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Transcriptional and Epigenetic Mechanisms of Addiction

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Preface

Investigations of long-term changes in brain structure and function that accompany chronic exposure to drugs of abuse suggest that alterations in gene regulation contribute importantly to the addictive phenotype. We review multiple mechanisms by which drugs alter the transcriptional potential of genes, from the mobilization or repression of the transcriptional machinery to epigenetics — including alterations in the accessibility of genes within their native chromatin structure and the regulation of gene expression by non-coding RNAs. Increasing evidence implicates these various mechanisms of gene regulation in the lasting changes that drugs of abuse induce in brain, and offer novel inroads for addiction therapy.

Introduction

Drug addiction exacts an enormous medical, financial, and emotional toll on society in the form of overdose and health complications, family disintegration, loss of employment, and crime. NIH's National Institute on Drug Abuse (NIDA) estimates that the total cost of drug abuse in the U.S. exceeds \$600 billion annually, and it is particularly alarming to note a sharp increase in abuse of prescription drugs and in teenage drug abuse in general (www.nida.nih.gov/). These data substantiate the need for increased study of the neuronal effects of drugs of abuse and the mechanisms of addiction in the expectation of uncovering novel targets for treating and preventing addictive disorders.

Although most individuals are exposed to abused drugs, only a subset experience the loss of control over drug use and compulsion for drug seeking and taking that defines the addicted state. Entrance into this state is strongly influenced by both an individual's genetic constitution and the psychological and social context in which drug exposure occurs^{1–3}. Although the genetic risk for addiction is roughly 50%, the specific genes involved remain almost completely unknown. The addictive phenotype can persist for the length of an individual's life, with drug craving and relapse occurring even after decades of abstinence. This persistence suggests that drugs induce long-lasting changes in the brain that underlie addiction behaviors.

The many cells of an individual organism, though they contain essentially identical complements of DNA, differentiate to form distinct tissues and organs through regulated changes in the transcriptional potential of each gene based on environmental cues, cell-to-cell signals, and probably random factors⁴. It is becoming apparent that many of the same processes of gene regulation involved in this normal differentiation of cells and tissues during development are also engaged in the adult organism to mediate cellular adaptation to environmental stimuli^{5,6}. The processes involved in the regulation of transcriptional potential are varied and highly complex, and include activation and inhibition of

transcription factors, modification of chromatin and DNA structure, and induction of non-coding RNAs. Increasing evidence supports the hypothesis that each of these mechanisms of epigenetic regulation is directly affected by drugs of abuse, and that such adaptations are one of the main processes by which drugs induce highly stable changes in the brain that mediate the addicted phenotype. This Review summarizes the findings that support this hypothesis, and highlights areas where future research will extend this fundamental knowledge of addiction and exploit it for new therapeutics.

Drug Action and Gene Transcription

A seemingly equivalent syndrome of addiction can occur with exposure to a wide variety of chemical substances or even rewarding activities, from cocaine to gambling to sex. One common mechanism across these various forms of addiction is thought to be activation of the brain's reward circuitry, which centers on dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and their projections to the limbic system, in particular, the nucleus accumbens (NAc; also known as ventral striatum), dorsal striatum, amygdala, hippocampus, and regions of prefrontal cortex (Figure 1)^{7–9}. This reward circuitry is activated by stimuli or pursuits that promote evolutionary fitness of the organism, like nutrient-rich foods, sex, and social stimulation. As drugs of abuse activate this circuitry far more strongly and persistently than natural rewards, and without association with productive behavioral outcomes, chronic exposure to drugs modulates brain reward regions in part through a homeostatic desensitization that renders the individual unable to attain sufficient feelings of reward in the absence of drug. An alternate, but not mutually exclusive, hypothesis focuses on sensitization, whereby drugs alter the reward circuitry to cause increased assignment of incentive salience to drug cues, effectively making drug-associated environmental stimuli more difficult to ignore and leading to intense drug craving and relapse¹⁰. Pathological drug-induced changes in the reward circuitry further impair behavioral control.

Virtually all rewarding drugs or activities increase dopaminergic transmission from the VTA to the NAc and other target limbic regions, though they each employ partly distinct mechanisms and in some cases involve other neurotransmitter systems as well^{7–9}. The actions of drugs on the NAc are further complicated by the cellular heterogeneity of this brain region (BOX 1). Although drugs differ in their acute mechanisms of action, the common syndrome of addiction suggests that chronic exposure to these distinct acute mechanisms induces some shared molecular adaptations in brain reward regions that mediate the lasting nature of the addictive phenotype.

We and others have long hypothesized that changes in the transcriptional potential of genes, through the actions of transcription factors, chromatin modifications, and noncoding RNAs, contribute importantly to many of the neuroadaptations that result from chronic exposure to drugs of abuse (Figure 2)¹¹. We know that many mRNAs display altered expression in brain reward regions after chronic drug exposure, which suggests that transcription of individual genes is differentially regulated under these conditions. Over the past ~5 years, studies at the chromatin level have confirmed the involvement of such transcriptional mechanisms *in vivo*. Moreover, beyond stable changes in steady-state mRNA levels, this work has demonstrated that the "inducibility" of a gene—its ability to be induced or repressed in response to the next drug exposure or some other environmental stimulus—is also altered by chronic drug exposure and that such gene "priming" or "desensitization" is mediated by stable druginduced changes in the chromatin state around individual genes (Figure 3).

This transcriptional and epigenetic model of chronic drug action provides a plausible mechanism for how environmental influences during development can increase (or

decrease) the risk for addiction later in life. For example, there is mounting evidence that stress during adolescence increases the risk of addiction, and that exposure to drugs *in utero* increases the risk in adolescence and adulthood ^{12,13}. Long-lasting changes in gene transcription or in the potential for transcription that result from early-life stress or drug exposure — mediated at the chromatin level in the absence of genetic differences in primary DNA sequence — might render an adult brain more vulnerable to the addictive process. As alterations in transcriptional potential can last for many years, this model also explains how relapse can occur despite decades of abstinence.

Recent studies of rodent models of addiction have provided considerable support for this hypothesis and have contributed importantly to our understanding of *in vivo* transcriptional and epigenetic regulation in the brain. Here, we highlight key examples of transcriptional and epigenetic mechanisms of drug action, and identify some of the novel potential targets for therapeutic intervention during the addiction process.

Transcription Factors in Addiction

The classic mechanism for regulation of gene expression is through the actions of transcription factors: proteins that, in response to cell signaling pathways, bind to specific sequences of DNA, generally in the promoter or enhancer regions of target genes, and increase or repress their expression by promoting or blocking the recruitment, respectively, of the RNA polymerase-II transcriptional complex. Transcription factors operate as part of large protein complexes, with their mechanisms of action eventually involving alterations in chromatin structure (see below). Although neurons contain hundreds of transcription factors, studies of adaptations induced by drugs of abuse have focused primarily on a small subset.

ΔFosB

 ΔFosB^{14} is encoded by the *fosB* gene and shares homology with other Fos family transcription factors. It heterodimerizes with Jun family proteins to form activator protein 1 (AP-1) complexes that bind to AP-1 sites in responsive genes to regulate transcription. There is some evidence from *in vitro* studies that Δ FosB may also homodimerize 15 . Although all Fos family proteins are induced transiently by acute drug exposure, chronic administration of virtually any drug of abuse induces the long-lasting expression specifically of Δ FosB^{14,16,17}, a process most robust in the NAc and dorsal striatum, but also seen in several other reward-related brain regions including prefrontal cortex¹⁶. ΔFosB induction in the NAc and dorsal striatum by drugs of abuse, whether investigator-administered or selfadministered, occurs only in the subtype of medium spiny neuron (MSN) that expresses D1 dopamine receptors (D1-type MSNs)¹⁴. ΔFosB — a C-terminal truncation of full-length FosB that is generated by alternative splicing — lacks the two degron domains that are present in the full-length protein and conserved among all other Fos family proteins. This absence results in a four-fold increase in protein stability 18 . In addition, Δ FosB is phosphorylated in vivo at serine 27, and at several other sites, and this phosphorylation further stabilizes the protein by roughly 10-fold, both in vitro and in vivo 19,20. This intrinsic and regulated protein stability is a particularly interesting feature of the molecule, as it provides a molecular mechanism by which drug-induced changes in gene expression can persist for weeks after drug intake stops.

 Δ FosB has been linked directly to several addiction-related behaviors. In adult bitransgenic mice where removal of doxycycline induces Δ FosB overexpression specifically in D1-type MSNs of the NAc and dorsal striatum, such induction causes increased locomotor sensitivity to cocaine²¹, increased conditioned place-preference to cocaine and morphine^{21,22}, and increased cocaine self-administration²³. Meanwhile, viral-mediated overexpression studies show that cocaine induction of Δ FosB in orbitofrontal cortex (OFC), a subregion of

prefrontal cortex, mediates the ability of chronic cocaine to induce tolerance to the cognition-disrupting effects of acute drug exposure 24 . Such overexpression also enhances impulsivity during drug withdrawal, and both of these effects further promote drug self-administration 24,25 . Importantly, genetic or viral overexpression of $\Delta JunD$, a dominant negative mutant of JunD which antagonizes $\Delta FosB$ - and other AP-1-mediated transcriptional activity, in the NAc or OFC blocks these key effects of drug exposure $^{14,22-24}$. This indicates that $\Delta FosB$ is both necessary and sufficient for many of the changes wrought in the brain by chronic drug exposure. $\Delta FosB$ is also induced in D1-type NAc MSNs by chronic consumption of several natural rewards, including sucrose, high fat food, sex, wheel running, where it promotes that consumption $^{14,26-30}$. This implicates $\Delta FosB$ in the regulation of natural rewards under normal conditions and perhaps during pathological addictive-like states.

Progress has been made in identifying the broad range of transcriptional targets (some activated, some repressed) through which $\Delta FosB$ these produces various behavioral phenotypes in response to drug exposure 31,32 . By regulating numerous genes related to dendritic spine architecture, including synaptotagmin, microtubule associated proteins, activity-regulated cytoskeleton-associated protein (ARC), actin-related proteins, cyclin-dependent kinase-5 (CDK5), and kinesin $^{31-33}$, among others, $\Delta FosB$ is important for the structural plasticity that is induced in NAc by cocaine $^{34-36}$: it is both necessary and sufficient for cocaine-induced increases in the dendritic spine number of NAc MSNs 37 (BOX 2). As will be seen below, $\Delta FosB$ controls the activity of several other transcriptional and epigenetic regulatory proteins, which then further influence NAc dendritic arborizations, suggesting that $\Delta FosB$ serves as one of the master control proteins governing this structural plasticity. $\Delta FosB$ also regulates proteins that are important for glutamatergic synaptic function and plasticity, including AMPA receptor subunits 21,38 and 24 -calmodulin-dependent kinase II (CaMKII) 31,39 , consistent with the hypothesis that it mediates key aspects of the synaptic plasticity exhibited by MSNs after drug exposure 34,40 .

Though $\Delta FosB$ is far more stable than all other transcription factors linked to addiction to date, drug relapse can occur after decades of abstinence, a timescale dwarfing even phosphorylated $\Delta FosB$'s prolonged turnover rate. The question of whether $\Delta FosB$ can remain stably linked to individual gene promoters for longer periods of time, or whether $\Delta FosB$ can induce longer-lasting changes to the chromatin structure of individual genes (see Gene Priming and Desensitization below), to influence relapse behavior long after total cellular levels of the protein return to baseline remains a major focus for the field.

CREB (cAMP response element binding protein)

CREB forms homodimers that can bind to genes at cAMP response elements (CREs), but primarily activates transcription after it has been phosphorylated at Ser133 (by any of several protein kinases), which allows recruitment of CREB-binding protein (CBP) that then promotes transcription (see below)^{41,42}. The mechanism by which CREB activation represses the expression of certain genes is less well understood. Both psychostimulants (cocaine and amphetamine) and opiates increase CREB activity, acutely and chronically — as measured by increased phospho-CREB (pCREB) or reporter gene activity in CRE-LacZ transgenic mice — in multiple brain regions, including the NAc and dorsal striatum^{41–43}. Experiments involving the inducible overexpression of CREB or a dominant negative mutant in bitransgenic mice or with viral vectors have shown that CREB induction in the NAc, which occurs in both D1- and D2-type MSNs⁴¹, decreases the rewarding effects of cocaine and of opiates^{44,45}, an effect that promotes drug self-administration presumably via negative reinforcement⁴⁶. In contrast to cocaine and opiates, CREB shows more complicated and varied responses to other drugs of abuse or rewards. For example, chronic nicotine⁴⁷ or ethanol^{48,49} administration reduces pCREB levels in the NAc. On the other

hand, CREB activity appears necessary for nicotine to establish a place preference 50 , exposure to $\Delta 9$ -tetrahydrocannabinol (the active compound in marijuana) increases pCREB in the prefrontal cortex and hippocampus 51 , and stimuli associated with natural reward increase pCREB in the NAc 52 . In addition, other CREB family proteins, such as ICER (inducible cAMP repressor) and ATFs (activating transcription factors), have been implicated in the long-term actions of drugs of abuse and require further study 53 .

CREB activity has been directly linked to the functional activity of NAc MSNs. CREB overexpression increases, whereas dominant-negative CREB decreases, the electrical excitability of MSNs⁵⁴. Possible differences between D1- and D2-type MSNs have not yet been explored. The observation that viral-mediated overexpression of a K⁺ channel subunit in the NAc, which decreases MSN excitability, enhances locomotor responses to cocaine suggests that CREB acts as a break on behavioral sensitization to cocaine by upregulating MSN excitability⁵⁴. Numerous target genes for CREB have been identified that mediate these and other effects on NAc MSNs^{31,32,42,44,55}. Prominent examples include the opioid peptide dynorphin which feeds back and suppresses dopaminergic signaling to the NAc^{41,44}, as well as certain ion channels and glutamate receptor subunits which control NAc excitability^{54,55}. It is interesting to compare these effects of CREB in the NAc to similar data from the locus coeruleus, where CREB has also been found to increase neuronal excitability and thereby mediate aspects of drug tolerance and dependence (BOX 3).

NFκB (nuclear factor κB)

NFkB, a transcription factor that is rapidly activated by diverse stimuli, was studied initially for its role in inflammation and immune responses, and linked more recently to synaptic plasticity and memory 56 . NFkB has been demonstrated to be induced in the NAc by repeated cocaine administration, where it is required for cocaine's induction of NAc MSN dendritic spines (Box 2) and sensitization to the rewarding effects of the drug 57 . It has also been associated with nicotine dependence in human populations 58 . A major goal of current research is to identify the target genes through which NFkB causes this cellular and behavioral plasticity. Interestingly, cocaine induction of NFkB is mediated via Δ FosB 14 , illustrating complex transcriptional cascades involved in drug action. The role of NFkB in MSN spinogenesis has recently been extended to stress and depression models 59 , a finding of particular importance considering the comorbidity of depression and addiction, and the well-studied phenomenon of stress-induced relapse to drug abuse.

MEF2 (myocyte enhancing factor-2)

Multiple MEF2 proteins are expressed in brain, including NAc MSNs, where they form homo- and heterodimers that can activate or repress gene transcription depending on the nature of the proteins they recruit (i.e., p300, a coactivator, *vs.* class II histone deacetylases [HDACs], co-repressors [see below]). Recent work outlines a possible mechanism by which chronic cocaine suppresses striatal MEF2 activity in part through a D1 receptor–cAMP-dependent inhibition of calcineurin, a Ca²⁺-dependent protein phosphatase⁶⁰. Cocaine regulation of CDK5, which is also a target for cocaine and ΔFosB as stated earlier³³, may be involved as well. This reduction in MEF2 activity is required for cocaine induction of MSN dendritic spine number, but seems to inhibit behavioral sensitization to cocaine⁶⁰. Although these data suggest that MEF2 plays an important role in the structural and behavioral changes resulting from repeated cocaine administration, they also demonstrate an apparent inconsistency between MSN spine increases and behavioral sensitization to cocaine that merits further study³⁴. Although ethanol has been shown to decrease MEF2 expression in rat cardiomyocytes⁶¹, little is known about the effects of other drugs of abuse on MEF2 function in the brain.

Additional Transcription Factors

The transcription factors listed here are the ones most extensively studied in addiction models, however, increasing evidence links several others to drug exposure, including the glucocorticoid receptor, nucleus accumbens 1 transcription factor (NAC1), early growth response factors (EGRs), and signal transducers and activators of transcription (STATs)^{11,14}. For example, glucocorticoid receptor expression is required in dopaminoceptive neurons for cocaine seeking⁶² but not morphine responses⁶³, and this gene may be associated with initiation of alcohol abuse in teenagers⁶⁴.

Epigenetics of Addiction

Over the past decade, research into the regulation of transcriptional potential through modification of DNA and chromatin structure has exploded. As it became clear that epigenetic change underlies adaptations in the adult organism, investigations of epigenetic mechanisms have proven fruitful in numerous fields, including drug addiction^{65,66}. Here, we introduce three major mechanisms of epigenetic regulation — histone tail modification, DNA methylation, and microRNAs — and summarize the major findings that have linked each of these mechanisms to addiction.

Histone Tail Modification

Most DNA in eukaryotic cells is densely packed in chromatin, where 147 base pairs (bp) are wrapped around a nucleosome core in ~1.7 superhelical turns⁶⁷. Nucleosomes are composed of octamers that contain four histone dimers, one each of histones H2A, H2B, H3, and H4, with H1 binding to spans of non-nucleosomal DNA. Numerous types of posttranslational modifications of the N-terminal tails of histones alter chromatin compaction to create more "open" (euchromatin, which is transcriptionally permissive) *vs.* "closed" (heterochromatin, which is transcriptionally repressive) states⁶⁸ (Figure 3).

Many residues in the tails of histones are covalently modified in numerous ways, resulting in a complex "code" that is thought to control the accessibility of individual genes to the transcriptional machinery⁶⁹. Histone acetylation, which negates the positive charge of lysine residues in the histone tail, is associated with transcriptional activation. This process is controlled by histone acetyltransferases (HATs) and HDACs, each of which comprises multiple enzyme classes whose expression and activity are exquisitely regulated⁶⁷. Histone methylation has been associated with both transcriptional activation and repression depending on the particular residue and the extent of methylation^{70,71}: both lysine and arginine residues can be methylated by several families of histone methyltransferases (HMTs), and this reaction can be reversed by equally diverse histone demethylases. Histone tail modifications also include phosphorylation, ubiquitination, sumoylation, ADP ribosylation, among many others⁶⁷. The prospect of deciphering the histone code is daunting, given the seemingly infinite number of possible patterns of histone modifications, and the possibility that a particular pattern may have varying meaning depending on the individual gene involved. Nevertheless, new tools are accelerating progress in mapping the epigenetic state of individual gene promoters and the genome as a whole, and future research will determine the feasibility of identifying functionally meaningful chromatin codes⁷².

Multiple drugs of abuse induce changes in histone acetylation in brain, and evidence has begun to accumulate that these modifications underlie some of the functional abnormalities found in addiction models^{66,70}. First, global (i.e., total cellular) levels of H3 and H4 acetylation are increased in the NAc after acute or chronic exposure to cocaine^{65,73}, and gene promoters that show increased H3 *vs.* H4 acetylation have been mapped genomewide³². Despite these global increases, many genes show decreased histone acetylation after

chronic cocaine, raising a key question as to what governs gene-specific acetylation changes in the face of global modifications. Another key question concerns the precise intracellular signaling cascades through which cocaine induces changes in histone acetylation — there is some information that such changes may be specific to D1-type MSNs and involve regulation of growth factor-associated kinases^{74,75}. Second, alcohol withdrawal has been demonstrated to increase HDAC activity and reduce histone acetylation in the mouse amygdala⁷⁶, and the commonly abused inhalant benzyl alcohol regulates potassium channels that are tied to alcohol tolerance via H4 acetylation in *Drosophila*⁷⁷. Third, exposure to Δ^9 -THC, the active ingredient in marijuana, increases HDAC3 in trophoblast cells⁷⁸. However, this alteration was absent in a genome-wide screen of brain tissue from Δ^9 -THC-treated mice⁷⁹, demonstrating that experiments on cell lines can yield effects that are very different from those found in a complex heterogeneous tissue like the brain. These data highlight the need for further research to define the effects of drugs of abuse on histone acetylation in brain in a region- and cell type-specific manner and to identify the specific HAT and HDAC subtypes and intracellular signaling pathways that mediate this regulation *in vivo*.

Experimental alterations in histone acetylation potently affect addiction-related behaviors. Short-term administration of non-specific HDAC inhibitors, either systemic or intra-NAc, potentiates place conditioning and locomotor responses to psychostimulants and to opiates^{65,73,80}. More prolonged HDAC inhibition has been reported to induce changes in the opposite direction^{81,82}, perhaps through adaptations that oppose initial enzyme inhibition. Studies of specific HDAC isoforms have yielded interesting information: overexpression of HDAC4 or HDAC5 decreases behavioral responses to cocaine^{73,80}, whereas genetic deletion of HDAC5 hypersensitizes mice to the chronic (but not acute) effects of the drug⁸⁰. Likewise, mutant mice with reduced expression of CBP, a major HAT in brain, exhibit decreased sensitivity to chronic cocaine⁸³. Much additional work is needed to define the influence of specific HAT and HDAC subtypes on addiction-related phenomena.

The potential complexity involved is indicated by recent findings on sirtuins, which are considered Class III HDACs but in reality influence many non-histone proteins. Genomewide studies of chromatin alterations in the NAc after chronic cocaine revealed upregulation of two sirtuins, SIRT1 and SIRT2. Pharmacological inhibition of sirtuins decreases cocaine place preference and self-administration, whereas activation increases rewarding responses to cocaine³². SIRT1 and SIRT2 induction is associated with increased H3 acetylation and increased Δ FosB binding at their gene promoters³², suggesting that sirtuins are downstream targets of Δ FosB. Work is now needed to identify the proteins that are affected by cocaine-induced regulation of these sirtuins. For example, sirtuins deacetylate several transcription factors such as forkhead box (FoxO) proteins, and serve scaffolding functions by contributing to transcriptional repressive complexes^{84,85}, processes which now warrant study in cocaine models. These findings illustrate the ability of genome-wide efforts to identify fundamentally new mechanisms involved in drug action.

Histone methylation is directly regulated by drugs of abuse as well: global levels of histone 3 lysine 9 dimethylation (H3K9me2) are reduced in the NAc after chronic cocaine³⁷ and a genome-wide screen revealed alterations in H3K9me2 binding on the promoters of numerous genes in this brain region³²; both increases and decreases were observed, indicating again that epigenetic modifications at individual genes often defy global changes. The global decrease in H3K9me2 in the NAc is likely mediated by cocaine-induced downregulation of two HMTs, G9a and G9a-like protein (GLP), which catalyze H3K9me2³⁷. These adaptations mediate enhanced responsiveness to cocaine, as selective knockout or pharmacological inhibition of G9a in the NAc promotes cocaine-induced behaviors, whereas G9a overexpression has the opposite effect. G9a likewise mediates the ability of cocaine to increase the spine density of NAc MSNs³⁷ (Box 2). Interestingly, there

is a functional feedback loop between G9a and Δ FosB: Δ FosB seems to be responsible for cocaine-induced suppression of G9a, and G9a binds to and represses the *fosb* promoter, such that G9a downregulation may promote the accumulation of Δ FosB observed after chronic cocaine³⁷. In addition, G9a and Δ FosB share many of the same target genes.

Chronic cocaine also downregulates H3K9me3, a mark of heterochromatin, specifically in the NAc and this change is associated with a decrease in the total amount of heterochromatin in NAc MSN nuclei and an increase in the volume of these nuclei⁸⁶. Genome-wide mapping of H3K9me3 after chronic cocaine indicates that most of the cocaine regulation of this mark occurs at non-genic regions, including at repetitive line elements, which are consequently induced by cocaine⁸⁶. Although the functional implications of this regulation are not yet known, these findings highlight the profound effects that cocaine exerts on the genome within NAc neurons.

Studies are now needed to examine the actions of other drugs of abuse on these histone endpoints, as well as the effect of drugs on many other types of histone modifications known to regulate eukaryotic gene expression in other systems, in addiction models. Examples include recent, preliminary observations of chronic cocaine regulation of histone arginine methylation and poly-ADP ribosylation, of several families of chromatin remodeling proteins, and of histone variant subunits in the NAc, all of which illustrate the complexity of epigenetic changes associated with drug exposure⁸⁷–90.

Moreover, it will be important to relate drug-induced modifications of histones, occurring at specific drug-regulated genes, with the recruitment of numerous additional proteins that ultimately constitute the transcriptional activation or repression complexes that mediate such regulation. For example, early studies have demonstrated that cocaine induction of CDK5 in the NAc involves a cascade of events which include binding of Δ FosB to the *Cdk5* gene promoter, followed by the recruitment of CBP, increased H3 acetylation, and the recruitment of specific chromatin remodeling factors, such as transcription activator BRG1⁷³ (Figure 4). Such activation also involves reduced repressive histone methylation at this promoter, which is mediated via cocaine suppression of G9a. In contrast, a very different cascade mediates chronic amphetamine repression of the c-fos gene. Here, Δ FosB binds to the c-fos promoter and recruits HDAC1 and SIRT1, and presumably numerous other proteins⁹¹. Also, chronic amphetamine induces increased repressive histone methylation at the c-Fos promoter, perhaps mediated via increased G9a binding³⁷. It is interesting that such increased G9a binding occurs despite the global decrease in G9a expression, once again highlighting genespecific changes that occur on top of global modifications. Understanding the molecular basis of such gene-specific modifications — e.g., why ΔFosB triggers a cascade of transcriptional activation when it binds to one promoter, but a cascade of transcriptional repression when it binds to another — is a crucial goal of current research. To date, these efforts have been pursued on a protein-by-protein basis, which is experimentally painstaking. A major need in the field is to develop tools to analyze the complete protein complexes recruited to individual genes in concert with drug exposure.

DNA Methylation

Methylation of DNA occurs at the 5' position of cytosine nucleotides, with the resulting methyl group projecting into the major groove of the DNA double helix 92 . In mammals, this occurs almost exclusively in 5'-CpG-3' sequences and methylation is common throughout the genome — ~3% of all cytosines in human DNA are methylated 93 — with proper cytosine methylation required for normal development, genetic imprinting, and X-chromosomal inactivation 94 . CpG sequences are not evenly dispersed throughout the genome, but rather concentrated in regions termed CpG islands. These are CG-rich regions that overlap with the promoters of 50–60% of human genes and are typically methylated to a

much lower extent than CpG dinucleotides found outside of islands⁹⁵. CpG methylation is catalyzed by a family of enzymes termed DNA methyltransferases (DNMTs), some of which are responsible for maintenance of DNA methyl states whereas others perform *de novo* CpG methylation^{92,93}. The process of demethylation is less well understood, and may utilize DNA repair mechanisms, such as growth arrest and DNA damage-inducible protein GADD45 (Gadd45)⁹³ and methylcytosine dioxygenase Tet1^{96–98}. A variant of DNA methylation, 5-hydroxycytosine methylation, also seems to be important in gene regulation^{99,100} but has not yet been investigated in addiction models.

DNA methylation is generally considered to repress gene transcription through recruitment of corepressor complexes (e.g., HDACs, HMTs) that can sterically hinder the transcriptional machinery or modify nucleosome structure. Such complexes involve several DNA methylbinding domain proteins (MBDs)⁹³, which are required for normal cell growth and development. Indeed, mutations in methyl CpG binding protein 2 (MeCP2), a prominent MBD, cause the majority of Rett Syndrome cases and are found in a small number of patients with other autism spectrum disorders⁹⁴.

There are multiple known links between DNA methylation and addiction. Cocaine self-administration increases MeCP2 expression in the NAc¹⁰¹ and dorsal striatum¹⁰², and lentiviral knockdown of MeCP2 in the dorsal striatum (but not the NAc) decreases drug intake under extended but not limited access conditions⁶⁶. Hypomorphic *Mecp2* mutant mice show reduced locomotor sensitization and place conditioning with chronic amphetamine¹⁰³, however, the same study reported that viral knockdown of MeCP2 in the NAc increases amphetamine place conditioning whereas local overexpression decreases this behavioral response¹⁰⁴. The reasons for this discrepancy are unclear, but it seems likely that developmental abnormalities in the mutant mice, or the effects of reduced *Mecp2* expression in other brain regions, explain these differences. These findings therefore emphasize the importance of utilizing inducible and brain region-specific tools.

Two possible mechanisms for the actions of MeCP2 in drug reward have been proposed. First, a reduction in MeCP2 prevents amphetamine-mediated increases in NAc dendritic spine density while increasing the number of GABAergic synapses ¹⁰³. This is complemented by a GABAergic interneuron-specific increase in MeCP2 phosphorylation in the NAc, which regulates its transcriptional activity and correlates strongly with behavioral sensitization to amphetamine ¹⁰³. An alternative model suggests that MeCP2 represses the transcription of specific microRNAs (see below), resulting in reduced repression of brain-derived neurotrophic factor (BDNF)¹⁰⁵, which is also a target for CREB. BDNF has previously been described to promote cocaine self-administration ¹⁰⁶, consistent with the MeCP2 data. Though these models are not mutually exclusive, further work is necessary to integrate them with our growing understanding of the multiple brain regions and cell types involved in reward behaviors.

A direct link between CpG methylation and addiction involves DNMT3a. Repeated cocaine administration dynamically regulates DNMT3a expression in the mouse NAc, with decreases seen during early phases of withdrawal and sustained increases seen at later time points^{82,107}. Experimental reduction of DNMT3a activity in the adult NAc, achieved either via viral-mediated local knockout in floxed Dnmt3a mice or via local infusion of a DNMT inhibitor, increases behavioral responses to cocaine, whereas DNMT3a overexpression in this region decreases these responses, but also has the paradoxical effect of increasing NAc MSN spine density¹⁰⁷, similar to the effects of MEF2 manipulation in this brain region⁶⁰. A major goal of current research is to identify the specific genes whose methylation status changes in response to chronic cocaine and consequently regulates cellular and behavioral adaptations to the drug.

These observations that chronic cocaine alters DNMT3a and MBDs in the NAc and dorsal striatum raise the possibility that drug-induced changes in DNA methylation might also occur in germ cells and be passed onto to subsequent generations to regulate the propensity of the offspring for addictive behaviors. Such trans-generational transmission of DNA methylation changes and resulting behavioral plasticity remains highly speculative, although recent research has demonstrated robust effects of adult cocaine exposure in rats on cocaine responses in their progeny ¹⁰⁸.

Gene Priming and Desensitization

Ongoing studies of chromatin regulation in addiction models support the view that epigenetic modifications at individual genes, in addition to underlying stable changes in the steady-state levels of mRNA expression of certain genes, alter the inducibility of many additional genes in response to some subsequent stimulus in the absence of changes in baseline expression levels. Although such studies are still in relatively early stages of development, these types of latent epigenetic changes can be viewed as "molecular scars" that dramatically alter an individual's adaptability and contribute importantly to the addicted state.

Such priming and desensitization of genes is evident in a recently published microarray study³⁷. Numerous desensitized genes were identified: $\sim 10\%$ of genes whose transcription is induced acutely in the NAc by cocaine are no longer induced by a cocaine challenge after prior chronic exposure to the drug (Figure 3A). Conversely, numerous genes are primed: genes that are not affected by acute cocaine become inducible after a chronic course of cocaine, with ~ 3 -fold more genes being induced in cocaine-experienced animals. The mechanisms underlying such gene desensitization and priming remain incompletely understood; our hypothesis is that epigenetic mechanisms are crucial (Figure 3B). A subset of primed genes show reduced binding of G9a and H3K9me2 at their promoters in the NAc, suggesting the involvement of this epigenetic mark³⁷. Desensitization of the *c-fos* gene in the NAc, discussed above and depicted in Figure 4, involves stable increases in the binding of Δ FosB, G9a, and related co-repressors, which—although not affecting steady-state levels of *c-Fos* mRNA—dramatically repress its inducibility to subsequent drug exposure⁹¹.

A major need for the field is to now investigate many additional chromatin mechanisms that are recruited by drug exposure to mediate gene priming and desensitization and to understand the detailed mechanisms that target those particular genes. The goal of such studies would be to identify "chromatin signatures" that underlie such long-lasting regulation. The prominence of gene priming and desensitization indicates that studies of steady-state mRNA levels *per se* would miss important aspects of drug regulation that are not captured at the particular time point examined. For example, the aforementioned microarray study³⁷ measured mRNA levels 1 hr after a cocaine challenge, and preliminary evidence suggests that a partly distinct set of genes show evidence of priming and desensitization at 4 hr. These observations highlight the unique utility of genome-wide assays of chromatin regulation, as such assays would reveal priming and desensitization more globally³².

MicroRNAs

Increasing attention has focused on a variety of non-coding RNAs that are important in biological regulation ¹⁰⁹. These include microRNAs, which are generally around 22 bp long, are found in all mammalian cells, and are post-translational regulators that bind to complementary sequences on target mRNAs to repress translation and thus silence gene expression. Like histone modifications and DNA methylation, expression of microRNAs can alter the transcriptional potential of a gene in the absence of any change to the DNA

sequence, and thus can be considered an epigenetic phenomenon. Several recent studies have implicated microRNAs in addiction behaviors, and miRNAs altered by drugs of abuse have been shown to regulate the expression of many proteins strongly linked to addiction ¹¹⁰.

Cocaine self-administration in rats reportedly increases expression of the microRNA miR-212 in striatum, and experimentally increasing miR-212 levels in this region decreases cocaine reward¹¹¹. The actions of miR-212 depend on upregulation of CREB, which is known to decrease the rewarding effects of cocaine (see above), and more recent work demonstrates that MeCP2 may interact homeostatically with miR-212 to control BDNF expression and cocaine intake¹⁰⁵. It has been proposed that this CREB-miR-212-MeCP2-BDNF mechanism is at least partially responsible for cocaine tolerance and escalating intake. miR-124 and miR-181a are also regulated in brain by chronic cocaine, where they are decreased and increased, respectively 112. miR-124 overexpression in the NAc reduces cocaine place conditioning, whereas overexpression of miR-181a has the opposite effect 113. suggesting that drug regulation of these microRNAs may also act as mechanisms of tolerance and escalating intake. Like miR-212, miR-124 and miR-181a may operate through the CREB-BDNF pathway, as miR-124 overexpression downregulates both of these genes. However, these microRNAs have also been shown to affect the expression of the dopamine transporter, so their mechanisms of action are likely to be complex¹¹⁴. Finally, arginine exporter protein ARGO2 — which is important in microRNA-mediated gene silencing along with several specific microRNAs have recently been implicated in cocaine regulation of gene expression selectively in the D2 subclass of striatal MSNs¹¹⁵.

Other drugs of abuse have been linked to microRNAs as well. Opioid receptor activation downregulates miR-190 in cultured rat hippocampal neurons in a beta-arrestin2-dependent manner lift, and the *let-7* family of microRNA precursors is upregulated by chronic morphine exposure in mice lift. Interestingly, the μ opioid receptor is itself a direct target for let-7, and the resulting repression of the receptor has been suggested as a novel mechanism for opiate tolerance lift. In zebrafish and in cultured immature rat neurons, morphine decreases miR-133b expression, and this might influence dopamine neuron differentiation lift. Additionally, both acute and chronic alcohol exposure upregulates miR-9 in cultured striatal neurons, and this may contribute to alcohol tolerance through regulation of large-conductance Ca²⁺ activated K⁺ (BK) channels lift. miR-9 seems to preferentially downregulate BK channel isoforms that are sensitive to alcohol potentiation, perhaps shifting BK channel expression toward more tolerant subytpes lift. miR-9 also targets the D2 dopamine receptor lift, and so probably influences alcohol reward.

In the future, next-generation sequencing of microRNAs in several brain regions after exposure to drugs of abuse will be essential to uncover regulation of specific microRNAs and eventually the genes they regulate. Indeed, this process has already begun, as such screens are revealing numerous mcicroRNAs regulated in the NAc after chronic cocaine ^{115,120}. For example, cocaine regulation of the miR-8 family suggests novel mechanisms for drug-induced alterations in the neuronal cytoskeletal and synaptic structure ¹²⁰. Exploring this mechanism in drug-induced regulation of NAc dendritic morphology is an important line of future investigation.

Future Directions

This Review has summarized the increasing array of findings that support a role for regulation of the transcriptional potential of myriad genes in the brain's maladaptations to drugs of abuse. The mechanisms of transcriptional and epigenetic regulation are themselves varied and highly complex, and future studies are needed to catalogue the vast number of regulatory events that occur as well as to understand the precise underlying mechanisms

involved. Key questions include: What controls the recruitment or expulsion of individual transcriptional regulatory proteins to a particular target gene? Our hypothesis is that the underlying epigenetic state of that gene is a crucial determining factor, but then what controls the formation and maintenance of distinct epigenetic states at particular genes? Also, what are the intracellular signaling cascades that transduce the initial drug action at the neurotransmitter-receptor level to the neuronal nucleus to regulate the epigenetic state of specific subsets of genes?

The existing literature on transcriptional and epigenetic mechanisms of addiction is limited in several key ways. Most studies to date have employed conditioned place preference and locomotor sensitization paradigms. While these behavioral assays provide useful insight into an animal's sensitivity to the actions of drugs of abuse on the brain's reward circuitry, they do not provide direct measures of drug reinforcement or addiction per se. Rather, the field needs to make greater use of drug self-administration and relapse assays, which are considered the best available animal models of addiction 121-123. Likewise, most studies have utilized experimenter-administered drugs of abuse, even though we know that drugs exert some distinct actions when self-administered or given within a particular environmental context. Work is also needed to move beyond the relatively short time frames of most current experiments to examine transcriptional and epigenetic endpoints after much longer periods of drug exposure and longer periods of withdrawal from drug exposure, as well as to extend what has largely been studies of cocaine action in NAc to studies of several other drugs and several other reward-related regions. Future studies of gene regulation will better inform drug discovery efforts as they increasingly incorporate experimental paradigms that better model human addiction.

Another limitation of the existing literature is the reliance of many studies on overexpression systems, viral or transgenic, which often induce levels of expression far greater than those seen under normal conditions or even after drug treatment. Such overexpression of transcription factors, chromatin regulatory proteins, or their dominant negative mutants can lead to artifactual changes in gene expression and subsequent alterations in cell morphology, physiology, or behavior. It is reassuring that many of the phenomena described here that utilize overexpression systems have been validated with other methods: e.g., those genes regulated by overexpression of $\Delta FosB$ in the NAc of inducible bitransgenic mice³¹ overlap extensively with genes that show enrichment of endogenous $\Delta FosB$ binding after cocaine³². Similar caveats exist for the use of constitutive knockout animals, where loss of a gene in early development and in all tissues makes it difficult to interpret any changes observed in drug regulation involving a single brain region of an adult animal. Ultimately, a truly accurate understanding of the transcriptional and epigenetic regulation of the addiction process will require the generation of novel tools that control protein expression with greater spatial, temporal, *and* accumulation precision.

Methodological advances in epigenetics are needed as well. Current levels of experimental proof of epigenetic mechanisms of drug action have to date involved the overexpression or deletion of a given epigenetic protein (HAT, HDAC, HMT, DNMT, etc.) within a brain region of interest. However, such manipulations affect the epigenetic states of perhaps thousands of genes without targeting those genes that are specifically altered by drug exposure. Being able to experimentally manipulate the epigenetic state of an individual gene within a discrete brain region of an adult animal would represent a major advance for the field. Tools such as artificially designed zinc-finger proteins ¹²⁴ or sequence-specific transcription activator-like effectors (TALEs)¹²⁵, designed to bind specific DNA sequences *in vivo*, would offer exciting possibilities for future studies. Similarly, all genome-wide studies of drug-induced epigenetic changes in brain have thus far utilized total extracts of brain regions, even though we know that drugs produce very different effects on distinct

> neuronal and non-neuronal cell types within a given region. Genome-wide epigenetic analyses in a cell type-specific manner are a critical need in addiction research 126.

Advances in bioinformatics are also needed. Genome-wide studies of transcription factor binding and chromatin modifications generate enormous datasets, which require the development of more optimal tools to effectively mine the resulting data. For example, it will be crucial moving forward to overlay such epigenetic analyses with genome-wide changes in RNA expression and to compare data obtained in animal models with those from human postmortem brain tissue. In a similar vein, the studies reviewed here on drug regulation of gene expression must be integrated over several other levels of analysis. How do individual differences in genome sequences relate to individual differences in epigenetic regulation? Do drug-induced epigenetic modifications occur in peripheral tissues such as blood and do any such changes reflect addiction-relevant phenomena? Recent studies, for example, have found altered levels of methylation of the monoamine oxidase-A (MAOA) and MAOB gene promoters in blood of smokers ^{127,128}. Additionally, altered methylation of the MAOA gene is associated with nicotine and alcohol dependence in women but not men¹²⁹, emphasizing the need for studies of sex differences in epigenetic regulation in addiction models, which heretofore have focused almost exclusively on male animals (BOX 4).

As information on transcriptional and epigenetic mechanisms of addiction accumulates, it is essential to integrate it with equally important information regarding posttranscriptional (translational and posttranslational) regulation to obtain a complete understanding of how chronic exposure to a drug of abuse changes the brain to cause addiction. The ultimate goal of this research is to understand basic principles of neuronal and behavioral adaptation and, ultimately, to identify new targets for the treatment of addictive disorders and new methods for their prevention.

Glossary

Conditioned place A behavioral test where animals learn to p	reter an
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environment associated with rewarding drug administration. It preference

provides an indirect measure of drug reward

Degron domain A specific amino acid sequence that indicates the start site for

degradation of a protein via proteasomal or other proteolytic

processes

Dendritic spine A small protrusion from a dendrite that is typically associated

> with synaptic input from a glutamatergic axon at its tip, but which may receive other inputs along its sides or neck

Dependence Altered physiological state that develops to compensate for

persistent drug exposure and that gives rise to a withdrawal

syndrome after cessation of drug exposure

Enzymes that methylate CpG residues in DNA

DNA

methyltransferases

(DNMTs)

Dominant negative

mutant

A mutant molecule that forms heteromeric complexes with the

wild type to yield a non-functional complex

Histone Enzymes that catalyze the acetylation of histone N-terminal acetyltransferases

tails

(HATs)

terminal tails

Histone deacetylases Enzymes that catalyze the deacetylation of histone N-terminal

(HDACs)

Histone demethylases Enzymes that catalyze the demethylation of histone N-

(HDMs)

Enzymes that catalyze the methylation of histone N-terminal

methyltransferases

metnyltransierases

tails

tails

(HMTs)

Histone

Hypomorphic a mutation that causes a wild-type gene product to be

produced at a reduced level

Limbic system A collection of cortical and subcortical structures important

for processing memory and emotional information. Prominent

structures include the hippocampus and amygdala

Medium spiny neurons

(MSNs)

The main cell population of the ventral and dorsal striatum; these GABAergic projection neurons form the two main outputs of these structures, called the direct (D1-type MSNs)

and indirect (D2-type MSNs) pathways

Nucleosome The basic building block of chromatin in which 147 base pairs

of DNA are wrapped (~1.65 turns) around a core histone

octamer

Self-administration A form of operant conditioning using a drug as a reward,

generally by administration through an intravenous line that is

controlled directly by the animal's actions

Sensitization Enhanced drug responsiveness with repeated exposure to a

constant dose

Sirtuins Categorized as Class III histone deacetylases, but serve as

protein deacetylases for many non-histone proteins as well as

part of transcription repressive complexes apparently

independent of catalytic activity

Tolerance Reduced drug responsiveness with repeated exposure to a

constant dose

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Box 1

Cellular Organizaton of Nucleus Accumbens

The nucleus accumbens (NAc) is composed of multiple neuronal cell types (see the figure), with each cell type apparently exhibiting different transcriptional responses to drugs of abuse and mediating distinct aspects of drug reward and addiction. Glutamatergic afferents from the hippocampus, prefrontal cortex, and amygdala, among other regions, excite all subtypes of NAc neurons³⁶, with such excitation differentially regulating drug reward and motivation, as shown by recent optogenetic experiments^{130,131}. These excitatory inputs are modulated by dopamine afferents from the VTA (ventral tegmental area), and psychostimulant drugs such as cocaine and amphetamine act by directly prolonging the effects of these dopamine signals. Excitatory inputs to the NAc are also modulated by endogenous opioid peptides that are both expressed locally and released by input neurons. Opiate drugs thus act directly on NAc neurons that express opioid receptors; they also promote dopamine release in the NAc indirectly by inhibiting VTA GABAergic interneurons. Cannabinoids also have a role in regulating NAc neurons. They act primarily by locally repressing the function of glutamatergic synapses.

Much work is needed to further parse the cellular specificity of drug action in the NAc. 95% of NAc neurons are GABAergic MSNs (medium spiny neurons), which can be further differentiated into those MSNs that express the D1 dopamine receptor (D1-type MSNs) and express dynorphin and substance P and those that express the D2 dopamine receptor (D2-type MSNs) and express enkephalin¹³². Drug induction of ΔFosB^{133,134}, and the effects of ΔFosB and G9a on cell morphology and behavior, differ between D1type and D2-type MSNs¹³⁵, and neuronal activity of these two cell types causes opposing effects on the rewarding properties of cocaine ¹³¹. In addition, acute cocaine causes extracellular signal-regulated kinase (ERK)-dependent phosphorylation of MSK1 (mitogen- and stress-activated kinase-1) and of histone 3 specifically in D1-type MSNs⁷⁵, although the functional consequences of this histone modification are not yet known. In contrast, the effects of cannabinoids seem to predominate at glutamatergic synapses on D2-type MSNs¹³⁶. About 1–2% of NAc neurons are aspiny large cholinergic interneurons, which have been shown to play an important role in cocaine reward¹³⁰, and a similar number are GABAergic interneurons, the function of which are less well understood.

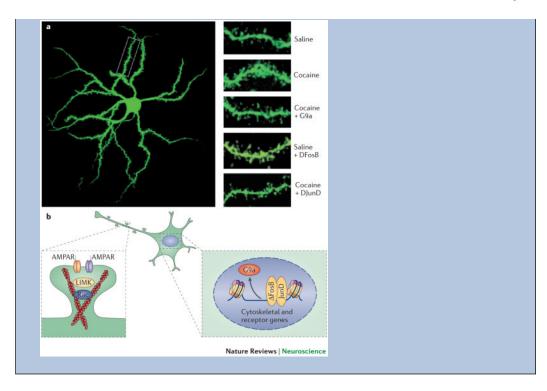
Although these studies are important, to date they have barely scratched the surface of what promises to be an important new focus in addiction research: to overlay the alterations in transcriptional potential of genes induced by chronic exposure to drugs onto the map of cellular subtypes in the NAc.



Box 2

Epigenetic Regulation and Dendritic Spine Plasticity

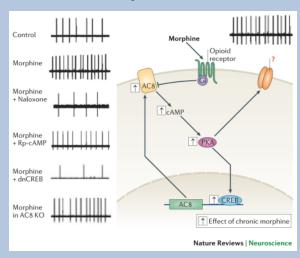
In order for changes in gene transcription and chromatin modifications to affect complex behaviors such as addiction, they must result in some functional output, such as a change in neuronal excitability (intrinsic membrane properties) or connectivity (synapse number or strength). Indeed, it is clear that nearly all drugs of abuse alter the structural connectivity of neurons in the reward circuitry, an effect most evident in changes in the number, shape, and size of dendritic spines on MSNs (medium spiny neurons, as depicted on the left) in the nucleus accumbens (NAc)^{34–36}. These changes seem to be behaviorally relevant, as they correlate with behavioral sensitization ¹³⁷, however, certain conditions that increase spine density cause the opposite behavioral effects^{60,107}. Moreover, the nature of these changes varies with the abused substance, time of withdrawal, and method of intake, even within a single brain region. For instance, experimenteradministered cocaine increases the number of thin spines on NAc MSNs during and shortly after chronic exposure, but increases mushroom spines and dendritic complexity during withdrawal^{34,36}. Moreover, opiates and psychostimulants both induce locomotor activity acutely and locomotor and reward sensitization chronically 138, whereas morphine consistently reduces NAc MSN spine density and complexity^{34,35}; resolving this discrepancy is an important future research goal. It is also likely that structural plasticity of the NAc plays a role in volition and decision-making, as self-administered drugs generally cause larger changes in spine density than the same doses administered by experimenters^{35,36}. Although the molecular underpinnings of these structural changes remain incompletely understood, several factors that control gene transcription and chromatin regulation have been implicated (as depicted in the example dendrites on the right). These include $\Delta FosB^{37}$, cAMP response element binding protein (CREB)⁴⁴, myocyte enhancing factor-2 (MEF2)⁶⁰, G9a³⁷, and DNA methyltransferase-3a (DNMT3a)⁷¹, each of which has been linked directly to cocaine regulation of NAc MSN spine density. A key goal is to now identify how these epigenetic factors control cytoskeletal and cytoskeleton-altering genes to regulate spine morphology and consequently changes in neuronal circuitry and addiction-related behaviors.



Box 3

Morphine Action in Locus Coeruleus: From Gene Transcription to Neuronal Excitability

The locus coeruleus (LC) is the major noradrenergic nucleus in the brain, and it has served as a useful model of opiate action^{11, 139}. Acute morphine decreases the firing rate of LC neurons, whereas chronic exposure to the drug allows the rate to return to baseline (a phenomenon known as tolerance), and withdrawal from morphine causes firing rates to increase dramatically over baseline (a phenomenon that is characteristic of dependence and withdrawal) (see traces on left of figure)^{140,141}. Chronic morphine exerts these effects on firing rate in part by upregulating the cAMP-cAMP response element binding protein (CREB) pathway – including induction of adenylyl cyclase type 8 (AC8) and CREB itself, (upward bold green arrows in the figure). As this pathway is acutely inhibited by the drug, cAMP-CREB upregulation can be seen as a classic negative feedback mechanism. These cellular and molecular effects of chronic morphine are independent of synaptic inputs and can be induced by direct activation of opioid receptors on LC neurons in brain slices ¹⁴¹. Moreover, the proposed role for CREB in LC, which was based originally on overexpression systems, has been validated more recently by the local knockout of endogenous CREB from LC neurons¹⁴¹. Activation of the cAMP-CREB pathway in LC neurons is behaviorally relevant, in that it contributes to symptoms of physical opiate dependence and withdrawal, which are mediated in part by LC activation. These studies establish the molecular details of a transcriptional mechanism of intrinsic homeostatic plasticity involved in the development of opiate tolerance and dependence, which have provided key insight into the chronic actions of opiates and of other drugs of abuse in several other CNS regions, including those directly related to reward, such as the nucleus accumbens and ventral tegmental area 11. PKA, protein kinase A; Rp-cAMP, a competitive inhibitor of cAMP-dependent processes; dnCREB, dominant negative CREB; AC8 KO, AC8 knock-out mouse.



Box 4

Sex Differences in Drug Addiction: Epigenetic Mechanisms?

Addiction research has historically neglected female subjects, particularly at the animal level, although both human and animal studies have found robust sex differences in drug responses 143,144. In self-administration studies with various drugs, female rats are more responsive in general, and exhibit particularly enhanced responses in the transition phases of acquisition or relapse compared to the maintenance phase 145,146. In addition, the locomotor effects of many psychostimulants are greater in female rats 147,148. Although, in general, ovariectomy reduces these differences and estrogen administration increases them, this is not true of all drugs of abuse, and some contradictory results have been reported 143. These data suggest that drugs of abuse have differential effects on the two sexes, and that the reward system may be different between men and women; clinical evidence bears out these hypotheses. For the most part, women have a later age of onset for substance abuse, although they progress to addiction more rapidly than men¹⁴⁹. In the specific case of cocaine, women report shorter periods of abstinence, have greater drug intake, and respond more strongly to cue-induced craving 143. These differences may be directly related to the brain's reward circuitry, as men have been reported to show greater striatal dopamine release than women in response to psychostimulant challenges 150. Interestingly, stress upregulates the expression of DNMTs (DNA methyltransferases) and MBDs (DNA methyl-binding domain proteins) in the NAc¹⁰⁷; these effects predominate in females and inhibition of DNMT3a in the NAc of female rats increases natural reward¹⁵¹, suggesting that the sexes may undergo differential epigenetic regulation of the reward circuitry. Furthermore, as we know that activation of the reward circuitry by sexual behavior induces $\Delta \text{FosB}^{27,29,30}$ and other regulators of transcription, there is little doubt that future studies will reveal further sexual dimorphism in the regulation of transcriptional and epigenetic mechanisms by drugs of abuse, findings which may have important consequences for treatment.

"At-a-Glance" Summary

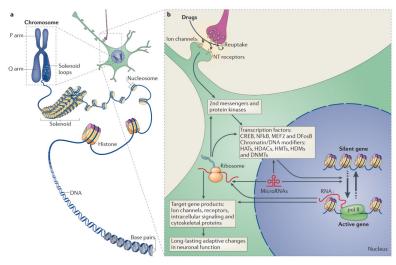
We hypothesize that changes in the transcriptional potential of genes, through
the actions of drug-regulated transcription factors, chromatin modifications, and
noncoding RNAs, contribute importantly to the neuroadaptations that underlie
addiction. This review highlights key examples of such transcriptional and
epigenetic mechanisms of addiction, and identifies some of the novel potential
targets for therapeutic intervention during the addiction process.

- The nucleus accumbens, a region central to the processing of reward and the
 addicting actions of virtually all drugs of abuse, contains a complex milieu of
 cell types and receives input from and sends signals to a variety of brain regions.
 Chronic exposure to drugs of abuse alters gene expression patterns, the
 morphology, and ultimately the functional activity of nucleus accumbens
 neurons, which neuroadaptations which contribute importantly to the addiction
 process.
- Chronic exposure to drugs of abuse alters the expression or activity of numerous transcription factors, including ΔFosB, CREB (cAMP response element binding protein), NFκB (nuclear factor κB), and MEF2 (myocyte-enhancing factor-2). Manipulation of these factors, specifically in the nucleus accumbens or other parts of the brain's reward circuitry, alters specific molecular, cellular, and behavioral responses in rodent models of addiction, which defines the functional role of these factors and their target genes in addiction.
- Epigenetic regulation underlies many adaptations of an adult organism to
 environmental stimuli, such as seen during drug addiction. Posttranslational
 modification of histone tails and direct modification of DNA, as well as altered
 levels or activity of a host of other chromatin remodeling proteins, mediate the
 ability of drugs of abuse, after chronic exposure, to alter the expression of
 specific genes in the brain's reward circuitry.
- Ongoing studies of chromatin regulation in addiction models support the view
 that epigenetic changes at individual genes alter not only the steady-state levels
 of their expression but also their inducibility in response to some subsequent
 stimulus. We propose that these latent epigenetic changes, termed "gene priming
 or desensitization," alter an individual's adaptability and contribute importantly
 to the addicted state.
- Several recent studies have implicated microRNAs in addiction-related behaviors in animal models, and several specific microRNAs, whose expression is altered by drugs of abuse in brain reward regions, have been shown to regulate the expression of several proteins strongly linked to addiction.
- Key questions include: What controls the recruitment or expulsion of individual transcriptional and chromatin regulatory proteins to a particular target gene? What controls the formation and maintenance of distinct epigenetic states at particular genes? How are the actions of drugs of abuse, all of which initially target the synapse, transduced to the neuronal nucleus to regulate the epigenetic state and transcriptional potential of individual genes?



Figure 1. Brain reward circuitry

The brain on the left depicts dopaminergic afferents (light blue arrows) which originate in the ventral tegmental area (blue) and release dopamine in the nucleus accumbens (red) and many other limbic targets. Also shown are other monoaminergic nuclei — the noradrenergic locus coeruleus (green) and serotonergic dorsal raphe (yellow) — which modulate drug reward and other actions. The brain on the right highlights glutamatergic regions that are important for reward: medial prefrontal cortex (green), orbitofrontal cortex (yellow), anterior cingulate cortex (dark blue), thalamus (purple), hippocampus (orange), and amygdala (aqua), all of which send excitatory projections to the nucleus accumbens (red). Drugs of abuse alter this reward circuitry in complex ways, which lead to addiction.



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Figure 2. Mechanisms of transcriptional and epigenetic regulation by drugs of abuse In eukaryotic cells, DNA is organized by wrapping around histone octomers to form nucleosomes, which are then further organized and condensed to form chromosomes (right). Only by temporarily unraveling compacted chromatin can the DNA of a specific gene be made accessible to the transcriptional machinery. Drugs (left) act through synaptic targets to alter intracellular signaling cascades, which leads to the activation or inhibition of transcription factors and of many other nuclear targets including chromatin regulatory proteins; the detailed mechanisms involved in the latter remain poorly understood. This leads to the induction or repression of particular genes, including those for noncoding RNAs; altered expression of some of these genes can in turn further regulate gene transcription. It is hypothesized that some of these drug-induced changes at the chromatin level are extremely stable and thereby underlie the long-lasting behaviors that define addiction. CREB, cAMP response element binding protein; DNMTs, DNA methyltransferases; HATs, histone acetyltransferases; HDACs, histone deacetylases; HDMs, histone demethylases; HMTs, histone methyltransferases; MEF2, myocyte enhancing factor-2; NFκB, nuclear factor κB; pol II, polymerase II.

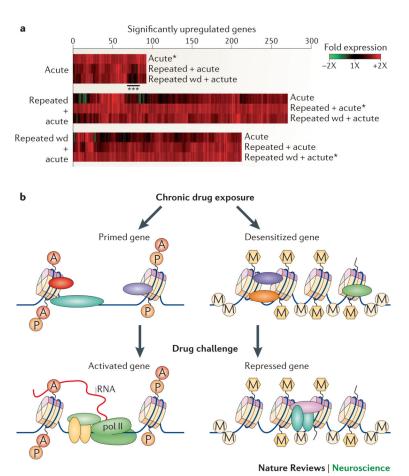
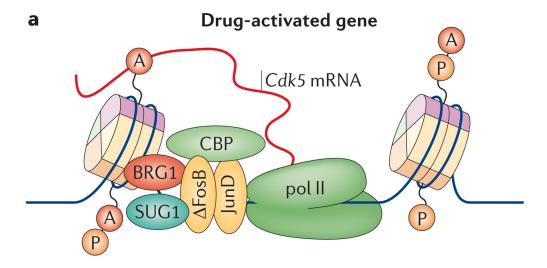
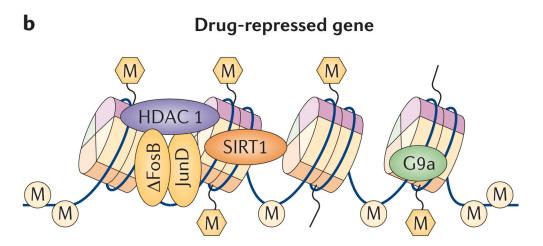


Figure 3. Gene priming and desensitization

In addition to regulating the steady-state expression levels of certain genes, cocaine induces latent effects at many other genes, which alter their inducibility in response to a subsequent stimulus. **A.** Analysis of mRNA expression after acute or chronic cocaine. Heat maps marked with an asterisk (*) show all genes that are upregulated in the NAc 1 hr after a cocaine challenge in naive animals (acute), in animals treated repeatedly with cocaine (repeated + acute), or in animals after 1 wk of withdrawal from repeated cocaine (repeated wd + acute). Associated heat maps show how the same genes were affected under the other two conditions. Desensitized transcriptional responses after repeated cocaine are indicated (***). **B.** Early evidence suggests that epigenetic mechanisms are important in mediating such gene priming and desensitization and that many such changes are latent, meaning that they are not reflected by stable changes in steady-state mRNA levels. Rather, such changes alter chromatin structure such that later drug challenge induces a given gene to a greater (primed) or lesser (desensitized) extent based on the epigenetic modifications induced by previous chronic drug exposure. A major goal of current research is to identify the chromatin signatures that underlie gene priming and desensitization.





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Figure 4. Epigenetic basis of drug regulation of gene expression

The figure is based on the mechanisms by which chronic cocaine, through $\Delta FosB$, activates the cdk5 gene (top) and represses the c-fos gene (bottom). Top: $\Delta FosB$ binds to the cdk5 gene and recruits several co-activators, including CBP (CREB binding protein) — a type of histone acetyltransferase (HAT) leading to increased histone acetylation, transcription factor BRG1 (also known as brahma-related gene 1) — a type of chromatin remodeling factor — and SUG1 (proteasome 26S ATPase subunit 5), another type of chromatin regulatory protein. $\Delta FosB$ also represses G9a expression, leading to reduced repressive histone methylation at the cdk5 gene. The net result is gene activation and increased CDK5 expression. Bottom: In contrast, $\Delta FosB$ binds to the c-fos gene and recruits several corepressors, including HDAC1 (histone deacetylase 1) and SIRT 1 (sirtuin 1). The gene also shows increased G9a binding and repressive histone methylation (despite global decreases in these marks). The net result is c-fos gene repression. As transcriptional regulatory complexes contain dozens or hundreds of proteins, much further work is needed to further define the activational and repressive complexes that cocaine recruits to particular genes to

mediate their transcriptional regulation and to explore the range of distinct activational and repressive complexes involved in cocaine action.