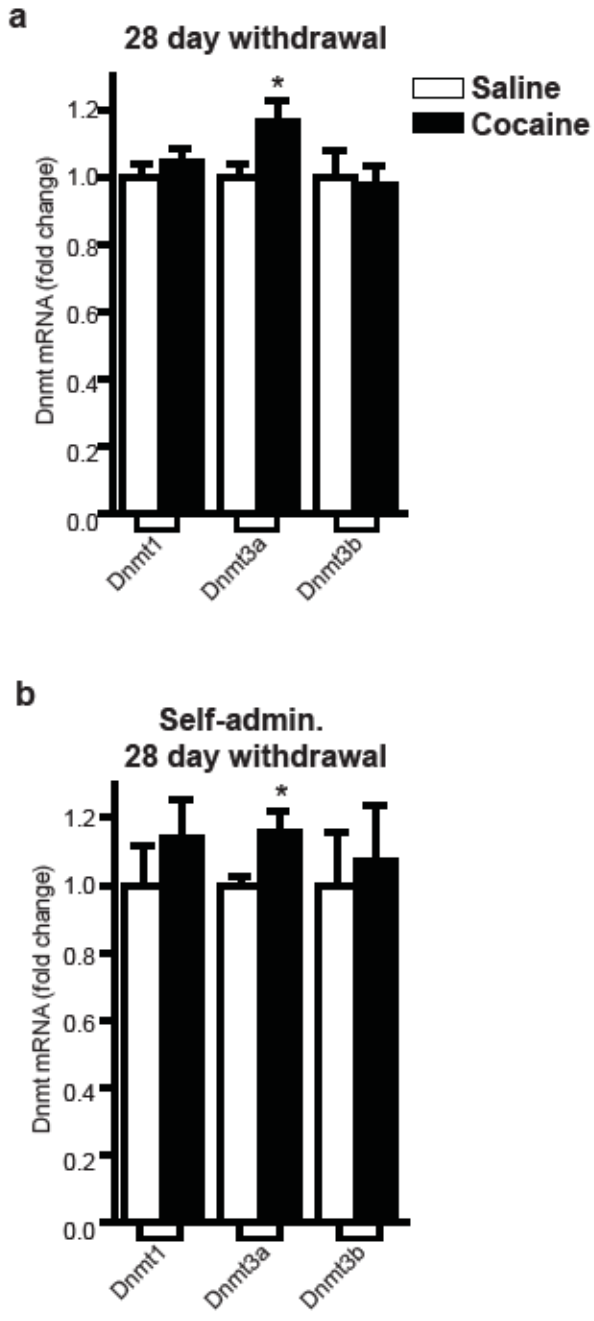

a
28 day withdrawal
Saline
Cocaine
Dnmt mRNA (fold change)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
Dnmt1
Dnmt3a
Dnmt3b
b
Self-admin.
28 day withdrawal
Dnmt mRNA (fold change)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
Dnmt1
Dnmt3a
Dnmt3b

Fig. 2-2. Prolonged induction of Dnmt3a by chronic cocaine after 28 days of withdrawal. **(a)** qPCR analysis of NAc of chronic (28 days) cocaine treated mice (20 mg/kg/day IP) that have undergone 28 days of drug withdrawal demonstrated an increase in Dnmt3a mRNA levels (*P<0.05, n=11). **(b)** Dnmt3a mRNA expression was significantly increased after 28 days of withdrawal in the NAc of chronic (3 week) self-administering rats (*P<0.05, n=6 control, n=7 self-administration).

Figure 3

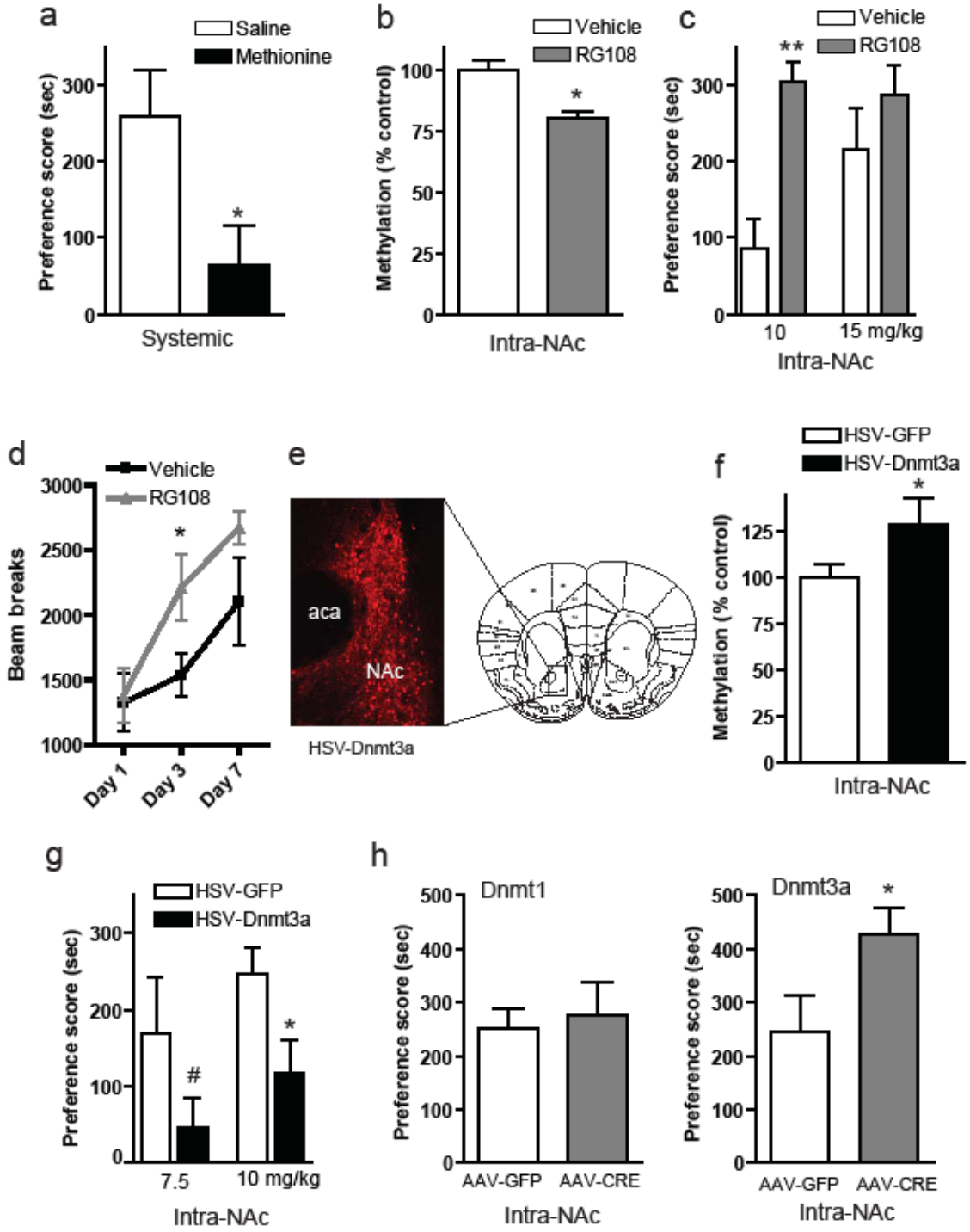

a
Saline
Methionine
Preference score (sec)
300
200
100
0
*
Systemic
b
Vehicle
RG108
Methylation (% control)
100
75
50
25
0
*
Intra-NAc
c
Vehicle
RG108
**
Preference score (sec)
300
200
100
0
10
15 mg/kg
Intra-NAc
d
Vehicle
RG108
Beam breaks
3000
2500
2000
1500
1000
*
Day 1
Day 3
Day 7
e
aca
NAc
HSV-Dnmt3a
f
HSV-GFP
HSV-Dnmt3a
*
Methylation (% control)
125
100
75
50
25
0
Intra-NAc
g
HSV-GFP
HSV-Dnmt3a
Preference score (sec)
300
200
100
0
#
*
7.5
10 mg/kg
Intra-NAc
h
Dnmt1
Preference score (sec)
500
400
300
200
100
0
AAV-GFP
AAV-CRE
Intra-NAc
Dnmt3a
*
Preference score (sec)
500
400
300
200
100
0
AAV-GFP
AAV-CRE
Intra-NAc

Fig. 2-3. DNA methylation regulates cocaine reward. (a) Chronic (7 days) methionine (0.78 g/kg/2x/day SC) diminished the rewarding effects of cocaine in the CPP paradigm (**P*<0.05, n=9). **(b)** Continuous intra-NAc infusion over 7 days of the DNMT inhibitor RG108 (100 μm) decreased global DNA methylation levels in NAc (**P*<0.005, n=6 vehicle, n=7 RG108), **(c)** increased cocaine CPP at 10 mg/kg IP (***P*<0.0005, n=9), **(d)** and enhanced the induction of locomotor sensitization to chronic cocaine (20 mg/kg IP) (**P*<0.05, n=8). **(e**) Verification of anatomical placement and viral infection in NAc after HSV-Dnmt3a-GFP injection; immunostaining for GFP is shown. Cartoon adapted from MBSC atlas, Figure 18, 1.54 mm Bregma. **(f)** HSV-Dnmt3a increased global DNA methylation levels (**P*<0.05, n=8 HSV-GFP, n=5 HSV-Dnmt3a). **(g)** Intra-NAc HSV-Dnmt3a significantly attenuated cocaine reward at 10 mg/kg cocaine (**P*<0.05, n=10), with a trend seen at a lower dose (#*P*=0.13, n=10). **(h)** Intra-NAc AAV-Cre injected into floxed-Dnmt3a mice significantly increased cocaine CPP at 7.5 mg/kg (**P*<0.05, n=14 AAV-CRE, n=18 AAV-GFP), with no effect seen in floxed Dnmt1 mice. All raw CPP data are provided in Supplemental Table 2-1.

Figure 4

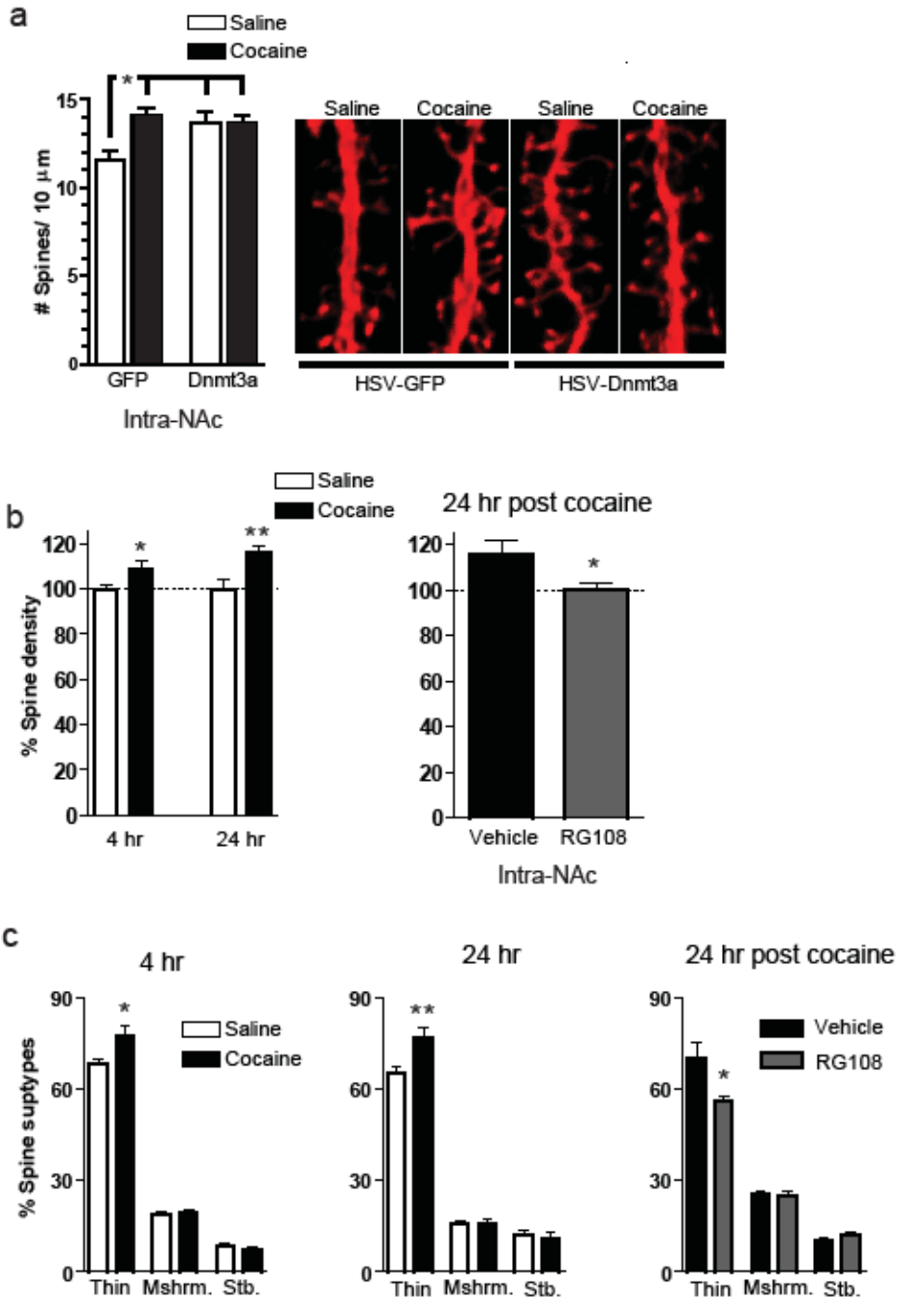

a
Saline
Cocaine
Spines/ 10 µm
GFP
Dnmt3a
Intra-NAc
Saline
Cocaine
Saline
Cocaine
HSV-GFP
HSV-Dnmt3a
b
Saline
Cocaine
% Spine density
4 hr
24 hr
24 hr post cocaine
Vehicle
RG108
Intra-NAc
c
4 hr
24 hr
24 hr post cocaine
% Spine suptypes
Saline
Cocaine
Vehicle
RG108
Thin
Mshrm.
Stb.

Fig. 2-4. DNA methylation regulates NAc dendritic spine density. (a) Dnmt3a overexpression mimicked cocaine's ability to increase dendritic spine density of NAc medium spiny neurons compared to saline GFP controls (*P<0.05, n=5 both HSV-GFP conditions, n=6 both HSV-Dnmt3a conditions). Representative confocal scans of GFP immunostained NAc neurons infected with either GFP or Dnmt3a-GFP viruses. To correspond with the shortened timescale of HSV expression, these analyses were performed 4 hr after 5 cocaine injections (20mg/kg IP). **(b)** Using an alternate method involving direct injection of Lucifer yellow into identified neurons, we found that chronic (7 days) cocaine treatment also significantly increased spine density at 4 hr (*P<0.05, n=7 saline, n=8 cocaine) and 24 hr (**P<0.01, n=8 saline, n=7 cocaine) after the last cocaine injection. Continuous (7 days) intra-NAc RG108 infusion significantly reduced spine density 24 hr after the last chronic cocaine injection (*P<0.05, n=4 Vehicle infused, n=5 RG108 infused). Data are expressed as % change in spine density relative to saline controls. **(c)** NeuronStudio spine type analysis of the same images from (b) revealed that chronic cocaine selectively increased thin spines both 4 and 24 hr after the last cocaine injection (*P<0.05, **P<0.01). Likewise, intra-NAc RG108 selectively reduced thin spines of chronic cocaine injected animals (*P<0.05). None of these conditions significantly affected the number of mushroom (mshrm) or stubby (stb) spines.

Figure 5

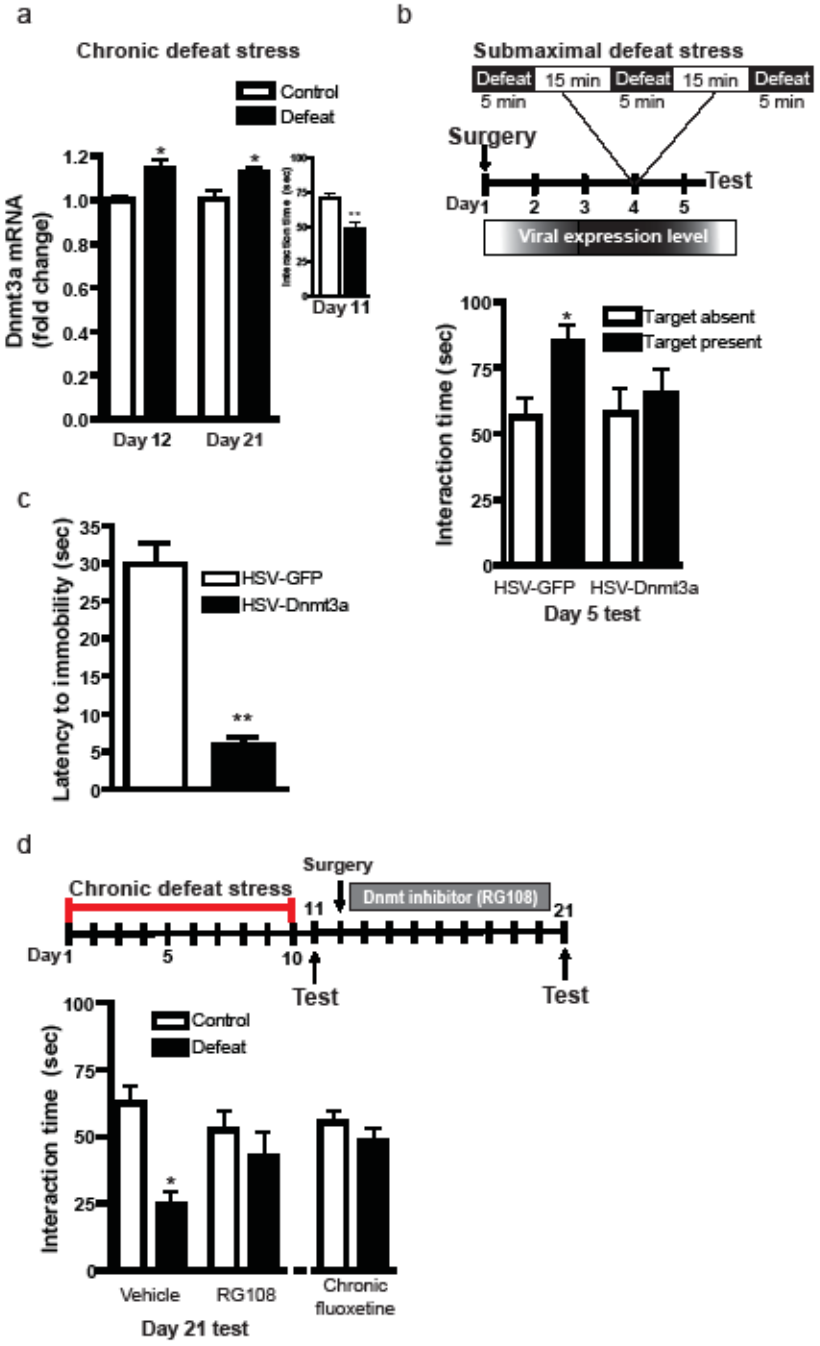
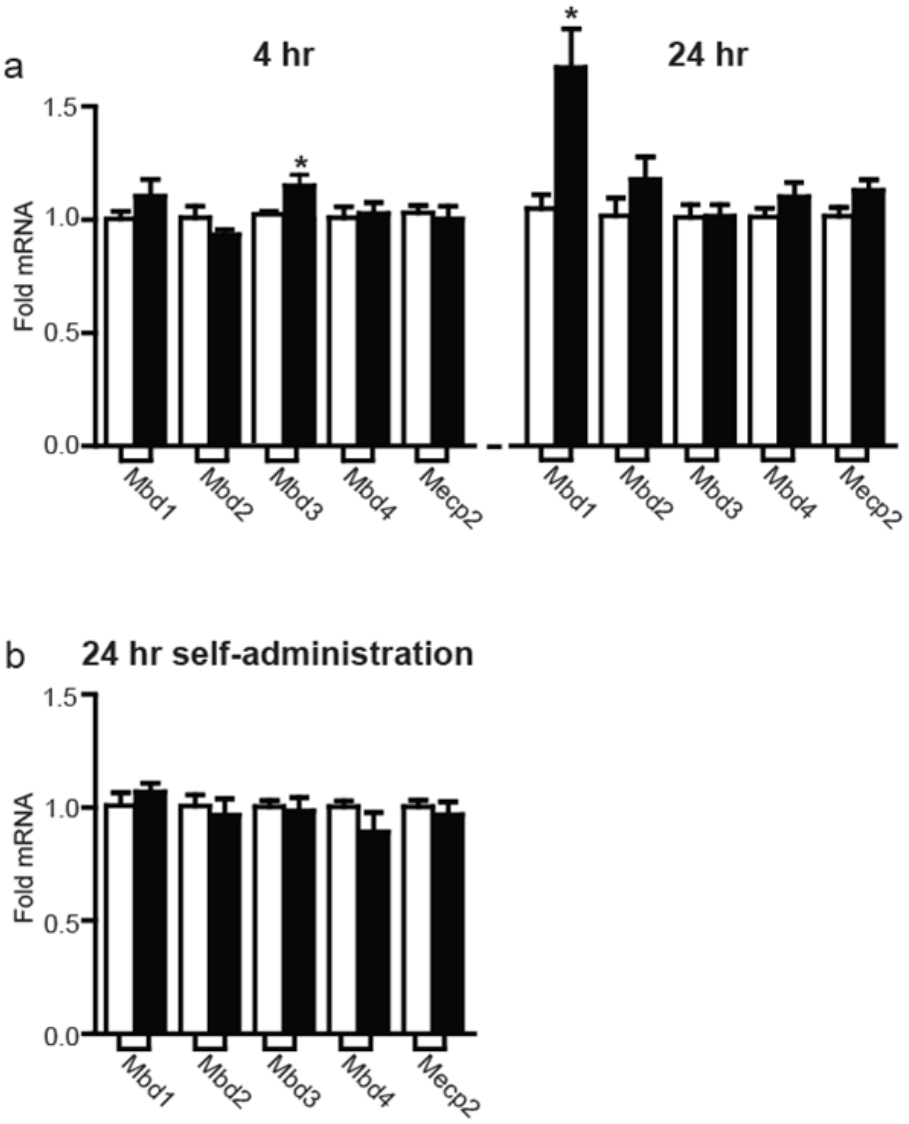

a
Chronic defeat stress
Control
Defeat
Dnmt3a mRNA (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Day 12
Day 21
Day 11
b
Submaximal defeat stress
Defeat
5 min
15 min
Defeat
5 min
15 min
Defeat
5 min
Surgery
Test
Day1
2
3
4
5
Viral expression level
Target absent
Target present
Interaction time (sec)
100
75
50
25
0
HSV-GFP
HSV-Dnmt3a
Day 5 test
c
Latency to immobility (sec)
35
30
25
20
15
10
5
0
HSV-GFP
HSV-Dnmt3a
d
Chronic defeat stress
Surgery
Dnmt inhibitor (RG108)
Day1
5
10
11
21
Test
Test
Control
Defeat
Interaction time (sec)
100
75
50
25
0
Vehicle
RG108
Chronic fluoxetine
Day 21 test

Fig. 2-5. Regulation of depression-like behavior by Dnmt3a. (a) qPCR of NAc taken 1 or 10 days after chronic (10 days) social defeat shows a significant increase in Dnmt3a levels at both time points (**P*<0.05). Inset displays social avoidance, a depressive-like behavior, observed in these mice (***P*<0.005, n=16 control, 26 defeated). **(b)** Naïve mice infused intra-NAc with HSV-GFP or HSV-Dnmt3a were subjected to a submaximal protocol of social defeat. HSV-GFP mice showed a significant increase in interaction with a social target ($F_{1,42}$ = 5.219, **P*<0.05) as would be expected from control mice[25], whereas HSV-Dnmt3a mice lacked this phenotype (*P*>0.05), a pro-depressive-like response. **(c)** Intra-NAc injection of HSV-Dnmt3a significantly decreased latency to immobility on Day 2 of the rat forced-swim test, also a pro-depressive-like response (***P*<0.001 n=7). **(d)** Conversely, intra-NAc infusion (10 days) of RG108, initiated one day after the last defeat episode, completely reversed the chronic social defeat-induced social avoidance exhibited by vehicle-infused mice ($F_{1,44}$ = 12.876 ***P*<0.001), an effect equivalent to that seen for chronic (20 mg/kg/day IP, 14 days) fluoxetine administration (*P*>0.05).

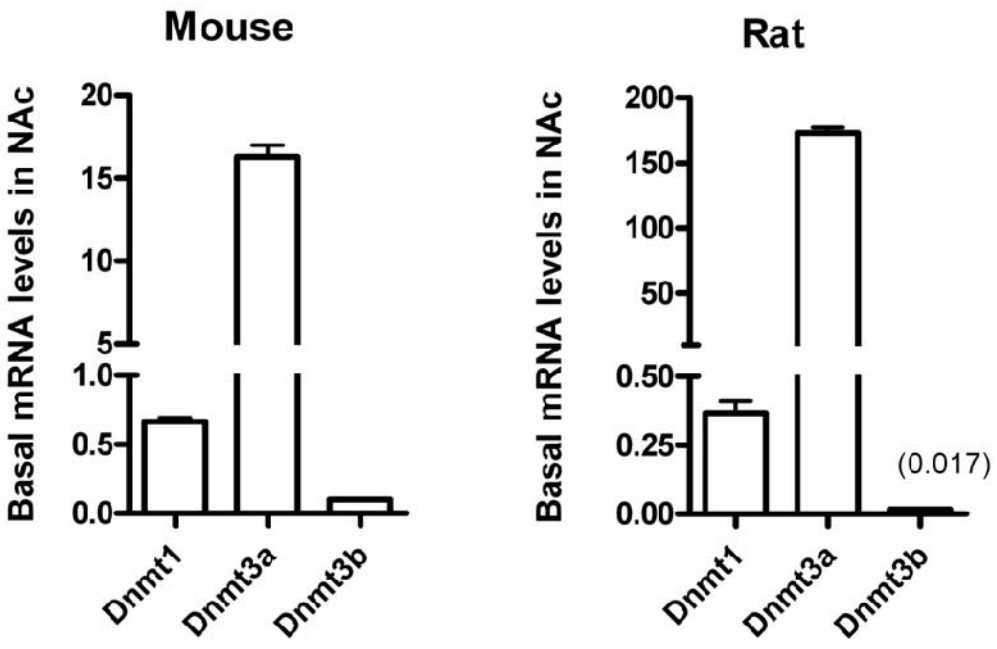
CHAPTER 2 SUPPLEMENTAL FIGURES

Supplemental Figure S1



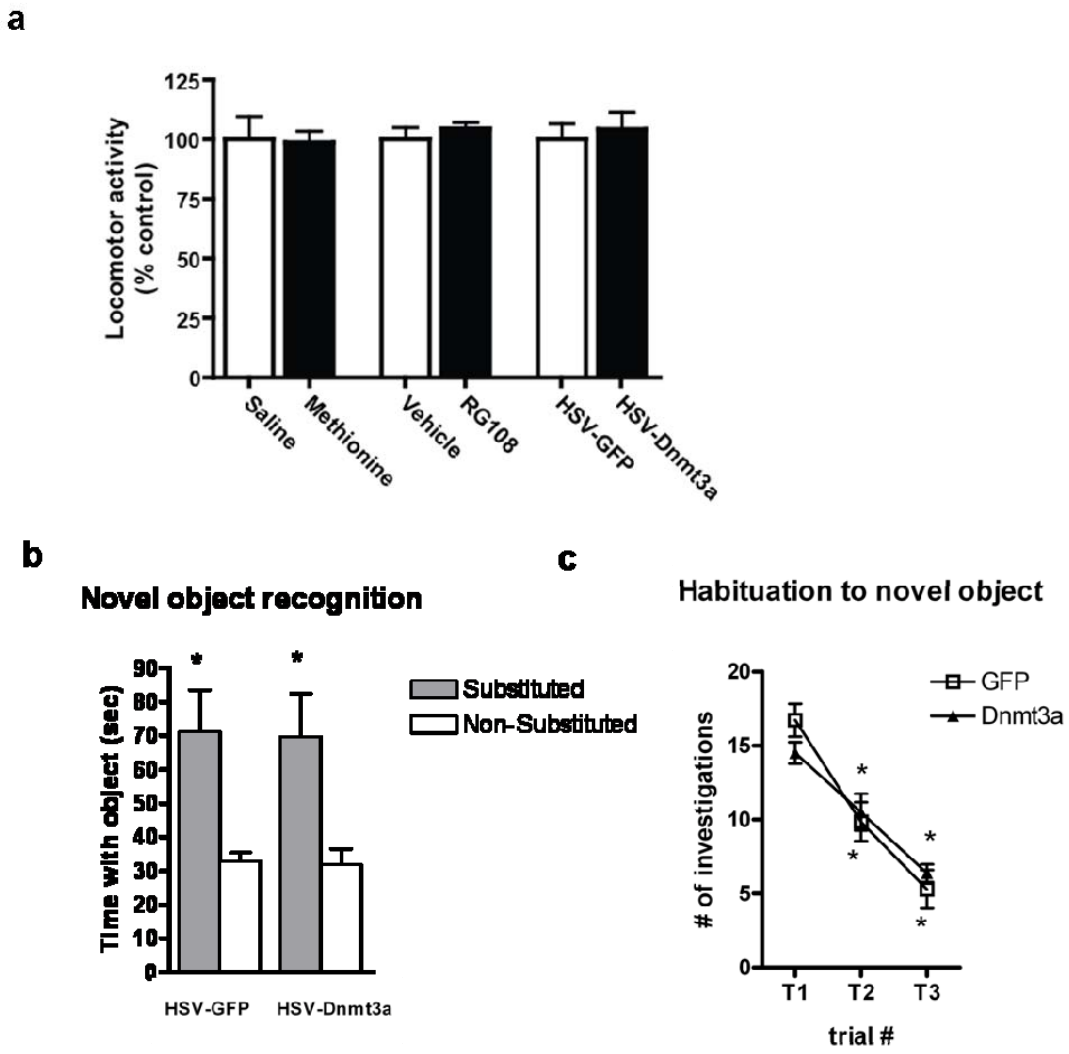
Supplemental Figure S2-1. **mRNA profiling of methyl-binding domain proteins (Mbd's) after chronic cocaine. (a)** qPCR analysis of NAc of chronic (7 days) cocaine treated mice indicated lack of regulation of Mbd's at 4 and 24 hrs, with the exception of a significant increase in Mbd3 at 4 hrs (*P<0.05, n=7) and Mbd1 at 24 hrs (*P<0.05, n=16). **(b)** However, Mbd1 and other Mbd's were unaltered in the NAc of self-administering rats examined 24 hours after the last cocaine dose.

Supplemental Figure S2



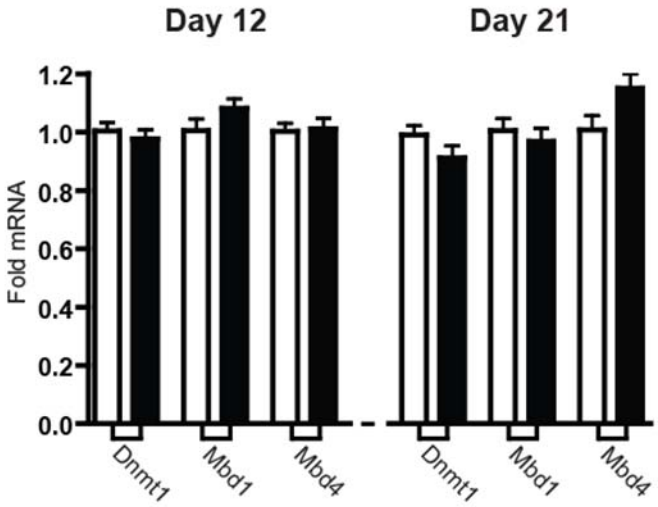
Supplemental Figure S2-2. **Relative Dnmt mRNA levels in NAc of mice and rats.** Data expressed as fold difference from the average level of all Dnmt's from 20 control mice and 6 control rats. Dnmt3a shows the highest expression in the NAc. Dnmt expression was measure by qPCR.

Supplemental Figure S3



Supplemental Figure S2-3. **Experimental manipulation of DNA methylation does not alter locomotor activity or learning. (a)** Locomotor activity was measured by photobeam breaks in the CPP chamber during the first day of saline training. We found no significant locomotor effects of subcutaneous methionine, intra-NAc RG108, or intra-NAc HSV-Dnmt3a. **(b)** Mice given intra-NAc injections of HSV-GFP or HSV-Dnmt3a spend equally more time with a novel, substituted object compared to non-substituted objects. Mice were repeatedly (3 times, 5 min each) exposed to 3 objects with 3 min intervals between test sessions. On the final trial, one object is substituted with a novel object. **(c)** HSV-GFP and HSV-Dnmt3a treated mice become equally disinterested in a single novel object upon reexposure. Mice were repeatedly exposed to a single object for 5 min with 1 hr intervals between test sessions.

Supplemental Figure S4



Supplemental Figure S2-4. **NAc mRNA profiling of Dnmt1, Mbd1, and Mbd4 following social defeat. (a)** qPCR analysis of NAc of mice subjected to chronic (10 days) social defeat taken 1 or 10 days after social interaction tests (Day 12 and 21) indicated lack of significant regulation of Dnmt1, Mbd1, and Mbd4 (P>0.05).

Supplemental Table 1

Fig. 3	Treatment	Mouse	Cocaine dose (mg/kg)	Pref score (sec)	SEM
a	METHIONINE	Wt	7.5	63.48	51.67
	SALINE	Wt	7.5	257.92	61.35
c	RG'08	Wt	10	304.58	26.05
	VEHICLE	Wt	10	85.17	39.78
	RG'08	Wt	15	286.35	39.32
	VEHICLE	Wt	15	214.58	55.91
g	HSV-DNMT3a	Wt	7.5	47.27	37.28
	HSV-GFP	Wt	7.5	170.17	71.69
	HSV DNMT3a	Wt	10	130.07	40.06
	HSV-GFP	Wt	10	246.85	35.14
h	AAV-Cre	floxed-Dnmt3a	7.5	428.48	46.77
	AAV-GFP	floxed-Dnmt3a	7.5	243.64	70.11
	AAV-Cre	floxed-Dnmt1	7.5	275.51	61.70
	AAV-GFP	floxed-Dnmt1	7.5	251.91	36.11

Pref, preference; wt, wildtype.

Supplemental Table 2-1. Raw CPP data from figure 2-3 (a-h).

CHAPTER 3
GENOME WIDE ANALYSIS OF COCAINE-INDUCED DNA METHYLATION

ABSTRACT

Studies in Chapter 2 establish that DNA methylation plays a key role in regulating drug reward and synaptic plasticity. The next major challenge lies in identifying the molecular mechanism of how DNA methylation exerts these effects. Thus, I optimized a technique called mDIP (methylated DNA Immunoprecipitation) for analyzing *in vivo* NAc DNA methylation levels across all gene promoters in the genome. Ultimately, these data point to a lack of detectable cocaine-induced regulation in gene promoters. However, a gene expression screen for alterations that occur in response to NAc Dnmt3a overexpression revealed that immediate early genes (IEG's) are specifically silenced by this alteration. Conversely, Dnmt inhibition robustly upregulates the expression of a large subset of genes that are also regulated in an activity-dependent manner. In addition to neuronal activity, IEG's are well-known to be upregulated by cocaine, however, the epigenetic mechanisms that mediate their return to basal levels are unclear. My discovery that DNA methylation selectively regulates IEG expression raises the possibility that DNA methylation may be involved in regulating cocaine induced IEG repression. Based on the mDIP-Chip data, the lack of differential DNA methylation at IEG gene promoter suggests that this regulation may be transient, occurring in a minority of cells, and/or occurring in genomic regions distal to IEG gene promoters.

INTRODUCTION

A hallmark of virtually all known mechanisms of long-lived neuronal plasticity is the dependence on transient intracellular signaling cascades. Thanks to major advances in biochemistry, over the past several decades neuroscientists have unraveled many mysteries of how neurons accomplish changes in synaptic function. On a molecular scale, key examples include insertion/removal of receptor subunits from synaptic membranes, alteration of synaptic protein function via posttranslational modifications, and the stimulation of the translation or degradation of proteins at the synapse. However, all these mechanisms occur at the protein level and are therefore subject to eventual turnover. Neuroscientists have long ignored the fact that the only permanent structure in any given neuron is its genome. Therefore, these transient intracellular signaling cascades that initiate all forms of neuronal plasticity must in some way be recorded in the genome. In order to adapt properly, each neuron must retain a “memory” of where it has been—what genes were expressed, and just as importantly what genes were not.

Because it is long-lived, methylation of DNA is a very likely candidate to assist neurons in achieving these putative ‘genomic records.’ In the past 5 years, several key studies have now implicated DNA methylation in regulating synaptic plasticity. For example, DNA methylation regulates activity-dependent functional synapses, long term potentiation (LTP), long term depression (LTD), as well as the formation of dendritic spines (Feng et al., 2010; LaPlant, 2009; Levenson et

al., 2006; Miller and Sweatt, 2007). Moreover, DNA methylation is implicated in playing a major role in several neurological diseases: autism, mental retardation, schizophrenia, addiction, depression, and disorders of memory formation. In parallel with these discoveries, over the past few years molecular and genomic techniques have been developed that allow one to analyze DNA methylation across all genes in the entire genome. In fact, one technique, mDIP-Chip (methylated DNA Immunoprecipitation on chip) has led to the identification of hundreds of gene promoters that are hypermethylated in cancer cells (Weber et al., 2007). And just two months ago (March 2010), an mDIP-chip study performed on hippocampal tissue from Dnmt1/3a double knock out mice identified 161 hypomethylated gene promoters (Feng et al., 2010).

The key next step will be to identify DNA methylation of genes that occur in wild type neurons in response to transient stimuli that are known to alter neuronal plasticity and behavior in a DNA methylation-dependent manner. To identify such genes, I employed a mouse chronic cocaine administration model. I focus on this model because I have found that DNA methylation plays an essential role in regulating dendritic spine homeostasis as well as behavioral responses to cocaine.

The specific goal of this work is to identify DNA methylation changes that occur in the mouse NAc genome in response to chronic cocaine. I used 3 approaches. First, I performed an mDIP-Chip experiment for analyzing DNA methylation in all promoters in the genome. To correlate putative methylation changes with changes in gene expression, I performed two gene expression

array experiments after chronic cocaine. In a second approach, I overexpressed Dnmt3a in NAc tissue and performed a gene expression screen of 100 genes. Third, I preformed a gene expression array after Dnmt inhibition of cultured neurons *in vitro*.

METHODS

Animals

We used adult male c57bl6j mice (Jackson laboratory) and Long-Evans rats (Charles River; self administration experiment). For all experiments, animals were habituated in our facility 1 week prior to experimentation and were housed on a 12 hr light-dark cycle with access to food and water *ad libitum*. All animal experiments were approved by the IACUC's of Mount Sinai School of Medicine and UT Southwestern.

Drugs

For chronic cocaine (Sigma) experiments, we used a standard cocaine injection which includes 7 daily intraperitoneal (IP) injections of 20 mg/kg cocaine. Control mice were injected with an equivalent volume of saline.

Striatal cultures

Embyronic striatal neurons (E18) were cultured from Long Evans rats (Charles river labs) as described previously without modifications (Pulipparacharuvil et al., 2008). The cells were kept for 10 days in two 6 well dishes with 8 x 10^6 cell/ well (12 total wells for 4 conditions ran in triplicate). On the 11^{th} day, 100 μm RG108 (in 0.2% DMSO) or DMSO (0.2%) was applied. After 24 hours, half of the wells were bathed in depolarizing solution (60 mM KCl) for 1 hour. RNA from each well was immediately harvested for microarray

analysis. Cells were monitored on a microscope daily to during the timecourse of the experiment to ensure that they remained healthy throughout the experiment. Neither RG108 nor KCl caused any overt change in cell number or morphology as observed by eye at high magnification.

Herpes simplex virus injections

We used the bi-cistronic p1005+ HSV vector, which expressed GFP alone or GFP with Dnmt3a. In this system, HSV infection occurs selectively in neurons. GFP expression is driven under the human immediate early cytomegalovirus (CMV) promoter, while the gene of interest, Dnmt3a, is driven by the IE4/5 promoter (Clark et al., 2002). The mouse Dnmt3a-1 plasmid (Linhart et al., 2007) was subcloned into the HSV vector, the vector was sequenced and then packaged into high-titer viral particles as described previously (Barrot et al., 2002). Stereotaxic surgery was performed on mice under general anesthesia with a ketamine/xylazine cocktail. Relative to Bregma, coordinates to target both the NAc shell and core were: 10° A/P +1.6 mm, M/L +1.5 mm, and D/V -4.4 mm. We bilaterally infused 0.5 µl at a rate of 0.1 µl/min. Mice were sacrificed 4 days post-op and 1 mm NAc tissue punches were taken after GFP visualization on a Leica fluorescent microscope.

DNA isolation

Frozen tissue punches were homogenized with a handheld pipette in 150 µL lysis buffer (20 mM Tris-HCL, 1mM EDTA, 400 mM NaCl, 1% SDS,

Proteinase K) and incubated for 3 hours at 65 degrees. Then 2 μL RNase A (Fisher Scientific) (100 mg/ml) was added and kept for 30 minutes at 25 degrees. One volume of Phenol:Chloroform:Isoamyl-Alcohol was added and briefly shaken in phase lock tubes (Qiagen Maxstract). Phase lock tubes were then centrifuged at 12,000 rmp for 10 min and aqueous phase was collected. Then standard ethanol precipitation is carried out with 100% and 70% ethanol using glycogen as a carrier. The pellet was dried and resuspended in 0.1x TE buffer. DNA amount and purity was then quantified using Nanodrop (Thermo Scientific).

Methylated DNA immunoprecipitation

i. Sonication of genomic DNA

Genomic DNA is randomly sheared by sonication to generate fragments between 300 and 1000 bp. First, 10-20 μg genomic DNA is diluted in TE in a 1.5 ml eppendorf tube (10-20 μg DNA in 400 μl TE). Then sonicate 4 times 10 seconds (BRANSON digital Sonifier model 450, used with the tapered Microtip, amplitude 20%), with 1 minute intervals between pulses (keep the tube on ice during the sonication). 8 μl was then ran on an agarose gel to check for appropriate fragmentation. Precipitate the sonicated DNA with 400 mM NaCl, glycogen (1 μl) and 2 volumes 100% ethanol. Resuspend the DNA pellet in TE and measure DNA concentration

ii. Immunoprecipitation

Sonicated DNA was immunoprecipitated with a monoclonal antibody against 5-methylcytidine (5mC) (EUROGENTEC #BI-MECY-1000) or an IGG

control. A portion of the sonicated DNA is left untreated to serve as input control. Immunoprecipitation is performed as follows:

1. Dilute 4 μg of sonicated DNA in 450 μl 0.1xTE

2. Denature for 10 minutes in boiling water and immediately cool on ice for 10 minutes

3. remove 45 μL for Input.

4. Add 46 μl of 10x IP buffer (100 mM Na-Phosphate pH 7.0, 1.4 M NaCl, 0.5 % Triton X-100)

5. Add 2 μl of 5mC antibody or 2 μL IgG control

6. Incubate 2 hours at 4°C with overhead shaking

7. Pre-wash 40 μl of Dynabeads with 800 μl PBS-BSA 0.1% for 5 minutes at RT with shaking

8. Collect the beads with a magnetic rack and repeat wash with 800 μl PBS-BSA 0.1%

9. Collect the beads with a magnetic rack and resuspend in 40 μl of 1x IP buffer

10. Add Dynabeads to the sample

11. Incubate 2 hours at 4°C with overhead shaking

12. Collect the beads with a magnetic rack and wash with 700 μl 1x IP buffer for 10 minutes at RT with shaking

13. Repeat wash with 700 μl 1x IP buffer twice

14. Collect the beads with a magnetic rack and resuspend in 250 μl proteinase K digestion buffer

15. Add 7 μl proteinase K (10 mg/ml stock)

16. Incubate 3 hours at 50°C (use a shaking heating block 800 rpm to prevent sedimentation of the beads)

17. Prepare phase lock tubes: 14k-30 seconds. Add samples to tubes. Add 1 volume of phenol:chloroform:IAA (250 μl). Shake 30seconds. Spin 14k – 8min room temp.

18. Precipitate the DNA with 400 mM NaCl (20 μl NaCl 5M), glycogen (1 μl) and 2 volumes 100% ethanol (no more than 500 μl). Keep at -80° for 30 minutes then centrifuge samples at 14k rpm for 25min.

19. Resuspend the DNA pellet in 30 μl 0.1xTE and keep at –20°C until later use.

iii. mDNA analysis by PCR and Whole Genome Amplification (WGA) for microarray

Enrichments in the MeDIP fraction were measured by real-time PCR and by microarray analysis. For real-time PCR, I used 20 ng of total input DNA and 2 μl of MeDIP DNA per reaction. Enrichments in the MeDIP fraction are calculated relative to an unmethylated control, CSA, which is an unmethylated CpG island containing promoter. For genome-wide analyses, input and MeDIP fractions are differentially amplified using Sigma's Whole genome amplification kit without modifications. Labeled with Cy3 and Cy5 and co-hybridized to microarrays as a two-color experiment. The methylation level is measured as the intensity ratio of immunoprecipitated to input DNA.

iv. mDIP-Chip analysis

Samples were labeled with Cy3 (input) or Cy5 (meDNA enriched) and hybridized to NimbleGen MM8 mouse “promoter plus” arrays with 3 biological replicates used per condition. Each replicate consisted of bilateral NAc punches pooled from 10 mice. ChIP-chip analysis was performed as described previously without modification (Renthal et al., 2009; Wilkinson et al., 2009). Briefly, pre-processed, normalized probes are assigned an alteration score which is based on mean probe intensity relative to input, the difference in cocaine mean intensity compared to saline, and a moving average of this signal difference. Alteration scores that were more than 3.1 standard deviations from the mean were considered significant ($p<0.001$).

RNA isolation

Bilateral NAc punches were dissected from mice treated with the indicated experiment and frozen on dry ice. Frozen brain tissue was then homogenized in TriZol (Invitrogen) and processed according to the manufacturer's protocol. RNA was purified with RNeasy Micro columns (QIAGEN) and processed as indicated by the manufacturer. Spectroscopy confirmed that the RNA had 260/280 and 260/230 ratios >1.8. Reverse transcription of total RNA (1.2 μg) was carried out using iScript (Biorad) as indicated by the manufacturer. The cDNA is then diluted to 4 ng/μL (assuming 100% efficiency from RT reaction). qPCR was then run in 10 μL triplicate reactions on 384 well plate using approximately 4 ng of cDNA per reaction, 10 mM primers, and SYBR Green (ABI). Each reaction was quantified

using the ΔΔCt method as previously described (Tsankova et al., 2006). Using the above method for RNA isolation and RT, I have optimized these PCR conditions to allow for analysis of more than 100 genes from a single mouse NAc—effectively increasing the number of PCRs by 10 fold compared to previous work in mouse NAc (Renthal et al., 2007).

cDNA microarray analysis

RNA isolation was performed exactly as described above on bilateral punches that were pooled from 4 animals per replicate. For the 24 hour microarray, there were 6 replicates per group (cocaine vs saline) and for the 1 hour microarrays (mouse cocaine, and rat striatal culture); there were 3 replicates per group. Microarray processing and data analysis were performed as previously described (Covington et al., 2009; Krishnan et al., 2007). Briefly, purified RNA was checked for quality using Agilent's Bioanalyzer (Santa Clara, CA). Reverse transcription, amplification, labeling, and hybridization to Illumina MouseWG-6 V2.0 or RatWG-12 V2.0 arrays were performed using standard procedures by UT Southwestern's microarray core. Raw data were background subtracted and quantile normalized using Beadstudio software (Illumina, San Diego, CA). Normalized data were then analyzed using GeneSpring software (Agilent). The dataset was filtered to only analyze transcripts that were significantly detected ($p<0.01$) across all 18 microarray. 'Significant' genelists were generated using this filtered list in addition to significance criteria of 1.3 fold change cutoff coupled with a non-stringent p-value cutoff of $p < 0.05$. We have a

high degree of confidence in these data because the data analysis criteria used for our study are recommended by the MicroArray Quality Control (MAQC) project because these criteria have been validated to have the highest inter-site reproducibility and intra-platform reproducibility. Second, many genes that are well known to be cocaine regulated (*fos, fosB, fosbl2, junB, egr1, egr2, egr4, hspa1, npas4, per2*) were on these lists. Furthermore, independent PCR analysis validated differential expression of 12 genes from the 1 hour genelist (Maze et al., 2010) and 7 genes from the 24 hour genelist (table 3-1).

RESULTS

Optimization of methylated DNA IP in brain tissue

As a first step to identifying differential DNA methylation on a genome wide scale, I optimized methylated DNA immunoprecipitation (mDIP) in mouse NAc tissue. The greatest hurdle in genome wide studies is the development and utilization of appropriate statistical analysis. Therefore, mDIP was determined to be particularly suitable for my studies because this technique would allow for the usage of the exact same Nimblegen promoter arrays which have been optimized and developed for data analysis for similar ChIP-chip studies (McClung et al., 2005; Renthal et al., 2009; Wilkinson et al., 2009). Moreover, this technique was deemed appropriate since it was successfully used to identify differential methylation in the genomes of cancer cells compared to healthy cells (Weber et al., 2005; Weber et al., 2007). However, at the time of study, mDIP had only been performed on cell lines *in vitro*. Therefore, it was crucial to reproduce key basic findings from cell culture studies to mouse NAc tissue homogenates. To this end, I developed an "in house" mDIP protocol which combines aspects of the published mDIP method with the Nestler lab's ChIP protocol (see mDIP protocol, methods section). Importantly, I optimized sonication conditions which reproduce the suggested size fractionation range of 300 to 1000 bp (**Fig. 3-1a**). Second, using 3 positive control loci (IAP and H19.1 and H19.3) and 2 negative control genomic loci (β-actin and CSA), I optimized the antibody-to-DNA ratio to reproduce published enrichment levels of known methylated and unmethylated

genomic loci. IAP is a retrotransposible element that exists in approximately 1000 copies per cell and is 100% methylated. H19.1 and H19.3 are two adjacent loci of an imprinted control region that is also 100% methylated. Finally, β-actin and CSA have been shown to lack methylation using a variety of techniques (Weber et al., 2005). As shown in **Fig. 3-1b**, it turns out that the antibody-to-DNA ratio dramatically effects PCR enrichment of methylated genes. Higher concentrations of antibody non-specifically enrich unmethylated DNA, whereas the lowest concentration of antibody exactly replicated the fold enrichment of positive and negative controls (Weber et al., 2005; Weber et al., 2007). Moreover, the standard error in NAc tissue in these experiments is actually considerably lower than the data from cell culture in the published method (Weber et al., 2005; Weber et al., 2007). Third, I determined the appropriate conditions for whole genome amplification (WGA) to produce enough DNA for microarray hybridization without compromising the relative methylation enrichment of positive and negative controls (**Fig 3-1b**). Overall, these data demonstrate that mDIP-chip is applicable in brain tissue for analyzing differences in DNA methylation for up to two orders of magnitude. Finally, before carrying out the cocaine mDIP-chip experiment, I performed a fourth "proof-of-principle" experiment to demonstrate that it is possible to detect differences in brain DNA methylation. Here, based on published differences in the striatum (STr) compared to the olfactory bulb (Ob) of the mRNA expression of tyrosine hydroxylase (TH), dopamine receptor 1a (Drd1a), and the shaker-related voltage gated potassium channel (Kcna4), I analyzed methylation of these three genes

across these brain structures (**Fig. 3-1c**) (Lein et al., 2007). Despite having very high expression in the Ob and low expression in the STr, TH was heavily enriched (~20 fold greater than actin) in both brain regions. However, TH had significantly higher methylation in the STr. Conversely, Drd1a had significantly higher methylation in the Ob and overall had moderate (~5 fold greater than actin) methylation levels. Consistent with the fact that the majority of genes have minimal levels of methylation in their promoter regions, I found that kcna4 had undetectable methylation in both brain regions.

DNA methylation analysis in NAc tissue after chronic cocaine

To identify whether cocaine persistently alters DNA methylation in the NAc, I performed an mDIP-Chip experiment in mice that were injected with chronic cocaine (7 days, 20 mg/kg) that had undergone 24 hours of drug withdrawal. To maximize potential for comparative analysis, this design was used to match our ongoing ChIP-chip studies at this cocaine withdrawal timepoint (Renthal et al., 2009). The chips contain 385K probes that span a 2 kb region across approximately 19,000 gene promoters in the mouse genome. Of note, one half of the NAc's from this experiment were used for a cDNA microarray. Also for cDNA microarray analysis, I sacrificed a second group of mice 1 hour after the last chronic cocaine injection. The purpose of the two cDNA microarray studies is to assist in identifying transcriptionally relevant genomic loci.

For quality control analysis, I verified that methylation enrichment occurred at the expected genomic loci. As predicted, both TH and H19 were in the top 1st

percentile of methylated promoters (IAP promoter probes not available), Drd1a was in the top 20th percentile, and negative controls were in the bottom 30th percentile. Similarly, if I take the top 20th percentile of methylated genes (3894 genes) and analyze absolute mRNA expression, I find that the majority (>90%) of these gene promoters are associated with transcripts that are expressed at low levels (**supplemental Fig. S3-1**). These findings provide a high degree of confidence in the technical validity of this mDIP-Chip experiment.

Using standard parameters (Renthal et al., 2009; Wilkinson et al., 2009) for saline versus cocaine comparative chip analysis, I found that 1711 gene promoters (approximately 9% of all analyzed gene promoters) showed significant changes in methylation by chronic cocaine compared to saline treated animals (Fig 3-2a, $p<0.001$). Specifically, 1228 gene promoters were hypermethylated and 484 were hypomethylated. Moreover, analysis of differential gene expression at 1 hour and 24 hours after chronic cocaine treatment revealed that approximately 13% and 4.8% of genes significantly regulated at the mRNA level are also differentially methylated (**Fig. 3-2a**). However, despite their statistical significance, the vast majority of promoters exhibiting differential DNA methylation have small fold change differences compared to saline (generally ranging from 1.2 to 1.5 fold change). For example, if I further apply a 1.5 fold cut-off to the DNA methylation genelist, I find that only 60 of the 1711 are differentially methylated (Fig 3-1a). This observation raises concern because even if such methylation changes do represent biologically significant events, it will be experimentally challenging to prove that such changes occur.

To this end, I developed a candidate genelist of 53 candidate genes. In order to maximize the possibility of identifying differentially methylated genes, the genes on this list were carefully selected based on several different criteria. For example, seven genes in **Table 3-1** highlighted in blue were identified at the 24 hr or 1 hr microarray and were independently validated to have differential mRNA expression. Moreover, ten genes highlighted in orange have been previously identified as playing an important role in biochemical or behavioral responses to psychostimulants. Third, I selected 12 of the 60 genes that met the criteria of reaching both statistical ($p<0.001$) as well as fold change cutoff (>1.5 fold). In the field, the gold standard for validation of differential methylation is with bisulfite sequencing (Shames et al., 2007). Therefore, I utilized a high throughput mass-spectroscopy based bisulfite sequencing technique (called Sequenom analysis) (Coolen et al., 2007). The greatest hurdle in Sequenom analysis is in developing optimal PCR primers and parameters that produce a single pure DNA amplicon (Coolen et al., 2007). Therefore, as a screen, I designed Sequenom primers that matched to a 100-500 bp sequence corresponding to the differentially methylated region of each of these 53 gene promoters and then used the best 26 primer sets for Seqenom analysis. Unfortunately, even after using an aliquot from the same DNA that I used for my mDIP-chip experiment, Sequenom analysis of these genes did not identify significant regulation at any of these 26 loci. **Figure 3-3** displays a typical example of methylation analysis one of these genes, Htr3a, which encodes the serotonin 5HT3a receptor.

In-Vivo analysis of DNMT3a gene targets

The mDIP-chip data overwhelmingly point to a lack of differential DNA methylation occurring at promoters in the genome. However, sizeable evidence suggests that DNA methylation is functionally relevant to cocaine-induced behavior in the NAc (LaPlant et al., 2010; see Chapter 2). Dnmt3a, for example, is transiently upregulated by chronic cocaine and viral-mediated overexpression of DNMT3a results in significant increase in global DNA methylation levels, significant increase in dendritic spine density, and a significant decrease in cocaine conditioned place preference.

Since DNMT3a has such robust effects at a cellular and behavioral level, an alternative approach to finding cocaine induced methylation targets would be to see which genes have altered mRNA expression in response Dnmt3a overexpression. This experiment has an element of added benefit and specificity since HSV only infects neurons. Therefore, I collected NAc RNA and performed real-time PCR analysis from mice that were sacrificed during the time of maximal HSV-Dnmt3a or HSV-GFP overexpression (**Table 3-2**). Importantly, the majority of the 100 genes whose expression I profiled are known to play important roles in drug addition. Moreover, the genes that I selected span a variety of cellular processes throughout neurons (receptors, ion channels, kinases, neuropeptides, spine structural genes, chromatin modifying enzymes, and transcription factors). Overall, 18 genes were significantly regulated by Dnmt3a overexpression (6

upregulated, 12 downregulated) (**Fig. 3-4**). Specific examples of transcripts that were regulated include: Dicer, a key regulator of miRNA processing, Camk2a splice variants, Dnmt1, and the spine associated genes, Homer1a, and Psd-95. Of note, a striking number of regulated genes (10/18) belong to a gene family that is dynamically regulated by neuronal activity—immediate early genes. In this family, 83% (10/12 genes) of immediate early genes were downregulated. Notably, most of these genes, such a c-fos, fosB, and Arc, are well known to be cocaine regulated (McClung et al., 2004). However, revisiting the mDIP-Chip DNA methylation binding profiles specifically at these promoters, I observed a lack of cocaine-induced differential methylation (data not shown)—suggesting that DNA methylation may regulate cocaine induced gene expression at regions outside of IEG gene promoters (see discussion). An important experiment that is underway will be to look at DNA methylation levels at IEG genes after Dnmt3a overexpression.

DNA methylation regulates activity dependent gene expression

Immediate early genes represent the first wave of transcriptional activation in response to cocaine. On a basic level, these genes are regulated by a transient, orchestrated, calcium dependent response to neuronal activity. Since Dnmt3a overexpression blocks the expression of this gene family, I wanted to test how inhibiting DNA methylation influences activity dependent gene expression by depolarizing cultured striatal neurons—a well established model for assessing activity dependent gene expression (Greer and Greenberg, 2008).

Here, striatal neurons were bathed in the Dnmt inhibitor, RG108, or DMSO, for 24 hours and were then subjected to KCl induced depolarization or control solution. RNA was harvested for microarray analysis immediately after 1 hour of depolarization. This timepoint was chosen because it is when activity dependent genes are maximally activated (Greer and Greenberg, 2008). I found that RG108 at baseline significantly increased expression of 95 genes. Heatmap comparison to how these genes are regulated after depolarization revealed that the majority of these genes (>65%) are also regulated by KCl (**Fig 3-5a**). I found that a much larger number of genes were regulated by neuronal activity alone (131 genes). Interestingly, heatmap analysis also revealed that many of these genes which are regulated by activity are also upregulated by RG108 (>40%) (**Fig. 3-5a**). Taken together, these data suggest that transcriptional regulation of sizable subset of activity dependent genes share a functional link with DNA methylation dependent gene transcription. Surprisingly, however, RG108 in the presence of KCl does not appear to markedly sensitize (further increase) expression of KCl regulated genes nor does RG108 sensitize the genome (increase the total number of genes that are significantly regulated by KCl). Mechanistically, this suggests either a ceiling effect (genes are maximally regulated by KCl), or perhaps DNA methylation plays a less of a role in the induction of IEG transcription and perhaps a more prominent aspect in the repression or stabilization or IEG expression.

DISCUSSION

In summary, chronic cocaine does not cause overtly detectable differences in DNA methylation at gene promoter regions. This is surprising since behavioral adaptations to cocaine and cocaine-induced changes in spine density are mediated at least in part via DNA methylation. Since gene promoters cover only a small percentage of the genome, it's plausible that cocaine-induced DNA methylation may occur outside of promoter regions. To functionally prove that manipulating DNA methylation does effect gene transcription, I overexpressed Dnmt3a in the NAc and discovered that Dnmt overexpression results in a marked and significant decrease in 83% of immediate early genes analyzed. Moreover, this regulation was selective to immediate early genes, since 82% of transcripts analyzed were not significantly regulated by Dnmt3a overexpression. In the converse experiment, *in vitro* Dnmt inhibition revealed that the vast majority of genes regulated by DNA methylation are also regulated in an activity-dependent manner. Therefore future studies point to analyzing NAc DNA methylation levels following Dnmt3a overexpression, Dnmt inhibition, and cocaine treatment at genomic regions in and around immediate early genes—particularly at IEG enhancer elements (discussed below).

The selective role of DNA methylation in IEG expression is of broad significance in the neuroscience community because IEGs represent the gateway to the genome's response to neural activity. This gene family is arguably the most dynamically regulated of all genes. In response to activity, somehow neurons are capable of: 1) rapidly activating, 2) rapidly deactivating, and 3)

maintaining a silenced, but ready state of IEG gene expression until the next wave of neuronal activity. On top of these 3 processes, a multitude of examples exist where re-activation, or re-exposure to the same stimulus, results in enhanced (so called 'sensitized') or attenuated (so called 'desensitized') IEG expression. For example, our group has repeatedly observed that IEG's such as c-fos and fosB have desensitized striatal induction in psychostimulant experienced compared to naïve rodents (Alibhai et al., 2007; Renthal et al., 2008). Moreover, this desensitized induction persists in animals for a long as 1 month after drug withdrawal (LaPlant, 2009). Such sensitization or desensitization has largely been attributed to up-stream post-transcriptional mechanisms such as differences in phosphorylation, however, the role of DNA methylation has not been assessed (Edwards et al., 2007). Here, I have discovered that DNA methylation plays a key role in the regulation of IEG expression. In addition to identifying if this regulation is directly via DNA methylation, it will also be important for future research to further understand the role of DNA methylation in regulating the temporal dynamics of IEG expression. Similarly, an important line of future research will be to see if repeated neural activity and IEG activation may result in a type of 'genomic recording' via accumulation of DNA methylation at IEG regulatory elements.

Lack of cocaine-induced DNA methylation at gene promoters

There are three interpretations regarding my meDIP-chip data. 1. Cocaine-induced DNA methylation is not significantly occurring in the genome. 2.

Cocaine-induced DNA methylation is occurring but not in gene promoters. Or 3. Cocaine-induced DNA methylation occurs in gene promoters, but subtle changes are at or below the threshold of detection using state-of-the-art techniques. Based on the 24 hour microarray data, which had cocaine-induced expression changes typically 1.3-1.5 fold different from saline treated animals, it is not surprising that subtle changes may be occurring with DNA methylation as well. An improved approach for future research will be to analyze meDNA and mRNA in purified neurons or even specific neuronal subtypes. This approach has resulted in much greater fold differences in mRNA (Heiman et al., 2008). Another important point that warrants clarification is that bisulfite sequencing may be a weakness for validation because it was recently discovered that hydoxymethylation of DNA occurs specifically in neurons (Kriaucionis and Heintz, 2009). This caveat ultimately means that sequencing reactions cannot actually differentiate between methylated cytosine and hydroxymethylated cytosine. At this point, the extent of hydoxymethylation that occurs throughout the gene promoter region is not known. An important strength of the mDIP technique is that the antibody recognizes only meDNA. Therefore, it may be worth revisiting my validation experiment by using the mDIP technique. To truly answer the 1st and 2nd possibilities (whether DNA methylation occurs outside of promoter regions), the next major experiment will need to test DNA methylation levels throughout the entire genome using sequencing technology. A key weakness of my study was that the microarray only covered approximately 2 kb for all 19,000 ref-seq gene promoters. While this may sound like a lot, overall this is only about

1.5% of the mouse genome. Recently, several high profile whole-genome sequencing publications have emerged which suggest that DNA methylation is playing a much bigger role outside of CpG islands than in gene promoters. One study found that the region distal to CpG islands containing gene promoters-so called “CpG shores”-is subject to heavy differential DNA methylation (Irizarry et al., 2009). Of major interest, a histone methylation ChIP-sequencing study done in neuronal culture found that activity dependent gene expression is heavily linked to epigenetic regulation at enhancer elements several thousand base pairs away from the canonical promoter region (Kim et al., 2010). Moreover, this study found that while me3K4H3 was highly enriched at activity dependent gene promoter regions, me1K4H3 was highly enriched at enhancer elements of these genes (Kim et al., 2010). This has important implications for a role of DNA methylation in activity dependent gene regulation because it is well known that the level of methylation on K4H3 is inversely correlated with the level of DNA methylation (Weber et al., 2007). Taken together, these studies highly suggest that the most likely genomic target of DNA methylation is at IEG enhancer elements.

Link between Dnmt3a’s influence on gene expression and Dnmt3a’s known cellular and behavioral role

As previously mentioned, I found that intra-NAc Dnmt3a overexpression increases dendritic spine density and decreases cocaine CPP. A high degree of evidence exists which supports the notion that IEGs are critically linked to

cocaine reward. IEGs are well known to be induced by cocaine (Hope et al., 1994). Several studies have found that pharmacologic or genetic attenuation of IEG induction is correlated with reduced behavioral response to cocaine (Kumar et al., 2005; Maze et al., 2010). However, one disconnect between cocaine's and Dnmt3a's actions is that cocaine transiently induces IEG expression and this is associated with an increase in dendritic spine density whereas Dnmt3a overexpression reduces IEG expression and increases spine density. Clearly there are many genes that influence dendritic spine formation and spine elimination. Within the IEG gene family Arc and ΔFosB have both been implicated in dendritic spine plasticity (Greer and Greenberg, 2008; Maze et al., 2010). Increased Arc promotes internalization of AMPA receptors thereby weakening synaptic strength and making synapses more prone to elimination, whereas ΔFosB increases spine density through yet to be determined mechanisms. Although IEGs are transiently increased by psychostimulants, Freeman and colleagues have found that Arc and several other IEGs (but not FosB) are persistently downregulated for up to 100 days after the last self-administration session (Freeman et al., 2008). Such regulation fits perfectly with cocaine induced upregulation of Dnmt3a and increased density of dendritic spines at longer term timepoints.

CHAPTER 3 FIGURES

Figure 3-1

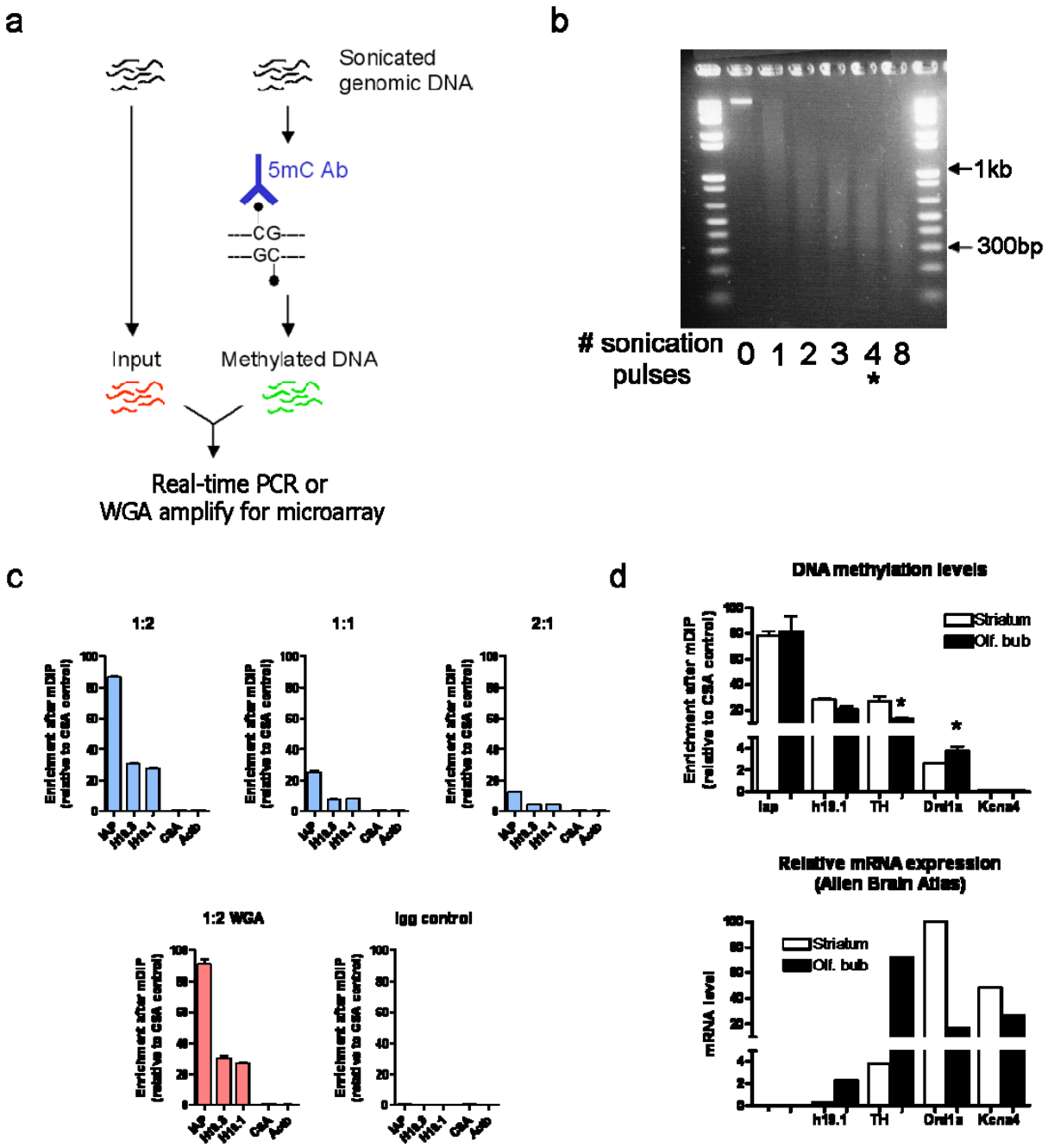
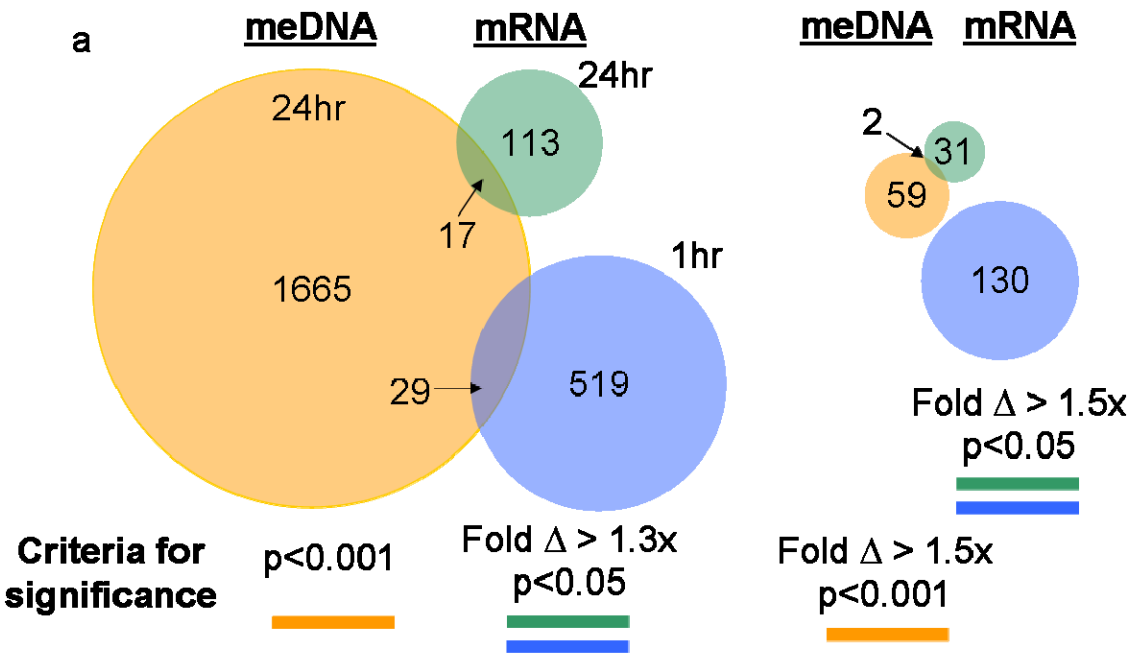


Fig. 3-1. Optimization of methylated DNA IP in brain tissue (a) mDIP overview. DNA is purified, sonicated, and immunoprecipitated using an antibody against methylated cytosine. After IP, samples can be analyzed via qPCR or amplified for microarray studies. **(b)** DNA size distribution after increase numbers of 10s sonication pulses. 4 pulses replicates the 300-1000bp size distribution reported in other studies. **(c)** PCR analysis of known methylated (IAP, H19.1, H19.3) and unmethylated (CSA, ActB) loci at varying IP conditions (antibody to DNA ratios) and after whole genome amplification (WGA). As the concentration of antibody is increased, the specificity for methylated DNA enrichment is lost. A 1:2 ratio exactly replicates finding from cell culture (90 fold enrichment of IAP, 30 fold enrichment of H19 loci). WGA under these conditions replicates these findings as well. **(d)** mDIP (top panel) comparisons of differentially expressed genes in striatum compared to olfactory bulb. Bottom panel displays relative mRNA expression of each of these differentially expressed genes as reported in the Allen Brain Atlas project. TH (tyrosine hydoxylase) and Drd1a (dopamine D1 receptor) are differentially methylated, with TH displaying generally high levels of methylation (20 fold enrichment) in both samples.

Figure 3-2


a
meDNA
mRNA
24hr
1hr
24hr
113
17
1665
29
519
Criteria for significance
p<0.001
Fold Δ > 1.3x
p<0.05
meDNA
mRNA
2
31
59
130
Fold Δ > 1.5x
p<0.05
Fold Δ > 1.5x
p<0.001

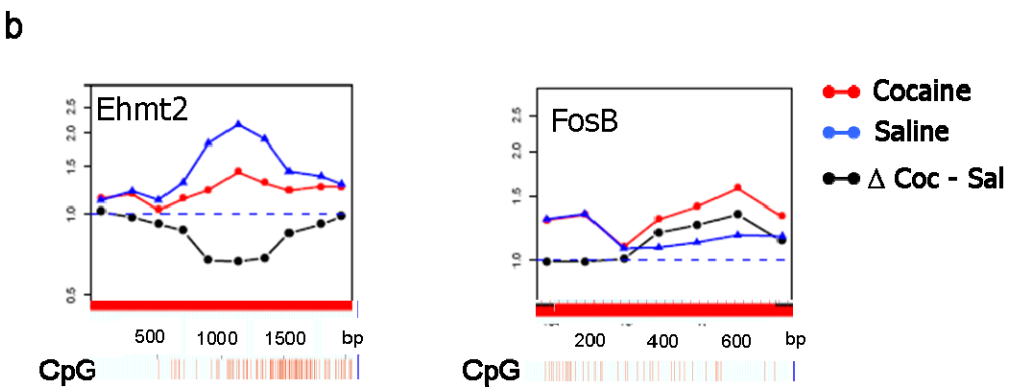
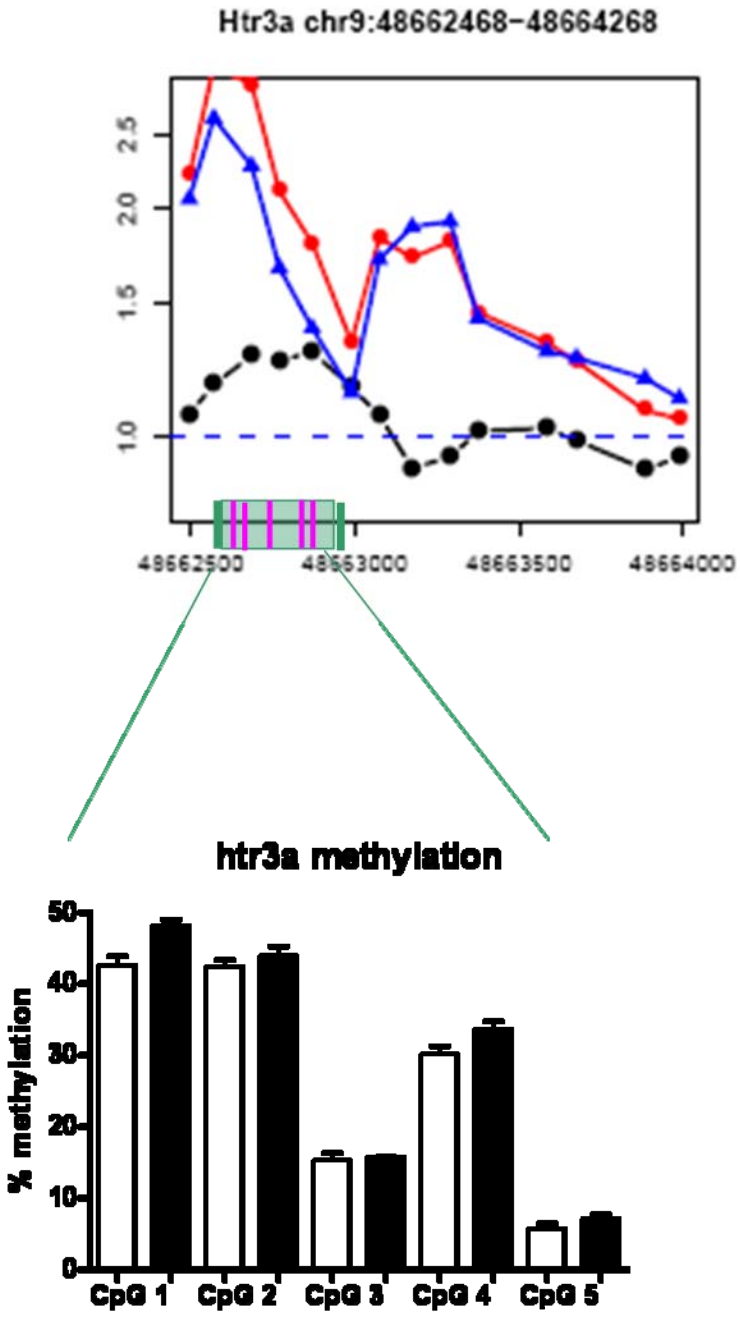

b
Ehmt2
500
1000
1500
bp
CpG
FosB
200
400
600
bp
CpG
Cocaine
Saline
Δ Coc - Sal

Fig. 3-2. Cocaine-induced mDIP-Chip analysis (a) Venn diagrams comparing significant mDIP genelist (1707 genes, $p<0.001$) to differentially expressed genes after 1 and 24hrs (544 and 130 genes, fold change (FC) cutoff 1.3x, $p<0.05$) **(b)** increasing significance cutoff to 1.5 fold dramatically reduces the number of significantly methylated genes (61 genes, FC 1.5x, $p<0.001$) as well as the number of overlapping differentially expressed transcripts. **(c)** Examples of promoter plots from genes from (a) with individual CpG sites annotated as vertical red lines. Red- enrichment with cocaine, blue- enrichment with saline, black- cocaine-induced fold change in enrichment.

Figure 3-3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2.5
2.0
1.5
1.0
48662500
48663000
48663500
48664000
htr3a methylation
% methylation
50
40
30
20
10
0
CpG 1
CpG 2
CpG 3
CpG 4
CpG 5

Fig. 3-3. Bisulfite sequencing example. Top panel is a promoter plot example of differential methylation at the Htr3a gene. Green box around promoter represents the amplicon that bisulfite sequencing primers were designed against. Pink vertical lines within box represent individual CpG sites. Bar graph below is quantification of % methylation.

Figure 3-4

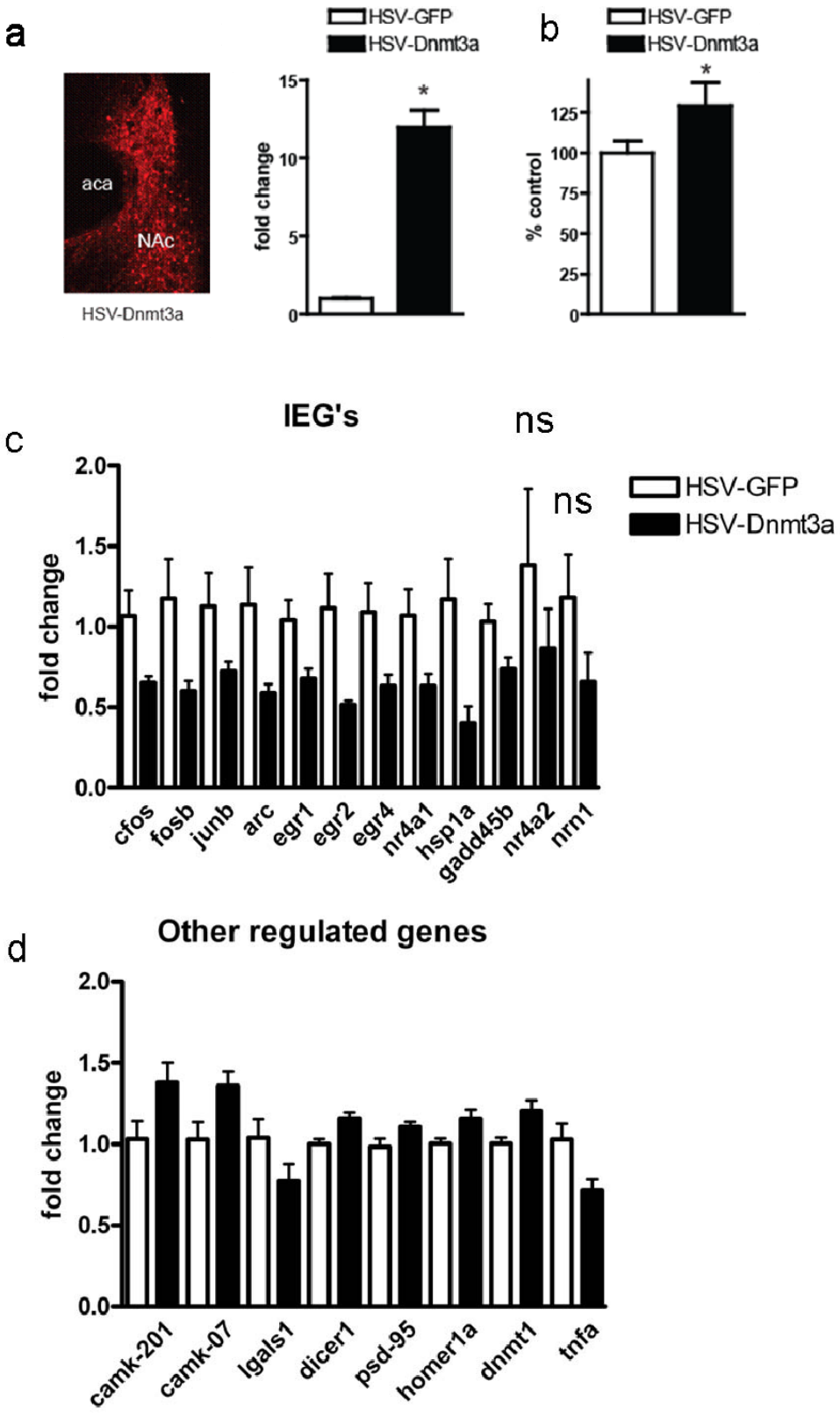

a
HSV-GFP
HSV-Dnmt3a
aca
NAc
HSV-Dnmt3a
fold change
15
10
5
0
*
b
HSV-GFP
HSV-Dnmt3a
% control
125
100
75
50
25
0
*
c
IEG's
ns
ns
HSV-GFP
HSV-Dnmt3a
fold change
2.0
1.5
1.0
0.5
0.0
cfos
fosb
junb
arc
egr1
egr2
egr4
nr4a1
hsp1a
gadd45b
nr4a2
nrn1
d
Other regulated genes
fold change
2.0
1.5
1.0
0.5
0.0
camk-201
camk-07
lgals1
dicer1
psd-95
homer1a
dnmt1
tnfa

Fig. 3-4. Intra-NAc Dnmt3a induced mRNA expression profiling (a) NAc tissue punches were taken from HSV-GFP or HSV-Dnmt3a-GFP positive tissue and mRNA overexpression of Dnmt3a relative to GFP was verified **(b)** global DNA methylation analysis shows that Dnmt3a overexpression increases total NAc DNA methylation levels. **(c)** mRNA expression profiling of 100 genes (see table 3-2) was carried out. Overall, 18 genes were significantly regulated. 10 out of 12 genes in the immediately early gene (IEG) family are significantly downregulated. **(d)** mRNA expression of 8 other genes that were significantly regulated by Dnmt3a overexpression.

Figure 3-5

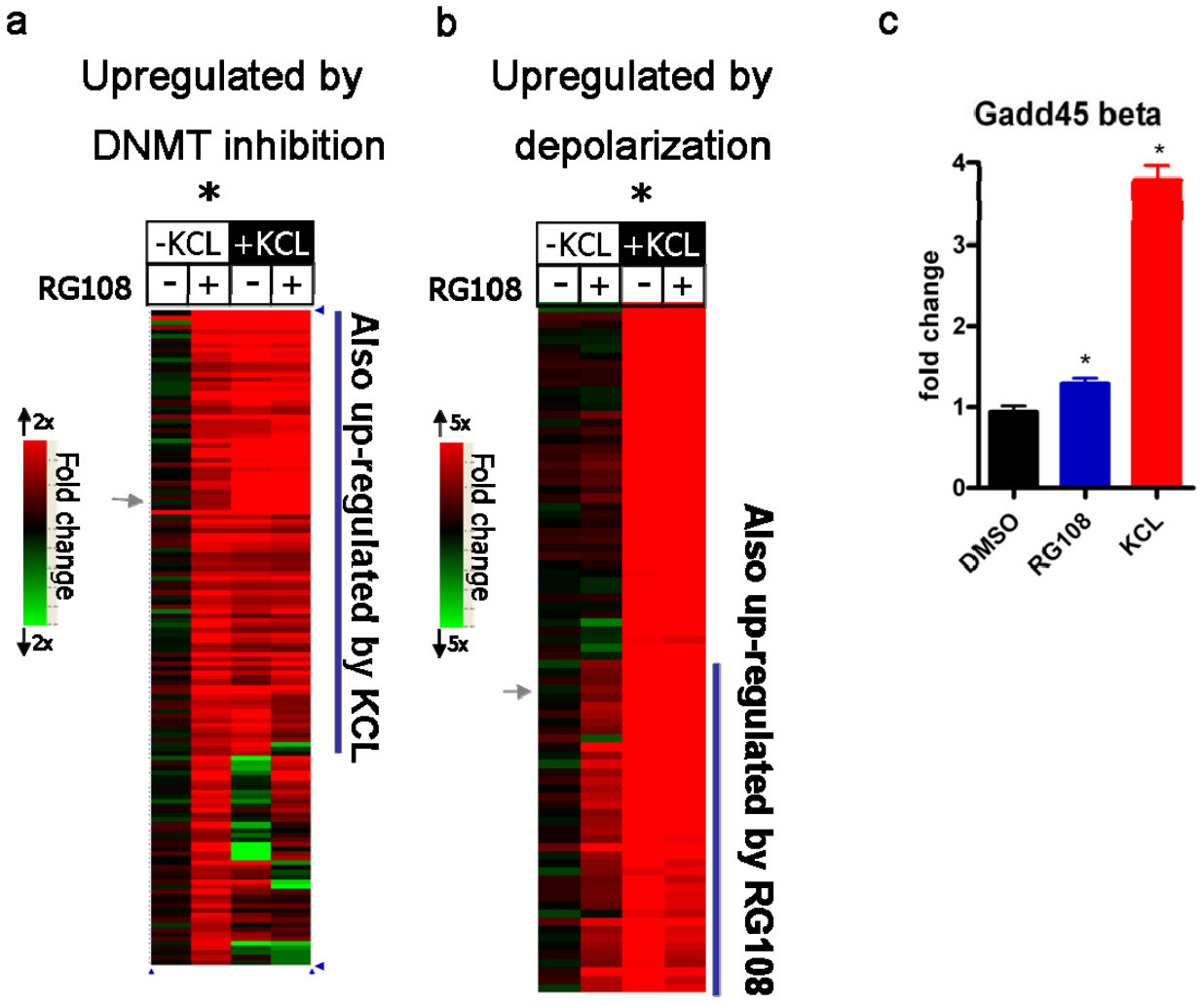

a
Upregulated by
DNMT inhibition
*
-KCL
+KCL
RG108
- + - +
2x
Fold change
2x
Also up-regulated by KCL
b
Upregulated by
depolarization
*
-KCL
+KCL
RG108
- + - +
5x
Fold change
5x
Also up-regulated by RG108
c
Gadd45 beta
fold change
4
3
2
1
0
*
*
DMSO
RG108
KCL

Fig. 3-5. RG108 induced mRNA expression profiling. 100 μM RG108 in 0.2% DMSO or 0.2% DMSO alone were applied to cultured E18 rat striatal neurons. After 24 hours, half of the samples were stimulated under depolarizing conditions (60 mM KCl) for 1 hour. RNA was then extracted and microarray analysis was carried out. **(a)** heatmap of 95 genes significantly upregulated by RG108 (*, FC >1.3x, $p<0.05$) and how these genes are regulated by KCl. More than 65% of these genes are also upregulated by KCl **(b)** heatmap of 131 genes significantly upregulated by KCl (*, FC >2x, $p<0.05$) and how these genes are regulated by RG108. More than 40% of these genes are similarly regulated by RG108 **(c)** Bar graphs of mRNA expression of Gadd45B. A gene that is significantly regulated on both heatmaps, as indicated by a "→" in (a) and (b).

Table 3-1. Candidate methylated genes

Top hypermethylated genes	Fold methylation	Validated fold mRNA expression	bisulfite sequencing validation?
Tph2	1.36		no
Syt4	1.38		
Sst	1.29		
Snca	1.39		no
Slc18a2	1.28		
Scnm1	1.27	0.9 (24hr)	no
Rhoc	1.27	0.91 (24hr)	no
Pml	1.26		
Pdgfrb	1.28	0.9 (24hr)	
Olig3	1.68		
Olfr196	2.46		
Myc	1.80		
Msh5	1.72		
Lmna	1.27	0.92 (24hr)	
Lim2	1.92		no
Lgals8	1.38		no
Lgals6	1.38		
Lgals4	1.38		no
Lgals1	1.31		no
Lcat	1.84		
Kcnu1	1.45		no
Kcnmb1	2.18		
Kcnj6	1.44		no
Kcnh6	1.28		
Htr4	1.29		no
Htr3a	1.29		no
Htr1d	1.33		
Hdac4	1.41		no
Hbp1	1.90		no
Gadd45gip1	1.68		no
fosB	1.31	1.64 (1hr)	no
Chrm4	1.31		
Cebpb	1.26		
Casp6	1.38		
Cart	1.28		no
Capg	1.30	0.85 (24hr)	
Camkk1	1.31		
Camk2a	1.30		no
Adrb3	1.27		

Top hypomethylated	Fold methylation	Validated fold mRNA expression	bisulfite sequencing validation?
Cask	0.79		
Dicer1	0.76		no
Dlgh4 (psd-95)	0.77		
Drd3	0.77		no
Ehmt2 (g9a)	0.73	0.75 24hr	no
Grin2d	0.76		no
Hdac7a	0.71		
Nrxn2	0.75		no
Ntrk2	0.78		no
Pah	0.68		no
Rab36	0.64		
Rorb	0.70	1.15 24hr	no
Strn4	0.65		
Tktl1	0.64		

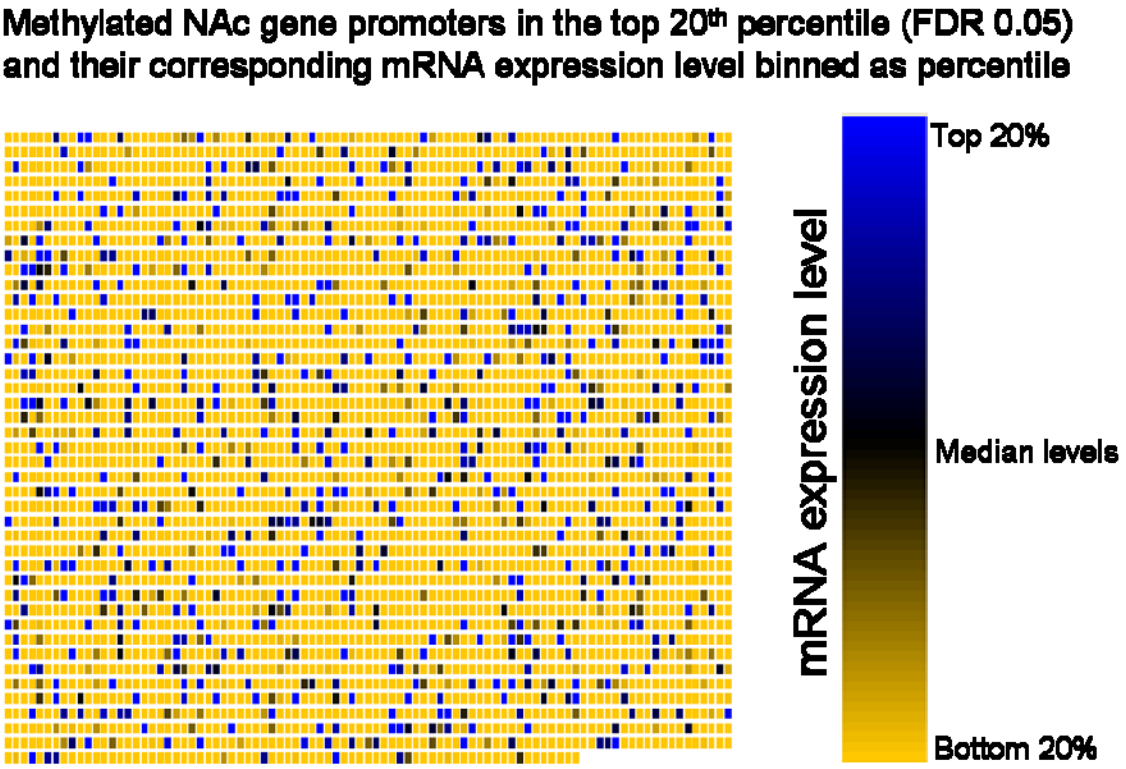
> 1.5 fold cutoff
Also on 1hr or 24hr microarray genelist
Implicated in cocaine reward

Table 3-2. NAc mRNAs analyzed after overexpression of Dnmt3a

Gene family	# genes analyzed	# genes significantly regulated
Metabotropic glutamate receptors	2	0
Ionotrophic glutamate receptors	5	0
Dopamine receptors	3	0
Gaba-a receptors	4	0
serotonin receptors	3	0
GPCR's (other)	2	0
Ion channels	3	0
Spine structural genes	3	2
Actin regulatory genes	6	0
Galnectins (ex: Lgals1)	3	1
Neurotophins/neuropeptides	13	0
Cytokines (EX: Tnfa)	1	1
Kinases (ex: Camk2a)	8	2
miRNA processing (ex: Dicer1)	1	1
Glucocorticoid family	2	0
NFkB signaling	3	0
Creb family	4	0
Histones	2	0
Chromatin modifying enzymes	15	1
Immediate early genes	12	10
Other	5	0
total	100	18

upregulation
downregulation

Suppl figure 3-1



Supplemental Figure 3-1. Comparison of DNA Methylation levels with mRNA expression levels. Displayed here is the mRNA expression level (shown as percentile) from a list of genes that lie in the top 20th percentile of enriched methylated genes (~3900 genes). One can see that the majority of genes promoters that have high methylation also have low mRNA expression.

CHAPTER 4
CONCLUSION

SUMMARY OF FINDINGS

DNA methylation and Behavior

Prior data on the behavioral role of histone deacetylases and repressive histone methyltransferases have found that these histone modifying enzymes attenuate the rewarding effects of cocaine (Kumar et al., 2005; Maze et al., 2010; Renthal et al., 2007; Wang et al., 2010). If transient covalent histone modifications play a role in addiction (a persistent illness), then what is the role of more stable covalent modifications such as DNA methylation? In this thesis, I present data demonstrating that Dnmt3a also reduces cocaine reward as measured by conditioned place preference. Therefore, these data provide the third line of evidence suggesting that mechanisms that promote global gene repression may serve as a therapeutic treatment for addiction. Conversely, the data from this thesis demonstrate the second line of direct evidence that blocking NAc gene repressive mechanisms is antidepressant (the other evidence comes from HDAC inhibitor studies by Covington et al., 2009).

Regulation of DNA methyltransferases

I found that Dnmt3a is persistently upregulated after prolonged cocaine withdrawal (30 days) or chronic social defeat stress (10 days after the last stressor). Contrary to what was expected, I also discovered that Dnmt3a is very

dynamically regulated (both up and down regulated) by the early effects of cocaine. This type of biphasic mRNA regulation (Fig 2-1a) of a chromatin modifying enzyme has never been found in the addiction field, nor has such persistent long-term regulation been observed in the NAc (further detailed in discussion, Chapter 2).

Gene targets

In the face of such complex regulation of Dnmt3a, what are the genomic loci that are regulated by methylation? Carefully standardized whole genome promoter analysis after chronic cocaine injections largely point to a lack of cocaine-induced DNA methylation at gene promoter regions. Two limitations of this experiment and its interpretation (also mentioned below) include the fact that this is only one timepoint in an obviously complex system and that the region analyzed (gene promoters) only assays ~1.5% of the mouse genome. In support of these caveats, it is clear that DNA methylation regulates transcription of specific genes. Using viral mediated overexpression and pharmacological inhibition of DNA methylation I discovered that DNA methylation either directly or indirectly regulates immediate early gene expression. Since immediate early genes represent the first wave of transcriptional activation in response to cocaine these data warrant more thorough DNA methylation analysis at various timepoints and at several genomic loci in and around immediate early genes (particularly at enhancer elements, as detailed in Chapter 3 Discussion).

DNA methylation and neural plasticity

In the addiction field, the most well documented persistent neuronal adaptation is cocaine's ability to increase dendritic spine density in the NAc. Therefore, does DNA methylation influence dendritic spine density? Viral-mediated Dnmt3a overexpression was found to be sufficient to increase dendritic spine density. Moreover, NAc specific pharmacological blockade of DNA methylation demonstrated that methylation is also necessary for cocaine induced increases in spine density. Finally, detailed spine morphology analysis brought a new understanding to the specific types of dendritic spines regulated by cocaine. I found that thin spines—which are thought to represent newly formed, immature spines—are the major subtype of spines regulated by cocaine and importantly, by DNA methylation.

DNA METHYLATION SPECIFIC FUTURE DIRECTIONS

The role of Gadd45β in addiction

As mentioned in the Chapter 1, Gadd45β is a putative DNA demethylase (Ooi and Bestor, 2008). Data in my thesis work have linked this gene to being regulated by cocaine as well as regulated by DNA methylation. Specifically, I have found that expression of this gene is reduced by Dnmt3a overexpression and increased by Dnmt inhibition. Adding to this complexity, I see that Gadd45β is also biphasically regulated by cocaine, with upregulation at 1 hour and

downregulation at 4 hours after a cocaine injection. Of note, this regulatory pattern precedes and inversely correlates with Dnmt3a's expression. Taken together, these data further suggest that cocaine induced DNA methylation is highly dynamic, and opens the possibility of cross-talk between Dnmt3a and Gadd45β. In light of these data, I have obtained both RNAi and overexpression constructs (generous gift Dr. H. Sun, Ma et al., 2009) and it will hence be important to test how manipulating Gadd45β influences cocaine induced dendritic spines and behavior.

Identifying cocaine-induced DNA methylation

As suggested above, I think the best strategy to identifying cocaine-induced methylation is a hypothesis driven approach. This includes careful timecourse and detailed genomic location analysis of the methylation status of immediate early genes such as Gadd45β, FosB, c-Fos, and Arc. In light of recent ChIP sequencing data (Kim et al., 2010), the most likely region of regulation of DNA methylation is in highly conserved enhancer elements. Since the gold standard for methylation analysis, bisifulte sequencing, cannot differentiate between cytosine methylation and the newly discovered brain hydroxymethylation, I do not think it is worthwhile to analyze DNA methylation via bisulfite sequencing, unless proven otherwise. The most convincing approach would be to perform both mDIP experiments in addition to chIP experiments with a methyl-domain binding protein, such as MeCP2. Since I have also found that FosB, c-Fos, and Arc (Gadd45β not assessed) have long-term (1 month

withdrawal) alterations in gene inducibility, it will also be very interesting to look at DNA methylation after long periods of withdrawal at these gene enhancers.

Role of DNA methylation in more long term behaviors

Now that the baseline manipulations of DNA methylation have been performed, the next key step will be to characterize how manipulating DNA methylation after cocaine experience influences cocaine craving and relapse behavior. Importantly, a collaboration with a self-administration lab has already been set up and experiments are underway!

GENERAL FUTURE DIRECTIONS

Regulation of chromatin structure has greatly advanced our basic understanding of the pathophysiology of the addicted and depressed state, and offers a fundamentally new approach for the development of more effective treatments for drug addiction and depression. Behavioral studies to date have left open major avenues of investigation as well as leaving key mechanistic questions unanswered. The hypothesis that 'blocking cocaine's ability to create a more permissive chromatin state serves as a potential therapeutic treatment for addiction' needs further investigation. A promising line of research would be to assess the behavioral function of enzymes that mediate transcriptional activation such as H3K4, H3K36, or H3K79 methyltransferases. To date, the majority of studies have focused on the enzymes that mediate transcriptional repression—such as HDACs, H3K9 methyltransferases, and DNA methyltransferases.

Moreover, despite having similar behavioral outcomes, the gene targets of HDACs, G9a, or DNMT inhibitors remain unknown. It is not known if these manipulations are affecting primarily cocaine-expressed genes (primed or desensitized genes) (Renthal et al., 2007) or completely novel genes—as was found in a recent study comparing target genes affected in the NAc by the HDAC inhibitor MS-275 or by an antidepressant medication (Covington et al., 2009). Furthermore, the degree of cross-talk and gene target overlap of each manipulation is not known. For example, since class III HDACs (sirtuins) regulate excitability of NAc neurons (Renthal et al., 2009), does this extend to other chromatin modifying enzymes such as DNA methyltransferases? Similarly, do manipulations that inhibit DNA methylation also affect histone acetylation, and *vice versa*, as has been shown in the learning and memory field (Miller et al., 2008)?

Similar to behavior manipulations, a more thorough analysis of cocaine's effects on chromatin structure and function demand attention. Two key weaknesses of the published genome-wide ChIP-chip study are that: 1) the immunoprecipitation utilized antibodies that were not specific to individual PTMs, and 2) analysis was localized only to gene promoter regions (an inherent limitation of ChIP-chip). For the next step in gaining insight into why, for example, cocaine globally reduces H3K9me2, it will be crucial to immunoprecipitate with a specific anti-H3K9me2 antibody in a completely unbiased genome-wide setting using ChIP-seq (ChIP-sequencing) technology.

In addition to more thorough genomic analysis and more thorough characterization of novel modifications, two crucial technical challenges exist in linking chromatin function with gene expression. First is differentiating causality from correlation. At best, the presence of a given PTM at a given gene promoter can only be associated with a gene's expression. Such correlation is even further confounded in examples such as the CART gene promoter, which has a bidirectional methylated H3 profile (see Fig. 1-1B). Even with pharmacological or overexpression studies, one cannot conclusively determine whether overexpression of Dnmt3a, for example, directly vs. indirectly influences transcription of the CART gene because such overexpression has global effects. To address this question, one must specifically target DNA methylation to the CART gene promoter. One possible route of accomplishing this very challenging task could be fusing a chromatin modifying enzyme to a protein that specifically binds to the CART gene promoter. This could be accomplished by knocking in specific 'targetable' sequences at a promoter, or perhaps by designing zinc-finger peptides to target specific sequences of DNA. This approach holds promise since it has proven successful in cell culture using a DNA methyltransferase fused to a zinc finger protein (Smith et al., 2008). If successfully applied *in vivo*, this could prove to be an extremely powerful tool in causally establishing the functional influence on transcription as well as providing insight in the *in vivo* half-life of a given PTM.

A second major technical challenge lies in further refining cell specific tools to analyze chromatin state within a given brain region of interest like the

NAc. The importance of this issue has overtones throughout this thesis. In the second chapter, I found that Dnmt3a is subtly (but significantly) regulated by cocaine and stress (**Fig 2-1a**) and, in the third chapter, I was ultimately unable to detect large differences in mRNA or any differences in methylated DNA expression in response to chronic cocaine (**Fig. 3-2a**). Here, I am left with wondering if perhaps *bona fide* regulation could be found in purified types of neurons. Because brain tissue is highly heterogeneous and contains many different classes of neurons and glia, data generated from ChIP and mRNA studies may be a poor reflection of what is actually occurring in specific neurons. Furthermore, because these analyses are carried out on tissue homogenates, it is difficult to ascertain whether differences reflect large changes that occur in absolute levels in a minority of cells, such as neuronal ensembles, or incremental changes that are occurring in a majority of cells in that region. Again referring to the CART promoter example (Fig. 1B), could this bidirectional profile be due to differences occurring in different populations of cells? This scenario is entirely plausible since NAc neurons which express Gs-coupled dopamine D1 receptors have vastly different baseline and cocaine-induced mRNA expression profiles compared to Gi-coupled D2 receptor containing neurons (Heiman et al., 2008; Lobo et al., 2006). Theoretically, since ChIP has been successfully combined with FACS (Flourescence Activated Cell Sorting) of brain tissue (by sorting NeuN+ cells to isolate neurons specifically), it should be possible to ChIP even further purified populations of cells (Jiang et al., 2008).

Altogether, such studies of chromatin open a new window on the molecular basis of drug addiction and depression. The hope is that as novel insight is achieved, it will be possible one day to take advantage of this information to develop better diagnostic tests for addiction and depression and to develop improved treatments and ultimately preventive measures.

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