



Published in final edited form as:

Mol Psychiatry. 2022 January ; 27(1): 687–709. doi:10.1038/s41380-021-01163-5.

Key Transcription Factors Mediating Cocaine-Induced Plasticity in the Nucleus Accumbens

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Abstract

Repeated cocaine use induces coordinated changes in gene expression that drive plasticity in the nucleus accumbens (NAc), an important component of the brain's reward circuitry, and promote the development of maladaptive, addiction-like behaviors. Studies on the molecular basis of cocaine action identify transcription factors, a class of proteins that bind to specific DNA sequences and regulate transcription, as critical mediators of this cocaine-induced plasticity. Early methods to identify and study transcription factors involved in addiction pathophysiology primarily relied on quantifying the expression of candidate genes in bulk brain tissue after chronic cocaine treatment, as well as conventional overexpression and knockdown techniques. More recently, advances in next generation sequencing, bioinformatics, cell-type-specific targeting, and locus-specific neuroepigenomic editing offer a more powerful, unbiased toolbox to identify the most important transcription factors that drive drug-induced plasticity and to causally define their downstream molecular mechanisms. Here, we synthesize the literature on transcription factors mediating cocaine action in the NAc, discuss the advancements and remaining limitations of current experimental approaches, and emphasize recent work leveraging bioinformatic tools and neuroepigenomic editing to study transcription factors involved in cocaine addiction.

Introduction: Cocaine-Induced Plasticity in the Nucleus Accumbens

Neurocircuitry analyses in humans and rodents identify the nucleus accumbens (NAc) as a critical brain region involved in mediating the reinforcing and addicting properties of drugs of abuse [1]. The NAc receives convergent glutamatergic inputs from several forebrain regions involved in regulating addictive behaviors, including the prefrontal cortex (PFC), basolateral amygdala, and ventral hippocampus, among other regions, in concert with mostly dopaminergic inputs from the ventral tegmental area (VTA). Medium spiny projection neurons (MSNs) comprise ~95% of the neuronal population in the NAc, with the remaining neurons consisting of several types of local interneurons. MSNs are divided into two main subpopulations depending on their predominant expression of dopamine (DA) receptor subtypes. DA receptor 1-expressing MSNs (D1 MSNs) and DA receptor 2-expressing MSNs (D2 MSNs) exhibit distinct transcriptional profiles [2,3], unique afferent

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Conflicts of interest:

None.

inputs and projection patterns [4,5], as well as different and often opposing contributions to addiction-related behaviors [6–12]. Several studies suggest that a small (<10%) subset of NAc MSNs express both receptor types, but their functioning and regulation is less well understood [3,13,14].

There is now a large literature that defines the effects of repeated cocaine exposure, including drug self-administration and prolonged withdrawal, on the functioning of the NAc and its constituent D1 and D2 MSNs [15–17]. Briefly, numerous adaptations have been characterized at the level of cell excitability, synaptic plasticity (function and morphology), and responsiveness to glutamatergic inputs [4,18–22]. While these neuroadaptations are likely mediated via a wide range of mechanisms, including protein modifications, trafficking, and local translation, increasing evidence supports the view that persistent changes in gene expression are also important contributors, especially to long-lasting drug-induced neuroplasticity that heightens the risk of relapse after prolonged abstinence [23–25].

Transcriptional Mechanisms Underlying Cocaine-Induced Plasticity

There are numerous mechanisms through which repeated exposure to drugs of abuse such as cocaine could induce changes in gene expression in NAc MSNs. The most straightforward scheme is that cocaine, by increasing levels of DA at the synapse, would increase activation of D1 and D2 receptors on MSN subpopulations, which through distinct intracellular signaling pathways would activate or suppress the activity of numerous transcription factors through changes in post-translational modifications or their total expression levels. Transcription factors are proteins that bind to specific sequences of DNA (known as response elements or motifs), usually 6-12 base pairs in length, within the regulatory regions (i.e., promoters and enhancers) of genes and then recruit numerous secondary regulatory proteins to the genes, with the result being the activation or suppression of transcription (Figure 1) [26]. Importantly, transcription factor binding may not affect the steady-state level of a gene's expression but instead alter the transcriptional response of the gene to a future stimulus through gene priming or desensitization [24]. Numerous studies have demonstrated such patterns of cocaine regulation of gene expression. For example, a recent study from our laboratory found that cocaine self-administration alters the expression of many hundred genes in the NAc, based on RNA-sequencing (RNA-seq) analysis of bulk tissue dissections, many of which are sustained after extended withdrawal or even become apparent only after extended withdrawal [27]. Moreover, a cocaine challenge after extended withdrawal results in the induction or suppression of hundreds of genes that are not affected by initial cocaine exposure—a clear demonstration of the prominence of gene priming and desensitization in this brain region in response to a distant history of cocaine self-administration (Figure 2) [27,28].

Studies over the past several decades—beginning in the early 1990s—have shown that rodents exposed chronically to cocaine—either investigator-delivered or self-administered—show differential expression or activity levels of numerous transcription factors in the NAc [25]. Those most studied to date include cAMP response element-binding protein (CREB), FOSB, nuclear factor κ B (NF κ B), and early growth response protein 3 (EGR3) (Table 1A). Cocaine-induced regulation of these transcription factors alters the expression of their

target genes, many of which are involved in long-term synaptic plasticity and modulating addiction-like behavior in rodents. As will be seen, technical advances introduced over the past few years have allowed a more unbiased and therefore comprehensive analysis of transcription factors involved in cocaine action, which is dramatically expanding the range of transcriptional mechanisms implicated in cocaine addiction [27].

In parallel, the field of epigenetics is providing a far more complete understanding of how the binding of a transcription factor to the regulatory region of a target gene influences that gene's steady-state transcription or its potential to be induced or suppressed (i.e., primed or desensitized) in response to a subsequent stimulus. Transcription factors bound to their response elements may recruit hundreds of enzymes that control a host of post-translational modifications of histones or methylation of the DNA itself, as well as numerous other proteins that control the spacing of nucleosomes (composed of histone octomers) along the linear span of DNA (Figure 1). In general, mechanisms that increase nucleosome spacing increase gene transcription, while mechanisms that reduce nucleosome spacing exert the opposite effect. Transcription factors also influence the three-dimensional (3D) structure of chromatin, which controls the activity of a gene in part by bringing enhancers and promoters in close proximity via chromatin looping [29,30]. Knowledge of these epigenetic mechanisms is refining our understanding of how drug-regulated transcription factors actually alter the steady-state or potential expression levels of a given target gene, although the precise changes to chromatin that occur when an active transcription factor binds to a given gene in response to cocaine exposure remain mostly unknown and a major focus of current research. Knowledge of epigenetic mechanisms has also suggested alternative pathways— independent of transcription factors—through which cocaine might alter gene expression, that is, through direct effects on chromatin regulatory proteins. Indeed, experimental manipulations of numerous epigenetic mechanisms, including histone acetyltransferases or deacetylases, histone or DNA methyltransferases or demethylases, and microRNAs (miRNAs) in NAc neurons has been shown to control cocaine-induced changes in gene expression and associated behavioral abnormalities. We refer the reader to several recent reviews of such noncanonical mechanisms of drug-induced regulation of gene expression [23,31–35].

Technical Advancements Improve Insights Into Transcription Factor Function

Historically, studies characterizing the functions of transcription factors in animal models of cocaine addiction have been hindered by the lack of unbiased experimental approaches to identify transcription factors driving cocaine-induced neuroplasticity, quantify genome-wide gene expression and transcription factor binding, and causally define the mechanisms downstream of transcription factor regulation.

First, earlier methods to quantify gene expression—from PCR to gene expression microarrays—are limited by the number of targets they could measure, thus introducing experimental bias by quantifying a small subset of candidate genes. This approach hindered the ability to identify the diversity of transcription factors and related proteins that underlie the lasting actions of cocaine and other drugs of abuse in the NAc and other brain reward regions. More recently, next-generation sequencing has become commonplace in

neuroscience laboratories and enabled the first unbiased, comprehensive (i.e., genome-wide) quantification of gene expression, protein-DNA interactions, epigenetic modifications on histones or DNA, and 3D chromatin architecture, among other properties of chromatin. These technologies are especially relevant for studies of transcription factors. For example, recent studies have employed RNA-seq to measure the effects of transcription factor manipulations on the NAc transcriptome [36,37]. Increasingly, RNA-seq is being applied to isolated subpopulations of neurons and even to individual cells [2,3]. In addition, chromatin immunoprecipitation paired with DNA sequencing (ChIP-seq) enables quantification of the binding of a single transcription factor across the genome. While early ChIP-seq protocols were limited by the requirement for large tissue quantities and high background signal, improved methods such as Cleavage Under Targets and Release Using Nuclease (CUT&RUN) [38] are amenable to quantifying transcription factor binding genome-wide in discrete regions of the mouse brain, even in selected cell types. Assay for transposase-accessible chromatin plus DNA sequencing (ATAC-seq) is a method to quantify open regions of chromatin (euchromatin), in which genes are accessible to transcription factors, chromatin-modifying enzymes, and transcriptional machinery for dynamic control of gene expression [39]. Interestingly, short DNA sequences occupied by transcription factors are depleted in ATAC-seq libraries, allowing researchers to deduce genome-wide transcription factor occupancy based on known transcription factor response elements [40]. Finally, genome-wide chromosome capture (HiC) is a technique that reveals the 3D arrangement of chromatin in the nucleus [41], which contributes to the regulation of gene expression through a variety of complex mechanisms including the formation of chromatin loops and topographically associated domains (TADs), regulating chromosome compartments, and localization within the nucleus [42]. Another technique, called chromatin interaction analysis using paired-end tag sequencing (ChIA-PET), combines genome-wide chromosome capture and ChIP technologies to identify gene segments in close proximity to chromatin-binding proteins, such as transcription factors [43]. Work in other tissues demonstrates that transcription factors regulate gene expression in part by altering chromatin topology [29,30,44], but this phenomenon remains unstudied in NAc MSNs.

Second, work from several laboratories has applied advanced computational approaches to derive more comprehensive biological insight from RNA-seq and other genome-wide datasets. For example, we recently analyzed RNA-seq data across numerous regions of the brain's reward circuitry in animals self-administering cocaine or saline at several time points, namely, early vs. late withdrawal \pm relapse [27]. This study revealed numerous transcription factors that are predicted to drive maladaptive behaviors during cocaine self-administration, many of which have not been studied in brain let alone in addiction-related phenomena. It is reassuring that most of the transcription factors identified in early studies using a candidate gene approach (e.g., CREB, FOSB, NF κ B, and EGR3) were also deduced as being important by unbiased bioinformatic analyses, suggesting that the field succeeded historically in identifying a subset of the transcription factors that are important for cocaine-induced plasticity, but neglected many others [27]. This work highlights the importance of leveraging unbiased, hypothesis-generating computational approaches to study the molecular basis of drug addiction.

Third, studies to characterize the causal relationship between an individual transcription factor binding to a single genomic locus and addiction-related molecular, neurophysiological, and behavioral endpoints have been hampered by the experimental techniques available to study gene function. Traditionally, such studies relied on overexpressing the transcription factor or a dominant-negative mutant, RNA interference (RNAi)-mediated suppression of RNA translation, or Cre recombinase-mediated knockout of the gene to study its downstream effects. However, these techniques—even when induced in the adult brain in a cell-type-specific fashion—alter total cellular levels of the transcription factor and therefore its binding at hundreds or thousands of genomic locations, which limits mechanistic insight for any individual target gene [45]. Previous studies have also shown significant off-target effects of RNAi, which remains a commonly used approach to study transcription factors in the brain, that further hinder insight into transcription factor function [46]. Additionally, these techniques typically induce changes in the expression levels of transcription factors that are far greater in magnitude than the changes observed in response to drugs of abuse, which may have distinct effects on downstream experimental measures than more physiologically relevant changes in gene expression [47]. The evolving field of locus-specific neuroepigenomic editing offers a powerful set of tools to vastly improve the quality of proof in studies of transcription factor function by selectively recruiting a transcription factor to its response element at a single target gene within a single type of neuron in a given brain region of interest (Box 1) [47–54]. Although still in early stages of development, this approach far more accurately establishes causal relationships between transcription factor binding at single gene and outcome measures at the transcriptional, molecular, cellular, circuit, and behavioral levels.

Integrating genome-wide next-generation sequencing approaches, cell-type-specific analyses, bioinformatic approaches, and neuroepigenomic editing represents an important frontier in elucidating the molecular basis of drug addiction. In the following sections, we synthesize the current literature on transcription factors driving cocaine-induced plasticity in the NAc, discuss new insights into the molecular mechanisms downstream of transcription factor regulation, and delve into the emerging literature using advanced computational methods and neuroepigenomic editing to delineate the function of novel transcription factors important in drug action.

Candidate Transcription Factors Mediating Cocaine-Induced Plasticity

CREB—CREB is a ubiquitously-expressed transcription factor implicated in diverse functions in the nervous system, including neurodevelopment, learning and memory, and neuronal plasticity [55,56]. CREB regulates the expression of several thousand target genes by forming homodimers and binding to cAMP response elements (CREs). It is generally thought that CREB is bound to target genes in its inactive state and, in response to a stimulus, phosphorylation of CREB at serine 133 enables the recruitment of CREB-binding protein (CBP), a histone acetyltransferase, and of numerous other regulatory proteins that ultimately facilitate CREB-mediated transcription. Notably, a recent study challenges this notion by demonstrating that transgenic mice lacking the serine 133 phosphorylation site retain certain CREB functions in the hippocampus [57]. These data suggest that the function of CREB phosphorylation at serine 133 may be context-dependent. Nonetheless, several

protein kinases phosphorylate CREB at serine 133, including protein kinase A (PKA), Ca²⁺/calmodulin-dependent kinase IV (CaMKIV), and ribosomal S6 kinase (RSK), the latter being downstream of extracellular signal-regulated kinase (ERK) signaling [56]. Conversely, CaMKII phosphorylates CREB at serine 143, which inhibits CREB function by dissociating the CREB homodimer and precluding CBP recruitment [58]. An interesting and as yet unanswered question is the upstream signaling pathway (i.e., cAMP, Ca²⁺/calmodulin, or BDNF-TrkB-ERK-RSK) that mediates cocaine or other drug-induced phosphorylation and activation of CREB in the NAc (Figure 3).

CREB was first implicated in drug addiction based on its known role as an effector for cAMP signaling and on evolving evidence that the cAMP pathway is upregulated in several brain regions in response to psychostimulant or opioid drugs of abuse [59]. Chronic cocaine administration was demonstrated subsequently to increase CREB activity within the NAc (Table 1A), with this induction mediating tolerance- and dependence-like effects to cocaine's behavioral actions (Table 2A). Specifically, transgenic- or viral-mediated overexpression of wildtype or constitutively active CREB (i.e., CREB^{S133D}) in NAc neurons was found to reduce rewarding responses to cocaine and elicit a negative emotional state, whereas overexpression of a dominant negative mutant form of CREB (i.e., CREB^{S133A}) exerted the opposite effect [45,55,60]. CREB induction in NAc neurons was also shown to drive increased cocaine self-administration and relapse presumably through a negative reinforcement mechanism [61], while antisense-mediated knockdown of CREB decreased cocaine self-administration [62]. One study using transgenic mice expressing CREB^{S133A} under the control of the D1 receptor promoter suggests that the tolerance-like effects induced by CREB activation are driven at least in part by D1-expressing neurons [63]. Effects of CREB in D2 MSNs, where it is also induced by drug exposure, have not yet been examined. CREB activation has been shown to play a similar negative reinforcement role with respect to opioid addiction [55,64], although its role for other drugs of abuse is not yet established with certainty.

CREB regulation in other brain regions.: Cocaine-induced regulation of CREB activity is observed in many additional brain regions. Specifically, previous work shows that chronic cocaine regulates the phosphorylation state of CREB in the dorsal striatum, basolateral amygdala, and central amygdala [65–67]. CREB phosphorylation in the CA1 and CA3 regions of the hippocampus remained unaffected. Another study demonstrated that chronic amphetamine administration regulates CREB activity in the olfactory bulb, thalamus, hypothalamus, motor cortex, somatosensory cortex, hippocampus, and amygdala, among other regions [68,69]. Although the functions of CREB in these brain regions have not been investigated in addiction models, one study investigated the effect of manipulating CREB activity in the VTA on cocaine reward [70]. CREB overexpression in the rostral VTA increases the rewarding effects of cocaine, while overexpression of mCREB induces an aversive response. Surprisingly, overexpression of CREB or mCREB in the caudal VTA has the opposite effect on cocaine reward. These data suggest that CREB oppositely regulates drug reward within subregions of the VTA. One possible explanation for this phenomenon is that transduced neurons in the caudal VTA were predominantly dopaminergic, which project

to the NAc and other forebrain structures, whereas in the rostral VTA the transduced cells were mostly GABAergic interneurons.

CREB target genes.: Earlier work relied on the use of microarrays to identify genes in NAc that are induced or suppressed upon overexpression of wildtype or dominant-negative mutant CREB [45], or gene promoters that bind endogenous CREB after chronic cocaine administration [71]. Many of the implicated genes suggested an important role for CREB in modulating glutamatergic neurotransmission. CREB facilitates the induction of the NMDA receptor (NMDAR) subunit 2B (NR2B) after chronic cocaine administration, which is necessary for the formation of silent synapses [72,73]. In addition, CREB increases brain-derived neurotrophic factor (BDNF) expression, a neurotrophin that binds to tropomyosin receptor kinase B (TRKB) and regulates intracellular signaling [74]. BDNF-TRKB signaling in NAc promotes cocaine intake [75] and increases expression of the AMPA receptor (AMPA) subunit, GLUA1, leading to increased expression of Ca²⁺-permeable AMPARs and to LTP induction at silent synapses [76]. As discussed previously, these molecular adaptations are essential for the development of incubation of craving during drug withdrawal. CREB was also shown to increase the intrinsic excitability of NAc MSNs, which was directly linked to decreased locomotor sensitization to cocaine and which is consistent with the hypothesis that CREB activation promotes drug tolerance and dependence [55,77]. Together, these studies suggest important molecular mechanisms by which CREB regulates behavioral responses to cocaine.

Another CREB target gene is prodynorphin (*Pdyn*) [60], which encodes an endogenous opioid peptide associated with the dysphoria experienced during withdrawal from drugs of abuse [78]. *Pdyn* was first considered as a CREB target in addiction models based on its known regulation by CREB in other systems and by the presence of a well-characterized CRE site in its promoter. Chronic cocaine was shown to induce dynorphin expression in NAc [60], an effect selective for D1 MSNs where *Pdyn* is expressed selectively [79]. Dynorphin is thought to exert its dysphoric effects by engaging in a negative feedback loop between D1 MSNs in the NAc and dopaminergic projections from the VTA [80]. According to this scheme, cocaine increases CREB-mediated dynorphin expression; dynorphin is then released onto DA neurons in the VTA where it activates G_{i/o}-coupled κ opioid receptors (KORs) and attenuates DA neuron firing. This mechanism is supported by data showing that intra-cerebroventricular administration of a KOR antagonist is sufficient to block the reduction in cocaine reward caused by CREB overexpression [60].

These earlier studies of CREB function and its target genes relied on the use of CREB overexpression and knockdown techniques, which fall short in defining causal relationships between CREB binding at a single target gene and downstream experimental measures. This is because these approaches alter CREB binding at hundreds or thousands of genes, as noted earlier [45]. Recent work from our laboratory developed a clustered regularly interspaced short palindromic repeats (CRISPR)-based neuroepigenomic editing tool that enables targeted recruitment of the constitutively active mutant of CREB (CREB^{S133D}) to a CRE site at a single target gene in a defined cell population in awake, behaving animals (Box 1) [37,47,48]. Targeting CREB^{S133D} to any of several target genes that contain a CRE site induces that gene selectively, an effect not seen using a CREB mutant lacking the serine

133 phosphorylation site (CREB^{S133A}), highlighting the specificity of this approach. Similar tools could be developed for any other transcription factor and used to study its downstream molecular mechanisms, but this has not yet been achieved. The earlier studies of CREB also fall short of defining the cellular specificity of CREB regulation and action, relying on overexpression or knockdown approaches that target all NAc neurons and on analysis of target genes in homogenized NAc tissue. A major need moving forward is greater attention to cell-type-specific analyses to define, for example, CREB targets selectively in D1 and D2 MSNs (Box 2).

FOSB— FOSB is a truncated variant of the full-length FOSB protein, generated by alternative splicing, which has been shown to be an important component of the transcriptional and behavioral responses to drugs of abuse [31,81]. The FOS family of transcription factors (cFOS, FOSB, FRA1, and FRA2) form heterodimers with members of the JUN family (cJUN, JUNB, and JUND) to form activator protein 1 (AP1) complexes that regulate transcription at their respective target genes. FOSB was first identified as being the only FOS or JUN family transcription factor that is induced in NAc in response to chronic cocaine exposure [82]. Since this initial demonstration, FOSB has been demonstrated to be induced in NAc by several classes of drugs of abuse (Table 1A) [83], and this induction is selective for D1 MSNs except for opioids which induce the protein equally in D2 MSNs as well (Table 1B) [84]. Elevated FOSB protein levels are also observed in the NAc of human patients with cocaine or opioid addiction [85,86]. Drug induction of FOSB is highly complex, with four transcription factors implicated: CREB, EGR3, serum response factor (SRF), and E2 factor 3a (E2F3a) (Figure 4) [87–89]. All four factors bind to response elements within a small upstream promoter region and there is evidence that they work cooperatively to induce the *FosB* gene. Additionally, several chromatin mechanisms have been identified to occur in concert with *FosB* gene induction, including dynamic regulation of histone acetylation and methylation [28,90–93] and DNA methylation [94]. In fact, the *FosB* gene is one of the most robustly primed genes after prolonged withdrawal from cocaine; identifying the “chromatin scars” that persist at *FosB* during withdrawal to mediate this priming is a high priority of current research. There is no evidence for cocaine regulation of *FosB* splicing. Rather, the progressive accumulation of FOSB protein, in the absence of accumulation of full-length FOSB, observed during chronic drug administration is attributed to its unique stability. FOSB lacks the degron domains present in full-length FOSB and all other FOS family proteins, which reduce its targeting for proteasomal and other proteolytic degradation [95]. The half-life of FOSB is increased further by its phosphorylation at serine 27, and perhaps other residues, by casein kinase 2 and CaMKII (Figure 3) [85,96].

Experiments that overexpress FOSB, or a dominant negative mutant of a JUN protein (cJUN or JUND) that antagonizes AP1-mediated transcription, in NAc neurons—by use of inducible genetic mutant mice or viral-mediated gene transfer—indicate that drug-induced accumulation of FOSB in D1 MSNs increases an animal’s sensitivity to the rewarding effects of cocaine and other drugs of abuse, increases cocaine self-administration, and promotes relapse, presumably through positive reinforcement (Table 2B) [31,85,97–101]. The functional consequences of FOSB induction in D2 MSNs, which occurs in

response to opioid drugs alone or to chronic consumption of natural rewards [84], remains a subject of active investigation. More recent work, utilizing neuroepigenome editing tools to control the expression levels of endogenous FOSB and thereby avoid the confounds of conventional overexpression approaches (Box 2), have validated some of these findings [102]. In any event, the unique temporal features of FOSB—as a stable product of an immediate early gene, along with its role in promoting drug reward and intake, support the notion that it serves as a “molecular switch” towards an addiction-like state [103].

FOSB regulation in other brain regions.: While the majority of studies on FOSB in addiction models have focused on the NAc, drugs of abuse induce FOSB expression in many other brain regions [83]. For example, chronic cocaine elevates FOSB expression in the PFC, orbitofrontal cortex (OFC), dorsal striatum, hippocampus, hypothalamus, amygdala, and VTA [83,104,105]. Among these brain regions, the functions of drug-induced FOSB are most well-characterized in the OFC and hippocampus.

Cocaine self-administration increases FOSB expression in the OFC [106], which is associated with increased impulsivity, cocaine intake, and locomotor response to cocaine [107,108]. An interesting difference between cocaine induction of FOSB in OFC vs. NAc is that drug self-administration causes much greater FOSB induction in OFC compared to forced-drug administration, whereas in NAc equivalent induction is seen with volitional and non-volitional intake. In any event, these results show that FOSB activation in the NAc and OFC increases addiction-like behaviors [99,107,108], suggesting that pharmacological inhibition of FOSB may be an effective treatment for cocaine use disorder (see Transcription Factors as Therapeutic Targets for Cocaine Use Disorder). Microarray analyses point toward many putative FOSB target genes in the OFC that could mediate these behavioral effects, including those encoding mGLUR5, GABA_A receptor subunits, and substance P [106].

In the hippocampus, FOSB is induced by cocaine and involved in learning and memory [109]. By contrast, human patients with cocaine addiction show decreased levels of FOSB and FOSB, as well as several FOSB target genes, in the hippocampus relative to matched control subjects [110]. Knockdown of FOSB-mediated transcription using JUND in this brain region is associated with decreased cocaine CPP, possibly by impairing the paired context association rather than reducing the reward salience of cocaine [105].

FOSB target genes.: As with CREB, earlier work used microarray techniques, in combination with FOSB or mutant cJUN/JUND overexpression in NAc neurons, to identify FOSB target genes [45,71]. These studies suggest that FOSB facilitates the cocaine-mediated induction of the GLUA2 AMPAR subunit in NAc MSNs [111]. Increased GLUA2 expression would be expected to reduce the number of Ca²⁺-permeable AMPARs and thus decrease MSN excitability, which is observed after FOSB overexpression in D1 MSNs [100]. This finding is consistent with evidence that FOSB induction is necessary and sufficient for the formation of immature spines in D1 MSNs in response to chronic cocaine exposure [28,85,100], which as stated above are a postulated required substrate for longer-term drug-induced behavioral plasticity. However, the range of target genes through which FOSB controls spine growth and morphology remains mostly unknown.

In parallel, microarray data pointed to hundreds of genes potentially being influenced by FOSB [45,71]. Some interesting examples include cyclin-dependent kinase 5 (CDK5), NFκB, CaMKII, G9a (a repressive histone methyltransferase), and sirtuin 1 (SIRT1), all subsequently validated as bona fide targets that control neural and behavioral responses to cocaine [28,85,112–114]. FOSB was also shown to suppress the induction of *cFos* in the NAc by recruiting histone deacetylase 1 (HDAC1) to the *cFos* promoter [115], which could contribute to the molecular switch—selective induction of FOSB over other FOS family proteins—in the chronic drug-treated state. HDACs catalyze the removal of acetyl groups from histones and other proteins.

Recent work from our laboratory seeks to capture more physiologically-relevant target genes for FOSB in a more comprehensive fashion by utilizing CRISPR-mediated, locus-specific neuroepigenomic editing to induce endogenous FOSB selectively in D1 MSNs, D2 MSNs, or both cell types and identify all regulated transcripts using RNA-seq [37]. This approach takes advantage of the tool, outlined above, that targets CREB^{S133D} to a single target—in this case *FosB*—and characterizes the downstream consequences. This study demonstrated partly distinct gene targets for FOSB in D1 vs. D2 MSNs, with large differences in gene targets seen in each cell for male vs. female mice as well. A major need for the field is to now overlap this RNA-seq approach with direct measures of endogenous FOSB binding to its genomic targets genome-wide. Such measures have proven challenging technically using ChIP-seq but appear more feasible with CUT&RUN given the latter method's greater sensitivity and selectivity [116].

Among the aforementioned gene targets for FOSB are several that themselves control gene expression (e.g., those encoding cFOS, NFκB, G9a, and SIRT1), which underscores the complexity of transcriptional regulation (Figure 3). For example, cocaine and morphine increase FOSB binding at the *Sirt1* gene promoter, a class III HDAC, in the NAc, which is associated with increased *Sirt1* gene expression in this brain region [112]. Pharmacological activation or overexpression of SIRT1 in NAc neurons increases cocaine-induced reward, locomotor sensitization, and dendritic spine density [71,112,117]. The target genes of SIRT1 were identified using ChIP-seq. This work revealed the forkhead transcription factor (FOXO) family as important SIRT1 target genes [117], which we will describe later in this review.

While this review focuses on NAc MSNs, it is worth highlighting that local interneuron populations also regulate the behavioral response to cocaine and influence neuronal plasticity at NAc MSNs, but the cocaine-induced transcriptional mechanisms within these cell populations remain poorly understood [118–121]. One study investigated such mechanisms in the somatostatin-expressing interneurons within the NAc by RNA-seq and deduced AP1-mediated transcription as a major upstream regulator of cocaine's effects on gene expression in this cell type. In particular, repeated cocaine selectively induced JUND, without induction of any other FOS or JUN family member [121]. Viral-mediated overexpression of a dominant negative mutant of JUND selectively in this cell type of NAc was shown subsequently to reduce locomotor responses to cocaine. This finding underscores a major conclusion of this review, which is the need for more cell-type-specific investigations of transcription factor action in drug abuse models (Box 2).

EGR3—The EGR family of transcription factors (EGR1-4) are products of immediate early genes that are implicated in synaptic and behavioral plasticity. This involvement prompted their study in addiction models where EGR3 was found to be induced in the NAc by chronic cocaine administration [122]. In this brain region, EGR3 is responsive to intracellular pathways downstream of DA and BDNF signaling, both of which are elevated by cocaine [89]. Subsequent investigation demonstrated that chronic cocaine increases EGR3 expression in D1 MSNs but reduces it in D2 MSNs (Table 1B) [89]. The regulation observed in D1 MSNs may be mediated by FOSB, which preferentially accumulates in D1 MSNs and exhibits increased binding at the *Egr3* promoter following chronic cocaine treatment [71], although direct functional validation of EGR3 regulation by FOSB has not yet been demonstrated. EGR3 overexpression in D1 MSNs in the NAc increases cocaine reward and locomotor sensitization, while overexpression in D2 MSNs has the opposite effect (Table 2B) [89]. Conversely, miRNA-mediated knockdown of EGR3 in D1 or in D2 MSNs reverses these behavioral phenotypes. Interestingly, EGR3 overexpression in D2 MSNs in the NAc delays extinction and promotes relapse-like behavior in male mice, whereas this manipulation increases extinction and attenuates relapse-like behavior in female mice [123]. This latter finding highlights a major need in the field, namely, to examine sex differences in transcriptional regulation in drug abuse models, something that has received relatively little attention to date. The data also indicate cell-type-specific actions of EGR3 in the NAc, further emphasizing the importance of greater focus on this variable in future studies of transcription factors (Box 2).

EGR3 target genes. Multiple synaptic plasticity-related proteins, transcription factors, and chromatin-modifying enzymes implicated in cocaine-induced plasticity exhibit altered EGR3 binding in the NAc after chronic cocaine administration [89,124]. Specifically, chronic cocaine increases EGR3 binding at the promoters of genes encoding CaMKII α , CREB, FOSB/ FOSB, SIRT1, nuclear receptor subfamily 4 group A member 2 (NR4A2), and peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC1 α). These same genes show increased expression in D1 MSNs following repeated cocaine administration. The promoter region of the gene encoding PGC1 α , a transcriptional coactivator, contains binding sites for multiple transcription factors discussed in this review, including CREB, FOSB, NF κ B, EGR3, and MEF2, which underscores the complexity and cooperative nature of transcriptional regulation [124]. PGC1 α overexpression in D1 MSNs in the NAc elevates cocaine reward and locomotor sensitization, while overexpression in D2 MSNs has the opposite effect [124].

By contrast, cocaine administration decreases EGR3 binding at the promoters of G9a and DNA methyltransferase 3a (DNMT3a), which is associated with reduced expression of these target genes in D1 MSNs [89]. G9a is also a target for FOSB which, as noted earlier, has been shown to robustly control cocaine-elicited plasticity [28,125,126]. Likewise, DNMT3a expression in the NAc is linked to neuronal and behavioral plasticity after chronic treatment with cocaine. DNMT3a expression is reduced in this region during the early stages of withdrawal from chronic cocaine, possibly due to reduced EGR3 binding, but then becomes elevated after prolonged withdrawal from the drug [89,127]. DNMT3a overexpression in NAc neurons decreases the rewarding effects of cocaine, but increases

dendritic spine density [127]. Interestingly, one study suggests that a specific DNMT3a isoform, termed DNMT3a2, is elevated after cocaine self-administration and regulates cue-induced reinstatement [128].

Another line of evidence suggests that EGR3 regulates physiological responses to chronic cocaine administration by regulating energy balance in NAc MSNs [129]. Chronic cocaine alters EGR3 binding and gene expression of multiple genes involved in mitochondrial function including those encoding dynamin-related protein 1 (*Drp1*), nuclear respiratory factor 2 (*Nrf2*), and DNA polymerase gamma subunit (*Polg*). Further, miRNA-mediated knockdown of EGR3 in the NAc blocks the cocaine-induced increase in small mitochondria in D1 MSNs. However, the consequences of mitochondrial regulation by chronic cocaine exposure on NAc MSN function remain poorly understood.

NF κ B—NF κ B is a transcription factor classically studied in the context of the immune system and inflammation, but more recent studies point to a role for NF κ B in drug-induced synaptic plasticity [113,130–132]. NF κ B transcription factors are composed of dimerized subunits (RELA/p65, RELB, cREL, p50, and p52) and bind to specific response elements called κ B sites. The first indication that NF κ B might be implicated in cocaine action came from a microarray study which demonstrated induction of an NF κ B subunit in NAc upon overexpression of FOSB [133]. Chronic cocaine administration was subsequently shown to upregulate the expression of the p65 and p50 subunits in the NAc, which is associated with increased permissive histone marks (Table 1) [71,113,133]. Expression of a constitutively active inhibitory- κ B kinase (IKKca), which activates NF κ B, in NAc neurons increases cocaine reward and dendritic spine density of NAc MSNs (Table 2A) [113].

NF κ B target genes.: Several target genes for NF κ B have been identified in the context of addiction models. One such target is *Bdnf*, which is discussed earlier in this review [74]. Other putative targets revealed by motif analysis are genes encoding the JUN family of transcription factors [134]. As discussed previously, JUN proteins heterodimerize with FOSB or other FOS family proteins to generate AP1 transcription factors, which suggests that NF κ B may indirectly regulate FOSB-mediated transcription via interactions with JUN proteins. In addition, several NF κ B target genes converge on the endogenous opioid system. NF κ B regulates genes that encode the δ opioid receptor (DOR), μ opioid receptor (MOR), and dynorphin, the primary ligand for KORs. The role of each of these receptor subtypes in addiction pathophysiology has been reviewed in detail elsewhere [78].

Studies of NF κ B in addiction models underscore a major weakness of the earlier literature: most NF κ B subunits in brain are highly enriched in microglia and endothelial cells [135], while efforts to date to manipulate their expression in adult brain in addiction models used neurotropic viruses. NF κ B signaling no doubt functions in neurons, but these observations emphasize the importance of now better defining the cell-type-specificity of transcription factor regulation by a drug of abuse within the NAc or another brain region, and to then study the causal consequences of this regulation by manipulating the factor selectively within that one cell type. Such studies are becoming increasingly feasible with advances in cell-targeting approaches (Box 2).

MEF2—The myocyte-enhancing factor 2 family of transcription factors (MEF2A-D) have been shown to play a role in regulating neuronal survival, actin remodeling, and synaptic plasticity [136–138]. Genome-wide analysis of putative MEF2 target genes point to multiple genes implicated in structural plasticity, including N-WASP, WAVE3, Homer1a, ARC, and protocadherins [136]. Experiments in cell culture suggest that MEF2 also targets several transcription factors discussed elsewhere in this review, such as FOSB, EGR3, and nuclear receptor 77 (NUR77; also known as NR4A1) [139]. Based on this knowledge, work from our laboratory interrogated whether MEF2 transcription factors might be regulated in NAc by cocaine (Table 1A). We found that acute and chronic cocaine administration increases inhibitory phosphorylation of MEF2A/2D in the NAc and, by use of viral manipulations of MEF2 levels in NAc neurons, that this suppression of MEF2A/2D activity is required for cocaine's induction of dendritic spine density of NAc MSNs. Expressing a constitutively active form of MEF2 (MEF2-VP16) was shown to elevate cocaine-induced locomotor sensitization and reward (Table 2A) [136].

CDK5 is an important regulator of MEF2 transcription factors. As noted earlier, after cocaine administration, FOSB promotes transcription of CDK5 in NAc, which catalyzes inhibitory phosphorylation of MEF2 proteins [136]. This once again underscores the complex web of interactions among transcription factors and their regulatory proteins. Blocking CDK5 activity in NAc is associated with reduced spinogenesis, but with increased cocaine place conditioning and locomotor sensitization [114,140–142]. Therefore, reduced CDK5 activity, which releases the brake on MEF2-dependent transcription, elicits similar phenotypes as MEF2 overexpression. Together, these studies suggest that cocaine potentiates CDK5 activity via induction by FOSB, which then attenuates MEF2 activity to further facilitate cellular and behavioral plasticity to cocaine (Figure 3).

Additional Transcription Factors Driving Cocaine-Induced Plasticity From Unbiased Sequencing Datasets

Bioinformatic-centric approaches to analyze sequencing datasets—The ever-increasing use of RNA-seq and other next-generation sequencing approaches in neuroscience laboratories has driven novel bioinformatic efforts to optimally utilize these vast datasets to derive meaningful biological insight.

Integrating next-generation sequencing and bioinformatics is particularly relevant for elucidating transcription factor function, as many transcription factors have known motifs (response elements) within regulatory regions of their target genes [26]. One such bioinformatic approach, known as hypergeometric optimization of motif enrichment (HOMER), scans the promoter and other regions of differentially expressed genes (DEGs) for the enrichment of known transcription factor motifs [143]. Recent work from our laboratory used HOMER to identify transcription factors in NAc and several other brain reward regions associated with addiction-like behavior after prolonged withdrawal ± relapse from cocaine self-administration, many of which had not been previously linked to cocaine addiction [27].

Another bioinformatic approach involves transcriptomic network analysis, such as weighted gene co-expression network analysis (WGCNA) and multiscale embedded gene expression

network analysis (MEGENA) [144,145]. Here, expressed RNAs are subdivided into distinct modules, a collection of genes exhibiting coordinated co-expression across conditions within a given dataset, with each module containing hub genes, or predicted drivers of other genes in the same module. By searching for modules enriched in DEGs after cocaine self-administration, or genes whose expression is correlated with addiction-like behaviors, researchers can deduce those genes that are most important in driving the molecular pathology of cocaine addiction. These methods have revealed novel molecular mechanisms underlying brain pathology in Parkinson's disease [146], Alzheimer's disease [147,148], and depression [48,149,150], and are now being applied in addiction research [151].

Below, we discuss recent work that has employed unbiased bioinformatic tools to identify previously unexplored transcription factors driving addiction-like behaviors. These efforts are providing compelling evidence that bioinformatic-centric approaches to analyzing large sequencing datasets can reveal novel biological insight into addiction pathophysiology. At the same time, we should note that the semantic distinction between candidate factors and deduced unbiased factors becomes arbitrary since the initial focus on NF κ B in addiction models was based on an open-ended microarray analysis of FOSB targets. It is likewise reassuring that the more recent unbiased analyses of upstream transcriptional regulators of cocaine-induced gene expression alterations implicate CREB, AP1 (FOS and JUN family proteins), and EGR family transcription factors [27], indicating that the earlier hunches of their involvement were indeed well placed.

FOXO3a—Our laboratory paired CHIP-seq with de novo motif analysis and demonstrated that the FOXO transcription factor family motif is enriched in genes bound by SIRT1 in the NAc after chronic cocaine administration [117]. Based on this insight, we went on to demonstrate that cocaine increases the expression of FOXO3a, the FOXO family member most ubiquitously expressed in the brain, as well as known FOXO3a target genes in the NAc [117]. However, other FOXO family members are expressed in the NAc and their role in cocaine-induced plasticity remains unexplored [152]. Viral-mediated FOXO3a overexpression in NAc neurons, like SIRT1 overexpression, potentiates the rewarding effects of cocaine [112,117]. Based on data demonstrating that cocaine induction of SIRT1 in NAc is mediated by FOSB [71,112], these results suggest that FOXO3a may be part of a larger FOSB-mediated network of transcriptional regulation (Figure 3).

SIRT1 overexpression in the NAc reduces acetylation of FOXO3a, possibly through direct deacetylation by SIRT1, which increases the transcriptional activity of FOXO3a (Figure 3). Decreased genomic binding of SIRT1 was also associated with elevated histone 4 lysine 15 acetylation (H4K15ac) in the NAc after chronic cocaine administration. Notably, SIRT1 itself does not bind DNA in a sequence-specific manner, but rather is recruited to specific genes through interactions with DNA-binding proteins, such as transcription factors. These data suggest that SIRT1 influences cocaine-induced plasticity in the NAc through two distinct mechanisms: activation of FOXO3a-dependent transcriptional programs through deacetylation of FOXO3a and regulation of H4K15ac levels. Further studies are needed to identify key target genes of FOXO3a in the context of cocaine exposure, which represents an interesting focus of future research given the implication of FOXO-mediated transcription in a wide range of nervous system functions.

E2F3a—Transcriptome analysis across the brain's reward circuitry after cocaine self-administration predicted the E2F family of transcription factors as key upstream regulators of genes whose altered expression by chronic cocaine or cocaine withdrawal is associated with addiction-like behavior in the NAc [27]. The E2F family was similarly implicated in a study that integrated ChIP-seq data from NAc for several prominent histone modifications after chronic cocaine administration, which suggested a role for E2F proteins not only in overall transcriptional regulation by cocaine but in cocaine regulation of alternative splicing as well [153]. These are striking findings in that E2F proteins had not previously been implicated in addiction-related phenomena and barely studied at all in neuronal function, yet the proteins are highly expressed in neurons.

The highest expressed E2F family member in NAc is E2F3, which undergoes alternative splicing to produce E2F3a and E2F3b isoforms that, based on work in other tissues, serve as transcriptional activators or repressors [154]. Animals treated with chronic cocaine show elevated expression of E2F3a exclusively in D1 MSNs, while E2F3b remains unchanged (Table 1) [36,88]. Viral-mediated overexpression of E2F3a in NAc neurons increased cocaine-induced locomotor sensitization and reward, while E2F3a knockdown had the opposite effect (Table 2A), with manipulation of E2F3b having no effect on cocaine responses [36].

At the transcriptional level, E2F3a overexpression in NAc neurons was sufficient to recapitulate a large subset of the changes in overall gene expression observed by RNA-seq after chronic cocaine administration, as well as changes in alternative splicing seen in response to the drug [36]. *Fgfr1*, *Tle2*, and *Ptbp1* are examples of genes regulated similarly by cocaine and E2F3a overexpression in this brain region. Together, these findings support an important role for E2F3a in mediating behavioral and transcriptional plasticity induced by chronic cocaine administration.

One mechanism by which E2F3a facilitates cocaine-elicited plasticity might be through its induction of FOSB. The *FosB* gene contains an E2F binding site ~500 base pairs upstream of the transcription start site (TSS) and the binding of E2F3, but not of other E2F family members, is increased at this site of the *FosB* gene in NAc after chronic cocaine. Further, E2F3a overexpression in this brain region increases *FosB* and *FosB* mRNA expression, while E2F3a knockdown partially blocks the induction of FOSB by cocaine [88], consistent with the involvement of several other transcription factors as stated earlier. These data support the scheme that E2F3a is an upstream regulator of FOSB in NAc, a further example of the inter-connected web of cocaine-elicited transcriptional mechanisms (Figure 3). Of note, cocaine regulates E2F3b, not E2F3a, in the PFC, where E2F3b exerts very different transcriptional and behavioral effects as seen for E2F3a in NAc [155]. These observations highlight the importance of characterizing transcriptional mechanisms of drug addiction in many other brain reward regions beyond the NAc.

Nuclear receptors—In the same transcriptomic analysis from animals self-administering cocaine and examined at different withdrawal time points ± relapse, numerous nuclear receptors were identified as upstream regulators of genes associated with addiction-like behaviors [27]. Most of these nuclear receptors have not previously been linked to addiction-

related phenomena. One such nuclear receptor is retinoic X receptor alpha (RXR α , also known as NR2B1), an isotype of the RXR family of nuclear receptors. RXR α is activated by retinoic acid and regulates the expression of its target genes as a homo- or heterodimer. RXR α provides a particularly powerful test of bioinformatic predictions because, unlike the other transcription factors discussed in this review, its own transcription in NAc is not affected by cocaine when measured at the bulk tissue level. Nonetheless, based on its deduction as an important upstream regulator, we proceeded to study RXR α in addiction models. We showed that RXR α is a potent, positive regulator of cocaine reward in NAc of both female and male rodents, and that overexpressing RXR α in neurons of this brain region modulates many of the same genes that are altered by cocaine self-administration [156]. While RXR α is expressed predominantly in microglia [135], it is also expressed in NAc interneurons [3]. Ongoing work on this nuclear receptor seeks to define whether its regulation of cocaine responses occurs via microglia or this neuronal cell type, or both, and to then identify the target genes mediating cocaine-induced neuroplasticity.

A recent study from Carpenter and colleagues identified NR4A1 (NUR77), as a predicted mediator of cocaine-induced neuroplasticity using RNA-seq following cocaine self-administration plus early or late withdrawal [157]. Chronic cocaine administration transiently increases NR4A1 expression, but the expression returns to baseline after extended withdrawal. However, there is an enrichment of permissive histone marks (H3K27ac and H3K4me3) at the gene after extended withdrawal, suggesting that *Nr4a1* remains primed for reactivation by cocaine. This study employed CRISPR activation and interference (CRISPRa/i) tools to bidirectionally regulate endogenous NR4A1 expression in the NAc; these tools are discussed in greater detail elsewhere in this review (Box 1). CRISPR-mediated activation of NR4A1 in the NAc attenuates cocaine reward and reinstatement after prolonged withdrawal, while CRISPR-mediated knockdown increases cocaine reward (Table 2A).

In addition, pharmacological activation of NR4A1, using peripheral administration of cytosporone-B (Csn-B), decreases cocaine reward, thus replicating the effect of CRISPR-mediated activation of NR4A1 in the NAc on cocaine reward. This study provides promising preliminary evidence that NR4A1 warrants further investigation as a possible treatment for cocaine use disorder. These data are further evidence that unbiased next-generation sequencing approaches are useful for understanding the molecular basis of drug addiction and that this work can be leveraged to develop novel treatments for drug addiction.

Transcription Factors as Therapeutic Targets for Cocaine Use Disorder

Research over the last decade suggests that transcription factors are viable targets for therapeutic intervention in human disease. Multiple approved medications are agonists or antagonists of glucocorticoid, mineralocorticoid, or gonadal steroid hormone receptors, all of which are transcription factors. Another class of compounds, called thiazolidinediones, are used to treat type II diabetes by modulating the peroxisome proliferators activated receptor (PPAR) nuclear receptor family [158]. Additionally, a selective inhibitor of AP1 transcription factors (T-5224) is under investigation for the treatment of rheumatoid arthritis [159], cancer [160], and intravertebral disc degeneration [161], among others. This

compound selectively disrupts DNA binding of AP1 complexes without affecting the activity of other transcription factors. Applying a similar approach to disrupt dimerization or the DNA binding properties of additional transcription factors may present a novel treatment avenue for drug addiction.

Identifying small molecule regulators of transcription factors for the treatment of cocaine use disorder represents a major challenge for the field. These compounds must be able to cross the blood-brain barrier and achieve sustained bioavailability in target brain structures to regulate transcription factor function without inducing peripheral toxicity or detrimental off-target interactions. In addition, many transcription factors, such as CREB, are ubiquitously expressed throughout the brain and peripheral organs, suggesting that small molecule regulators of these transcription factors would disrupt normal physiological function. By contrast, growing evidence suggests that small molecule inhibitors of FOSB may be effective treatments for cocaine use disorder because FOSB is highly enriched in striatal regions, FOSB overexpression in the NAc or OFC increases addiction-like behavior in preclinical models of drug addiction [99,107,108], elevated FOSB expression is observed in the NAc of human patients with cocaine addiction [85], and the long-term stability of FOSB is thought to contribute to heightened vulnerability to relapse after prolonged withdrawal from drugs of abuse [103]. However, evaluating the therapeutic efficacy of FOSB inhibitors for cocaine addiction will require careful investigation because

FOSB is also involved in important physiological functions such as learning and memory [109], resilience to chronic stress [162], motivation for natural rewards [163] and, in the periphery, osteoblast and adipocyte function [164]. To date, several compounds have been identified that disrupt the DNA binding of FOSB [165,166], but studies investigating the efficacy of optimized FOSB inhibitors in preclinical models of drug addiction are still needed. The ongoing efforts to develop small molecule regulators of transcription factors demonstrate that, beyond identifying innumerable targets for these transcriptional regulators that could serve as the basis for new addiction treatments, transcription factors themselves should be seen as a potential focus for therapeutic discovery for drug addiction.

Concluding Remarks

Cocaine and other psychostimulants increase neurotransmission at dopaminergic synapses, leading to altered intracellular signaling on postsynaptic neurons, such as NAc MSNs. Transcription factors respond to these intracellular messengers—being activated or inactivated in the cytoplasm and transported into the nucleus or having upstream protein kinases transported into the nucleus where they activate resident transcription factors. In the nucleus, transcription factors bind to specific response elements across the genome, within genic regions and at distant enhancers, to regulate the expression of hundreds or thousands of genes by recruitment of RNA polymerase II and its associated transcriptional machinery or, conversely, proteins that suppress gene expression (Figure 1). Drug-induced regulation of transcription factors, including alterations in their activation by upstream protein kinases or in their own transcription, mediates lasting changes in the steady-state expression, as well as in the expression potential (priming/desensitization), of a host of target genes which then underlie many of the maladaptations observed in the brain's reward circuitry that drive drug addiction.

The new tools of locus-specific neuroepigenomic editing are improving the level of proof that can now be obtained to directly link a transcription factor to its downstream functional—transcriptional, cellular, synaptic, and ultimately circuit—consequences (Box 1). This work is most advanced for cocaine, with similar approaches now required to better understand transcriptional responses to opioids and several other classes of abused drugs. We focused in this review on NAc, which is by far the most studied brain region, but parallel studies are needed for many other reward regions also implicated in drug addiction.

Despite the progress made to date in studying a small handful of cocaine-regulated transcription factors, advanced bioinformatic analyses of genome-wide datasets are identifying large numbers of additional transcription factors that are predicted to be key upstream regulators of differentially regulated genes after cocaine self-administration and withdrawal that remain unexplored in addiction models [27,157]. Further, the detailed molecular mechanisms that link receptor actions of drugs of abuse to downstream regulation of transcription factors remain in general poorly understood (Figure 3). Mechanisms mediating the induction of FosB in NAc by cocaine illustrate that such upstream pathways are highly complex and therefore especially difficult to delineate in vivo (Figure 4). Yet a focus on in vivo models is essential because it is well known that regulatory events that occur in vivo are often not captured in cultured neurons.

Likewise, a great deal more needs to be learned about the detailed changes in chromatin architecture at downstream target genes that work in concert with transcription factors to mediate gene regulation. For example, most transcription factors activate some target genes, but suppress others, and little is known about the mechanisms that determine such actions. There remains a paucity of studies examining transcription factor binding in discrete regions of brain, let alone in specific cell types in those regions, with ChIP-seq lacking the sensitivity to produce high quality datasets for most transcription factors in microdissected brain regions like the NAc. By contrast, alternative approaches, such as CUT&RUN, offer far greater sensitivity and are showing potential for providing such crucial data, as found recently for mapping cocaine-induced binding of endogenous FosB genome-wide [116]. Combinatorial analysis of such data with a host of other chromatin measures, such as ChIP-seq for histone modifications, whole genome bisulfite sequencing to quantify DNA methylation, or HiC to map the 3D structure of chromatin, will help address these outstanding questions. Combinatorial approaches are required as well to understand how cocaine regulation of multiple transcription factors, occurring simultaneously within the same cell type (e.g., D1 MSNs), is integrated to achieve the ultimate time-dependent effects of cocaine on gene expression. Moreover, it is increasingly essential to carry out such studies in a cell-type-specific manner since it is known from other tissues, and from early work in brain as outlined in this review, that the target genes of a given transcription factor differ dramatically from one cell type to another (Box 2).

Another challenge is to better understand the stimulus specificity that has been observed for some transcription factor actions. It was stated earlier that FosB mediates the ability of chronic cocaine and of chronic morphine to induce *Sirt1* in NAc [112]. By contrast, *Sirt2*, another sirtuin isoform, is also induced in NAc by cocaine via FosB, whereas it is not induced by morphine even though both drugs induce FosB to the same extent in

D1 MSNs. What mediates this stimulus specificity? Do the drugs induce FOSB in distinct subsets of D1 MSNs? Are there other transcriptional changes induced by cocaine in D1 MSNs that are permissive for *Sirt2* induction, or by morphine that preclude *Sirt2* induction?

These are just some of the outstanding questions that need to be answered in delineating the precise transcriptional steps through which cocaine or other drugs of abuse change brain reward mechanisms to drive an addicted state, particularly in the context of improved animal models capturing specific behavioral characteristics of substance use disorders [167]. Elaborating the likely scores of transcription factors that contribute to this transcriptional regulation, and the hundreds of target genes through which each factor acts to produce its downstream molecular, cellular, circuit, and behavioral plasticity, will fundamentally improve our understanding of the molecular basis of drug addiction and provide a template to guide efforts at developing improved treatment and prevention measures for substance use disorders.

Acknowledgements:

Several figures in this review were created using [Biorender.com](https://biorender.com)

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Box 1: Locus-Specific Neuroepigenomic Editing

Current genetic and pharmacological approaches to regulate gene function have several limitations that hinder experimental insight. First, overexpression or knockout techniques often induce changes in gene expression that fall outside of the range observed under physiological or pathophysiological conditions. This is crucially important because empirical data demonstrate that gene regulation outside of a physiologically-relevant range can have different, even opposite, experimental outcomes [47,141,168]. Second, these techniques alter transcription factor binding at hundreds or thousands of genomic regions, making it difficult to determine the role of individual target genes. Lastly, gene knockdown strategies, such as RNA interference (RNAi) and dominant negative approaches, are prone to significant off-target effects [46].

Locus-specific neuroepigenomic editing utilizes a highly specific DNA binding domain tethered to a chromatin-modifying protein, allowing researchers to model a specific epigenetic change at a single genomic locus in a discrete cell population in the brains of awake, behaving animals. Current neuroepigenomic editing platforms include zinc finger proteins (ZFPs), transcriptional activator-like effectors (TALEs), and clustered regularly interspaced short palindromic repeats (CRISPR). These tools are modified to lack functional nuclease domains and are instead tethered to a transcriptional effector domain. ZFPs and TALEs bind directly to their target DNA sequence via protein-DNA interactions, while CRISPR uses a guide RNA (gRNA) to direct nuclease-dead Cas9 (dCas9) binding.

A wide range of effector domains have been tested *in vivo* including histone acetyltransferases (HATs), histone methyltransferases (HMTs), DNA methyltransferases (DNMTs), and a transcription factor [47–54]. For example, work from our laboratory used the CRISPR platform to guide a phosphomimetic (active) form of CREB (CREB^{S133D}) to its response element at a single CREB target gene implicated in mediating resilience to chronic stress, zinc finger protein 189 (*Zfp189*) [48]. Unlike CREB overexpression experiments, this method causally links CREB binding at a single target gene to downstream experimental measures. A similar approach was used recently to target CREB^{S133D} to the *FosB* gene [37].

In a related strategy, neuroepigenome editing tools have been used to control bidirectionally the expression of an endogenous transcription factor. For example, coupling a *FosB*-targeted ZFP fused to a histone acetyltransferase or methyltransferase increases or decreases, respectively, levels of expression of endogenous FOSB and FOSB in the NAc [102,169,170]. While the induced FOSB/ FOSB can still influence all of its downstream targets, this approach avoids confounds associated with conventional overexpression and knockdown methods.

An important focus of future research is expanding the neuroepigenomic editing toolbox to study a wide range of transcription factors and better establish the molecular mechanisms downstream of transcription factor activation.

Box 2: Cell-Type-Specificity of Transcription Factor Actions

To date, the majority of studies on transcription factors in the context of cocaine-induced neural plasticity have involved analysis of bulk tissue and viral gene transfer techniques paired with neuron-specific promoters or neurotropic viruses. While these early studies have yielded a great deal of information on transcription factor function, they are inherently limited due to the rich heterogeneity of cell types in the brain and their unique contributions to brain function and pathophysiology. In the NAc, D1 and D2 MSNs differ in their transcriptional profiles [2,3], synaptic inputs and projection targets [4,5], cellular response to drugs of abuse [16], and effects on addiction-like behaviors [6–12]. In addition, local interneuron populations as well as glial and endothelial cells modulate the microcircuitry within the NAc to influence behavior. As a result, studies on homogenized brain tissue or with pan-neuronal gene manipulations are limited with respect to the insight they can provide into the cell-type-specific actions of transcription factors and their target genes.

For example, previous work demonstrated that FOSB overexpression in NAc neurons increases the expression of NF κ B—leading researchers to overexpress NF κ B in NAc neurons and study the downstream effects on cocaine-induced plasticity and behavioral phenotypes [113,133]. However, NF κ B is expressed predominantly in microglia and endothelial cells. There is no doubt that NF κ B signaling in neurons influences neuronal plasticity, but it remains unclear if the cocaine-mediated induction of NF κ B occurs in a subset of NAc neurons or rather in microglia or endothelial cells, suggesting that NF κ B overexpression in NAc neurons may not accurately portray the functions of drug-induced NF κ B.

Notably, there are some advantages to conducting initial analyses on bulk tissue rather than isolating specific cell types. A study from our laboratory showed that overexpression of zinc finger protein 189 (ZFP189), a CREB-regulated putative transcription factor, in PFC neurons dramatically alters the expression of genes in other cell types, especially endothelial cells [48]. By focusing on a single cell population, this finding would likely have been overlooked. Therefore, bulk analyses and cell-type-specific studies are often complementary, but to date the majority of studies on transcription factors in cocaine-induced plasticity in the NAc have not included cell-type-specific analyses.

Fortunately, there is now a growing toolbox for studying transcription factors in a cell-type-specific manner within a defined brain region of awake, behaving rodents. Several methods are available to isolate specific cell types from brain tissue for downstream analyses, including fluorescence-activated cell sorting (FACS), fluorescence-activated nuclei sorting (FANS), magnetic-activated cell sorting (MACS), and RiboTag affinity purification. Work from our laboratory has applied some of these techniques to study D1 vs. D2 MSN subtypes in the NAc [2,171]. Coupling these methods with RNA-seq, ChIP-seq, ATAC-seq, and other approaches can be leveraged to study the cell-type-specific effects of transcription factor manipulations on the transcriptome, genome-wide transcription factor binding, and chromatin accessibility, respectively. A major technical challenge for the field is improving experimental protocols to lower the amount of required input tissue for these protocols, which is very limited for samples of rodent brain

tissue after microdissection and cell sorting. As mentioned in the main text, CUT&RUN is an alternative method to ChIP-seq that is amenable to lower input samples [38]. In addition, viral-mediated gene transfer methods are available to manipulate transcription factors in an inducible manner and selectively in many types of neurons or glia [172]. An important avenue for the field moving forward is to leverage these technologies to study transcription factors in a cell-type-specific manner, which will ultimately lead to an improved understanding of the molecular changes in the brain that drive maladaptive, addiction-like behaviors and elucidate potential therapeutic targets for cocaine use disorder.

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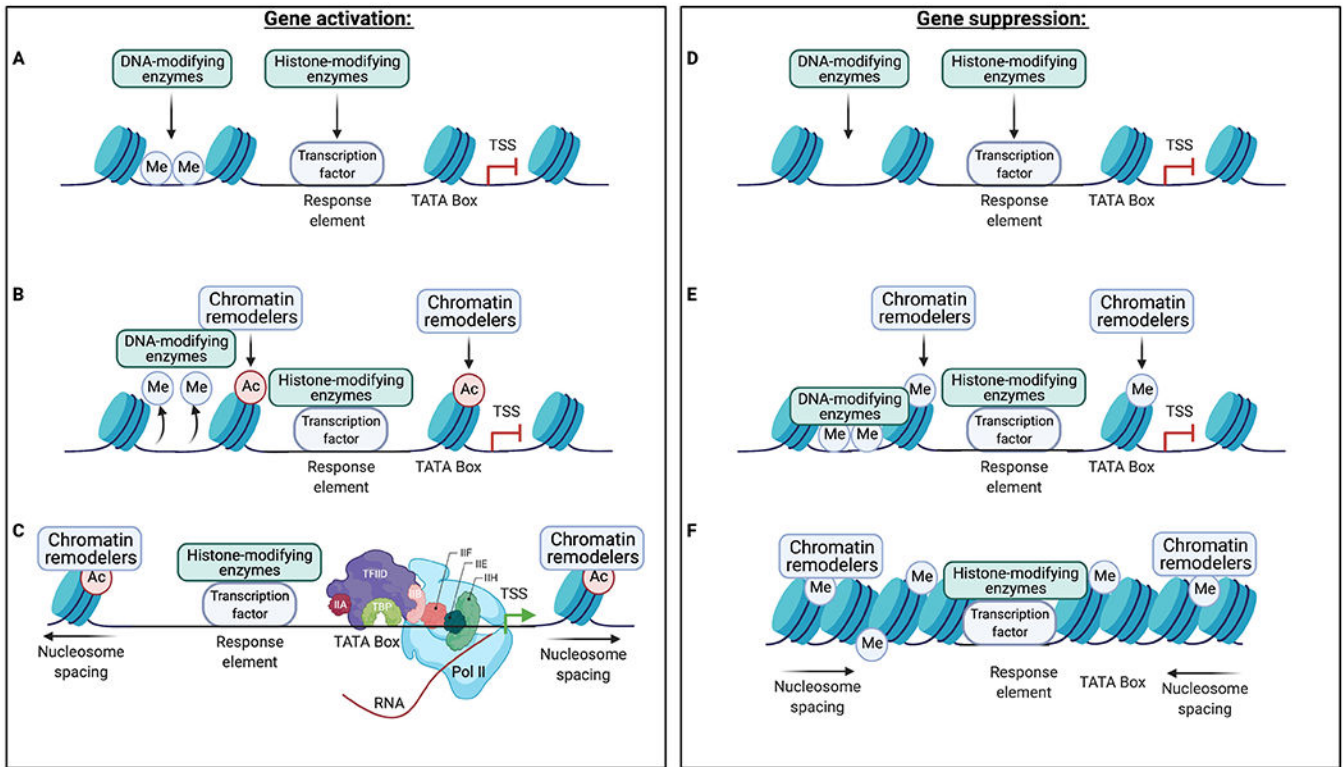


Figure 1: Theoretical mechanisms of gene activation or suppression by transcription factors. Transcription factors bind to specific DNA sequences, termed response elements, at their target genes and recruit secondary epigenetic enzymes and transcriptional machinery to regulate gene transcription. While many transcription factors either activate or suppress gene transcription at their target genes, some transcription factors may have opposite effects on transcription at different target genes via distinct mechanisms downstream of transcription factor binding. In this scheme, a transcription factor binds to its response element at a given target gene and recruits various chromatin-modifying enzymes (**A**). These enzymes remove repressive DNA methylation (Me) and deposit permissive modifications (e.g., acetylation [Ac]) on local histones (**B**), which are subsequently recognized by chromatin remodeling proteins that increase nucleosome spacing and enable recruitment of the transcriptional machinery (**C**). Conversely, a transcription factor bound to its response element (**D**) may recruit chromatin-modifying enzymes that deposit repressive modifications on local histones and DNA (**E**). These repressive modifications are then recognized by chromatin remodeling proteins that reduce nucleosome spacing and prevent the binding of transcriptional machinery (**F**). It is important to note that many additional proteins, chromatin modifications, and non-coding RNAs also regulate gene transcription, but these mechanisms remain poorly characterized in the context of drug-induced adaptations in NAc MSNs. TATA box denotes promoter region of the gene just 5' of the transcription start site (TSS). The purple and light blue shapes in (**C**) show subunits of the basal transcription machinery and RNA polymerase II (Pol II).

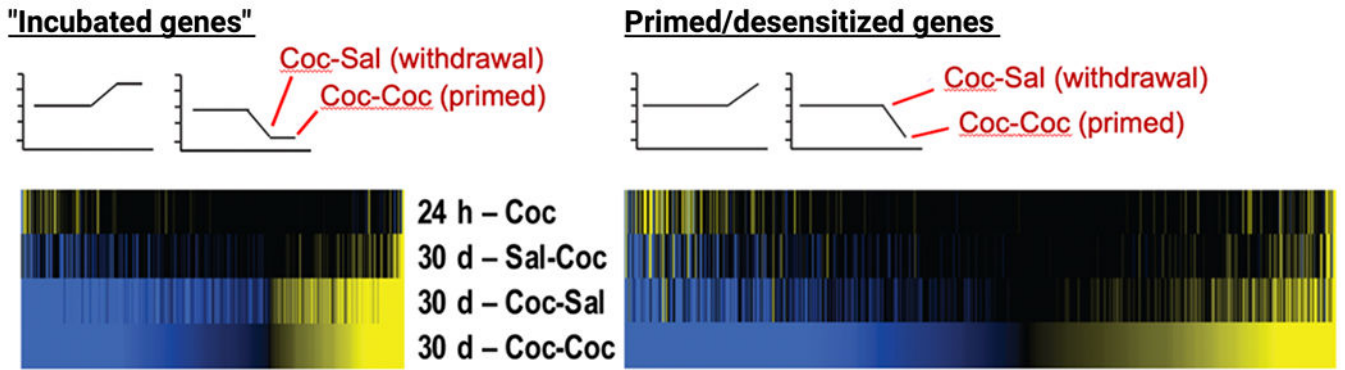


Figure 2: Priming and desensitization of gene expression in NAc after prolonged withdrawal from cocaine self-administration.

Mice self-administered saline (Sal) or cocaine (Coc) for 10 d. One cohort was analyzed 24 h after the last self-administration session (24 h - Coc). The other cohorts remained in their home cages for 30 d and then returned to the self-administration chambers and given an intraperitoneal injection of saline or cocaine yielding three groups: Sal-Coc (acute cocaine); Coc-Sal (30 d withdrawal, saline challenge: “Incubated genes”); Coc-Coc (30 d withdrawal, cocaine challenge: primed/desensitized genes). Heatmaps show RNAs whose expression levels exhibit statistically significant differential expression (yellow: upregulated; blue, downregulated) from all other conditions at both Coc-Sal and Coc-Coc conditions (left) or after the Coc-Coc condition (right). From Walker et al., 2018.

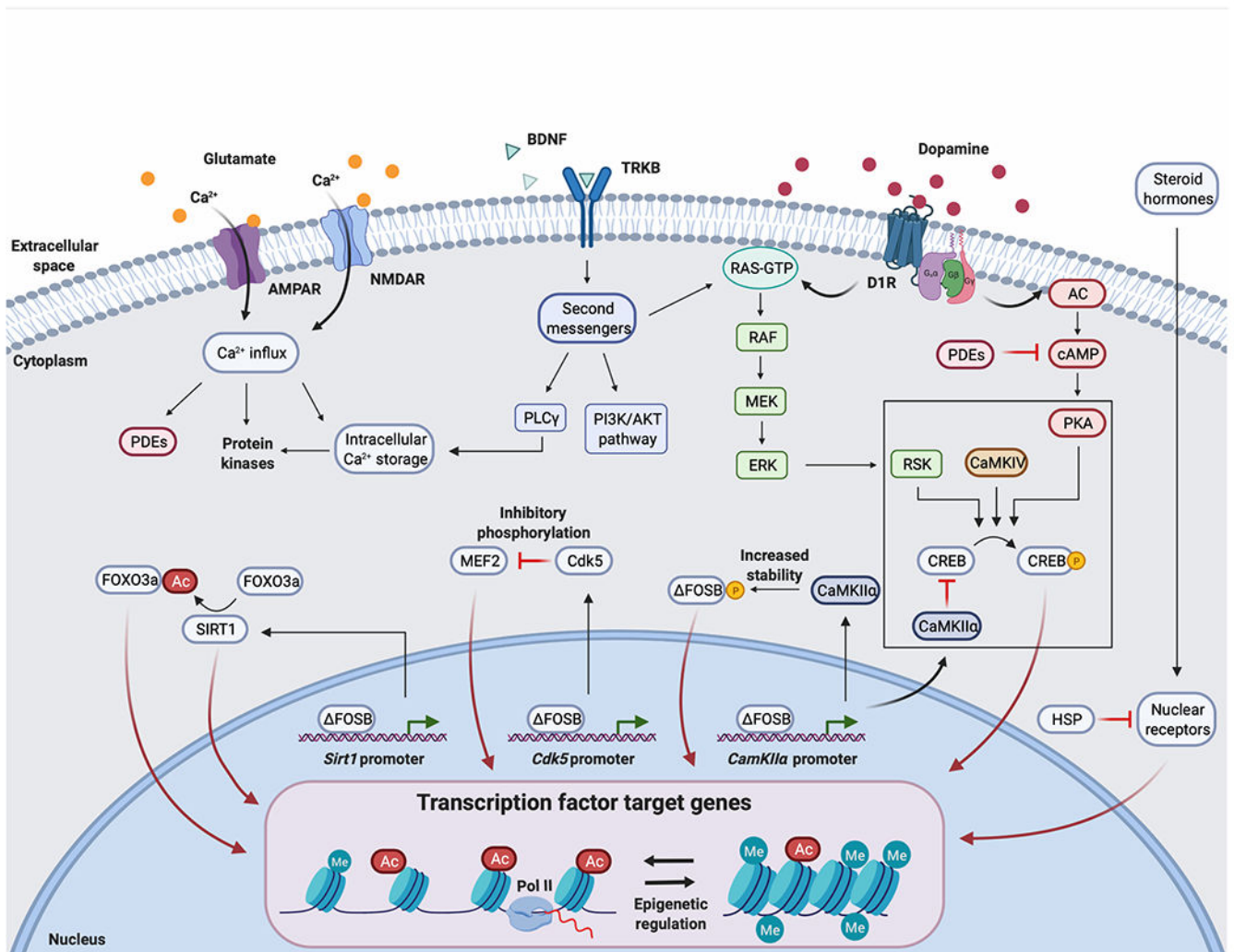


Figure 3: Intracellular signaling pathways in D1 MSNs after chronic cocaine administration. Chronic cocaine potentiates dopaminergic, glutamatergic, and BDNF-TRKB signaling in D1 MSNs in the NAc. Here, we represent several known intracellular signaling pathways that contribute to cocaine-induced regulation of transcription factor activity, but many additional pathways are involved in regulating these and other transcription factors, mechanisms which remain less well understood. Post-translational modifications of transcription factors are important regulators of their activity, and may have an inhibitory or activating effect depending on the type of modification (phosphorylation, acetylation, etc.) and site of modification. Each regulated transcription factor then controls the expression levels of a unique set of target genes. This is illustrated for Δ FOSB, the accumulation of which after chronic cocaine exposure activates or represses the expression of many intracellular signaling molecules, including CDK5, CaMKII α , and SIRT1, among many others. Each of these targets in turn influences the activity of many additional transcription factors. Delineating the complex web of interactions among intracellular messengers and transcription factors remains an important frontier in understanding the molecular basis of cocaine addiction. The box around CREB regulation denotes the fact that these

phosphorylation events occur in the cell nucleus, whereas it remains uncertain whether post-translational modification of the other transcription factors shown occurs in the nucleus or cytoplasm. Note that Ca^{2+} entry is shown for both AMPARs and NMDARs, although only AMPARs of particular subunit compositions flux Ca^{2+} . Ca^{2+} can also enter cells through voltage-gated Ca^{2+} channels (not shown).

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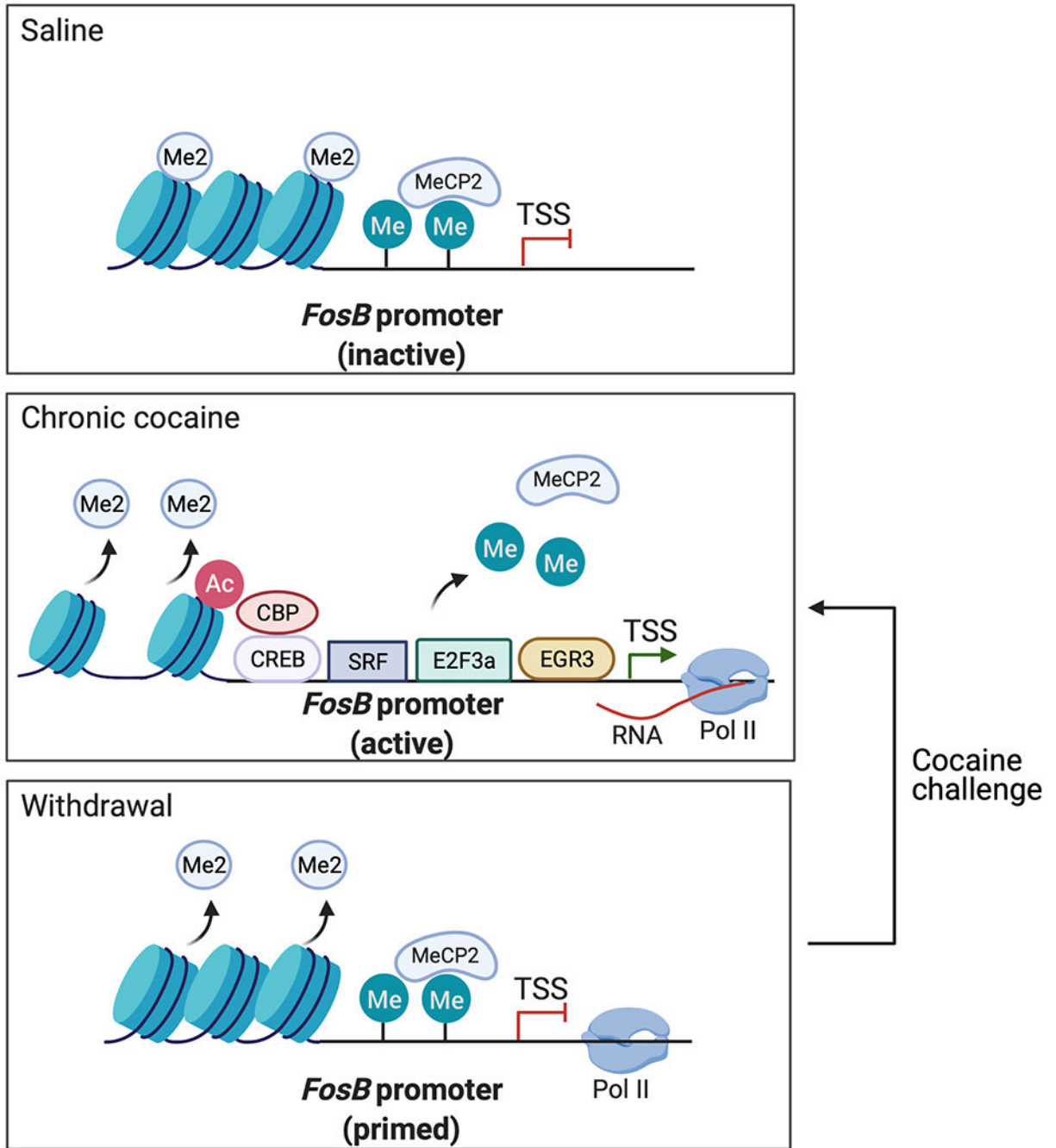


Figure 4: Postulated mechanisms regulating *FosB* gene expression after cocaine administration. Chronic cocaine exposure promotes the recruitment of four known transcription factors, permissive epigenetic modifications, and RNA Pol II to the *FosB* locus, in concert with the removal of repressive histone dimethylation (Me2) and repressive DNA methylation and MeCP2 binding. After prolonged withdrawal, the *FosB* locus remains in a “primed” state, as indicated by persisting, long-lived removal of repressive histone methylation marks and stalling of RNA Pol II near the gene’s transcription start site (TSS). Cocaine re-exposure

after prolonged withdrawal is thought to revert the *FosB* locus to the activated state, contributing further to the accumulation of FOSB protein in NAc D1 MSNs.

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Table 1:

Summary of studies investigating transcription factor gene expression or activity in bulk NAc tissue (A) or in a cell-type-specific manner (B).

Transcription factor	Treatment duration	Cocaine dose	Withdrawal duration	Effect on Transcription factor expression	Reference
FosB	1 day	0.5 mg/kg/inf	0 hours 24 hours	Increased FosB mRNA No effect on FosB mRNA	Larson et al. 2010
	1 day	10 mg/kg	24 hours	No effect on FosB protein	Robison et al. 2013
	7 days	10 mg/kg	24 hours	Increased FosB protein	Robison et al. 2013
	7 days	10 mg/kg	14 days	Increased FosB protein	Robison et al. 2013
	14 days	15 mg/kg x 2	18-24 hours	Increased FosB protein	Perrotti et al. 2008
	14 days	0.5 mg/kg/inf	18-24 hours	Increased FosB protein	Perrotti et al. 2008
CREB	18 days	0.5 mg/kg/inf	0 hours 24 hours 3 weeks	Increased FosB protein; no effect on FosB mRNA Increased FosB protein; no effect on FosB mRNA No effect on FosB protein	Larson et al. 2010
	1 day	30 mg/kg	0 – 1.5 hours	Increased pCREB	Nazarian et al. 2009
	1 day	10 mg/kg	20 minutes	Increased pCREB	Walters et al. 2003
	7 days	15 mg/kg	0 – 1 hour	Increased pCREB	Mattson et al. 2005
E2F3a	14 days	15 mg/kg x 2	0 – 1.5 hours	No effect on pCREB	Nazarian et al. 2009
	7 days	20 mg/kg	24 hours	Increased E2F3a mRNA Increased E2F3a protein	Cates et al. 2017
E2F3b	7 days	20 mg/kg	24 hours	No effect on E2F3b mRNA	Cates et al. 2017
	7 days	20 mg/kg	24 hours	Decreased EGR3 mRNA; Decreased EGR3 protein	Chandra et al. 2015
EGR3	10 days	1 mg/kg/inf	24 hours	Decreased EGR3 mRNA; Decreased EGR3 protein	Chandra et al. 2015
	10 days	1 mg/kg/inf	20 days	Decreased EGR3 protein in females No effect on EGR3 protein in males	Engeln et al. 2019
	7 days	20 mg/kg	24 hours	Increased FOXO3a protein	Ferguson et al. 2015
MEF2	1 day	20 mg/kg	4 hours	No effect on MEF2 P-S404/444 ¹	Pulipparacharuvil et al. 2008
	7 days	20 mg/kg	24 hours	Increased MEF2 P-S408/444 ¹	
	7 days	20 mg/kg	48 hours	No effect on MEF2 P-S404/444 ¹	
	7 days	20 mg/kg	4 hours	Increased MEF2 P-S408/444 ¹	
MEF2	7 days	20 mg/kg	24 hours	Increased MEF2 P-S408/444 ¹	Pulipparacharuvil et al. 2008
	7 days	20 mg/kg	24 hours	Increased MEF2 P-S408/444 ¹	

Transcription factor	Treatment duration	Cocaine dose	Withdrawal duration	Effect on Transcription factor expression	Reference
			48 hours	No effect on MEF2 P-S408/444 ¹	
NFκB	1 day	20 mg/kg	4-6 hours 24 hours	Increased NFκB-dependent transcription No effect on NFκB-dependent transcription	Russo et al. 2009
	1 day	20 mg/kg	4 hours	No effect on protein expression of p105, IκB-γ p70, or p65	Ang et al. 2001
	5 days	20 mg/kg	4-6 hours 24 hours	Increased NFκB-dependent gene transcription Increased NFκB-dependent gene transcription	Russo et al. 2009
	5 days	20 mg/kg	4-6 hours	Increased protein expression of p105 and p65 subunits No effect on protein expression of IκBβ and IκBα.	Russo et al. 2009
	14 days	20 mg/kg	4 hours	Increased protein expression of p105 Trending increase in protein expression of IκB-γ p70 and p65	Ang et al. 2001
NR4A1	21 days	0.7 mg/kg/inf	24 hours	Increased NR4A1 mRNA	Carpenter et al. 2020
	21 days	0.7 mg/kg/inf	28 days	No effect on NR4A1 mRNA	Carpenter et al. 2020

Transcription factor	Cell type	Treatment duration	Cocaine dose	Withdrawal duration	Effect on Transcription factor expression	Reference
FOSB	D1 MSNs	21 days	0.5 mg/kg/inf	24 hours	Increased FOSB protein	Lobo et al. 2013
		7 days	20 mg/kg	24 hours	Increased FOSB protein	Lobo et al. 2013
	D2 MSNs	21 days	0.5 mg/kg/inf	24 hours	No effect on FOSB protein	Lobo et al. 2013
		7 days	20 mg/kg	24 hours	No effect on FOSB protein	Lobo et al. 2013
CREB	D1 MSNs	7 days	20 mg/kg	24 hours	No effect on CREB protein	Lobo et al. 2013
	D2 MSNs	7 days	20 mg/kg	24 hours	No effect on CREB protein	Lobo et al. 2013
E2F3a	D1 MSNs	7 days	20 mg/kg	24 hours	Increased E2F3a mRNA	Cates et al. 2019
	D2 MSNs	7 days	20 mg/kg	24 hours	No effect on E2F3a mRNA	Cates et al. 2019
E2F3b	D1 MSNs	7 days	20 mg/kg	24 hours	No effect on E2F3b mRNA	Cates et al. 2019
	D2 MSNs	7 days	20 mg/kg	24 hours	No effect on E2F3b mRNA	Cates et al. 2019
EGR3	D1 MSNs	7 days	20 mg/kg	24 hours	Increased ribosome-associated EGR3 mRNA	Chandra et al. 2015
	D2 MSNs	7 days	20 mg/kg	24 hours	Decreased ribosome-associated EGR3 mRNA	Chandra et al. 2015

¹MEF2 activity was measured by levels of inhibitory phosphorylation.

Table 2:

Summary of studies analyzing behavioral and neuronal plasticity after transcription factor manipulation in NAc neurons (A) or in a cell-type-specific manner (B).

Transcription factor	Manipulation	Locomotor sensitization	Conditioned place preference	Self-administration	Dendritic spines	Electrophysiology	Reference
FOSB	Overexpression	Increased	Increased	Increased FR1 self-admin Left-shift dose response Increased breakpoint	Increased thin and stubby spines No effect on mushroom spines		Colby 2003, Kelz 1999, Robison 2013, McClung and Nestler 2003
FOSB	Knockdown	No effect	Decreased				Peakman 2003
CREB	Overexpression		Decreased	No effect on FR5 self-admin Left-shift dose response Increased breakpoint Increased reinstatement	Increased silent synapses	Increased excitability, upstate duration, and firing frequency	Brown 2011, Carlezon 1998, Dong 2006, Huang 2008, Larson 2011, McClung and Nestler 2003
CREB	Knockdown	Increased	Increased	No effect on FR5 self-admin Right-shift dose response Decreased breakpoint No effect on reinstatement	Blocks cocaine-induced silent synapses	Decreased excitability and action potentials per upstate, no effect on duration upstate	Vialou 2012, Larson 2011, Huang 2008, Dong 2006, Choi 2006, Carlezon 1998, Brown 2011
NF κ B	IKK overexpression	Increased			Increased spine density		Russo et al. 2009
NF κ B	IKK knockdown		Decreased		Decreased spine density		Russo et al. 2009
MEF2	MEF2-VP16 ¹	Increased	Increased		Blocks cocaine-induced increase in spine density		Pulipparacharuvil et al. 2008
MEF2	MEF2A/2D shRNA	Decreased			Increased spine density		Pulipparacharuvil et al. 2008
FOXO3a	Overexpression		Increased				Ferguson et al. 2015
FOXO3a	Knockdown		No effect				Ferguson et al. 2015
E2F3a	Overexpression	Increased	Increased				Cates et al. 2017
E2F3a	Knockdown	Decreased	Decreased				Cates et al. 2017

Transcription factor	Manipulation	Locomotor sensitization	Conditioned place preference	Self-administration	Dendritic spines	Electrophysiology	Reference
E2F3b	Overexpression	No effect	No effect				Cates et al. 2017
E2F3b	Knockdown	No effect	No effect				Cates et al. 2017
NR4A1	dCas9-VP64		Decreased	No effect on FR1 self-admin No effect on reinstatement (24 hr WD) Decreased reinstatement (28 d WD)			Carpenter et al. 2020
NR4A1	dCas9-KRAB		Increased				Carpenter et al. 2020
FOSB	Overexpression in D1 MSNs	Increased	Increased		Increased spine density	Decreased AMPAR:NMDAR	Gueter et al. 2013
	Overexpression in D2 MSNs	No effect	No effect		No effect on spine density	Increased AMPAR:NMDAR	Gueter et al. 2013
CREB	D1-A-CREB ²	Increased	Increased	No effect on FR1 self-admin			Bilbao et al. 2014
EGR3	Overexpression in D1 MSNs	Increased	Increased				Chandra et al. 2015
	Overexpression in D2 MSNs	Decreased	Decreased	Increased extinction and decreased reinstatement in females Decreased extinction and increased reinstatement in males			Chandra et al. 2015, Engeln et al. 2019
	Knockdown in D1 MSNs	Decreased	Decreased				Chandra et al. 2015
	Knockdown in D2 MSNs	Increased	Increased				Chandra et al. 2015

¹MEF2-VP16 is a constitutively active form of MEF2.

²D1-A-CREB represents the expression of a dominant negative mutant of CREB (A-CREB) under the control of the *Drd1* promoter.