CREB binding at the Zfp189 promoter within medium spiny neuron subtypes differentially regulates behavioral and physiological adaptations over the course of cocaine use


PII: S0006-3223(22)01478-0
DOI: https://doi.org/10.1016/j.biopsych.2022.07.022
Reference: BPS 14944

To appear in: Biological Psychiatry

Received Date: 12 January 2022
Revised Date: 6 June 2022
Accepted Date: 5 July 2022


This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2022 Published by Elsevier Inc on behalf of Society of Biological Psychiatry.
CREB binding at the *Zfp189* promoter within medium spiny neuron subtypes differentially regulates behavioral and physiological adaptations over the course of cocaine use

Collin D. Teague¹,*¹, Joseph A. Picone²,*¹, William J. Wright³, Caleb J. Browne¹, Gabriella M. Silva², Rita Futamura¹, Angélica Minier-Toribio¹, Molly E. Estill¹, Aarthi Ramakrishnan¹, Freddyson J. Martinez-Rivera¹, Arthur Godino¹, Eric M. Parise¹, Kyra H. Schmidt¹, Nathalia V. Pulido¹, Zachary S. Lorsch¹, Jee Hyun Kim¹,⁴, Li Shen¹, Rachael L. Neve⁵, Yan Dong³, Eric J. Nestler¹, Peter J. Hamilton²,*†

¹Nash Family Department of Neuroscience and Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA
²Department of Anatomy and Neurobiology, Virginia Commonwealth University School of Medicine, Richmond, VA, USA
³Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA
⁴IMPACT: The Institute for Mental and Physical Health and Clinical Translation, School of Medicine, Deakin University, Geelong, Australia
⁵Gene Delivery Technology Core, Massachusetts General Hospital, Cambridge, MA, USA.
*correspondence: peter.hamilton@vcuhealth.org
* These authors contributed equally to this work.

Short title: *Zfp189* expression diminishes cocaine reinforcement

Keywords: Cocaine, transcription factor, CRISPR, addiction, nucleus accumbens, *Zfp189*
Abstract:

Background:
Over the course of chronic drug use, brain transcriptional neuroadaptation are thought to contribute to a change in drug use behavior over time. The function of the transcription factor CREB within the nucleus accumbens (NAc) has been well documented in opposing the rewarding properties of many classes of drugs, yet the gene targets through which CREB causally manifests these lasting neuroadaptations remain unknown. Here, we identify zinc finger protein 189 (Zfp189) as a CREB target gene that is transcriptionally responsive to acute and chronic cocaine use within mouse NAc.

Methods:
To query the role of the CREB-Zfp189 interaction in cocaine use, we virally delivered modified CRISPR/dCas9 constructs, capable of selectively localizing CREB to the Zfp189 gene promoter in the NAc of mice.

Results:
We observe that CREB binding to the Zfp189 promoter increases Zfp189 expression and diminishes the reinforcing responses to cocaine. We show further that NAc Zfp189 expression is increased within D1 medium spiny neurons (MSNs) in response to acute cocaine, but increased in both D1 and D2 MSNs in response to chronic cocaine. CREB-mediated induction of Zfp189 potentiates electrophysiological activity of D1 and D2 MSNs – recapitulating the known effect of CREB on these neurons. Lastly, targeting CREB to the Zfp189 promoter within NAc Drd2-expressing neurons, but not Drd1-expressing neurons, was sufficient to diminish cocaine-conditioned behaviors.

Conclusions:
Together, these findings point to the CREB-Zfp189 interaction within NAc Drd2+ neurons as a molecular signature of chronic cocaine use that is causal in counteracting the reinforcing effects of cocaine.
Introduction:

Repeated drug use is correlated with persisting changes at the molecular, cellular, and circuit levels in the brain that are thought to give rise to the lasting behavioral maladaptations that define drug addiction (1,2). The development of drug tolerance – a phenomenon describing the reduced sensitivity to a drug after repeated exposures – is an important component of the pathophysiology of drug addiction that contributes to the dangerous escalation of drug consumption over time (1). Understanding the molecular sequence of events that contribute to a reduction in the reinforcing effects of addictive drugs over the course of drug use will inform novel interventions with the capacity to prevent or reverse some of the most damaging consequences of chronic drug use.

Previous research established that activation of cAMP response element (CRE)-binding protein (CREB) in the nucleus accumbens (NAc), a brain region involved in regulating reward and reinforcement, is a conserved mechanism capable of diminishing the reinforcing effects of addictive drugs across several drug classes (2–9). CREB is a ubiquitously expressed transcription factor involved in numerous nervous system functions including learning and memory, synaptic plasticity, and nervous system development (10,11). CREB regulates gene expression via binding to CRE motifs within the promoter or enhancer regions of target genes and recruiting CREB binding protein (CBP), a histone acetyltransferase (HAT), and the basal transcription complex to these genes (8,12). CREB was implicated initially in drug addiction based on the empirical observation that cocaine and morphine elevate adenylyl cyclase and cAMP-dependent protein kinase A (PKA) levels in the NAc(2,6). PKA and other protein kinases phosphorylate CREB at serine residue 133 to activate CREB-dependent transcription (11,13). In addition,
subsequent studies confirmed that CREB is activated by many drugs of abuse including stimulants, morphine, and nicotine (14–16), suggesting that CREB-mediated transcriptional regulation is a common molecular response to different classes of drugs (2).

Viral overexpression of CREB in rodent NAc neurons reduces conditioned place preference (CPP) for cocaine and increases cocaine self-administration (5,8,17,18). Both the reduction in cocaine conditioning and the seemingly paradoxical increase in cocaine self-administration are indicative of reduced cocaine reward and reinforcement in these animals. These data suggest that CREB function in the NAc represents a molecular mechanism that promotes a drug dependence-like behavioral state. However, studies using CREB overexpression and knockout approaches alter CREB regulation at all target genes, numbering in the hundreds to thousands (12), making it difficult to determine which specific target genes causally mediate the effects of CREB activation on drug reward and volitional intake. Earlier studies using gene expression microarrays as well as chromatin immunoprecipitation (ChIP) paired with promoter microarrays identified numerous putative CREB target genes that may mediate its effects on drug tolerance, but most of these genes remain unstudied (7,8). In addition, it remains unclear if the mechanisms downstream of CREB activation in the NAc differ between distinct neuronal cell types in this brain region.

In this study, we set out to test the hypothesis that the cell-type-specific action of CREB at key target genes differentially occurs over the course of drug use and drives the molecular neuroadaptations associated with chronic drug exposure. To address this hypothesis, we leveraged clustered regularly interspaced short palindromic repeats (CRISPR)-based locus-
specific epigenome editing to study the downstream effects of CREB binding to a single CREB target gene – zinc finger protein 189 (Zfp189), which was identified in our earlier work as a key driver of a gene network associated with responses to social stress (19). In this system, a nuclease-dead Cas9 protein is tethered to the phosphomimetic (constitutively active) form of CREB (dCas9-CREB\textsuperscript{S133D}) and directed to the Zfp189 promoter using a DNA-targeting single guide RNA (sgRNA) (20,21). Prior work suggests that cocaine experience elevates CREB binding at the Zfp189 promoter in the NAc (7). By directing dCas9-CREB\textsuperscript{S133D} specifically to the Zfp189 promoter, initially throughout NAc neuronal populations and subsequently in a medium spiny neuron (MSN) subtype-dependent manner, we show that increased CREB binding at Zfp189 causally increases Zfp189 expression and controls the behavioral and physiological responses to cocaine. These data suggest a cell-type-specific mechanism by which CREB acts through Zfp189 in the NAc to regulate the physiological and behavioral adaptations to cocaine exposure.
Methods and Materials:

See the Supplement for Methods relating to cocaine self-administration procedure, whole cell patch clamp electrophysiology, tissue collection, viral reagents, stereotaxic infusions, and RNAscope®.

Animals.

C57BL/6J male and female mice, aged 8-12 weeks, were acquired from The Jackson Laboratory. D1-Cre and D2-Cre bacterial artificial chromosome (BAC) transgenic mice (http://www.gensat.org/cre.jsp) were bred in-house. Animals were housed at 22–25°C in a 12-h light–dark cycle and provided food and water ad libitum. All tests were conducted during the light cycle. Animal procedures were performed in accordance with guidelines of the IACUC at the Icahn School of Medicine at Mount Sinai or the VCU School of Medicine.

Behavioral paradigms.

For conditioned place preference (CPP), mice were placed in a three-chambered CPP box for 20 min to assess pretest preferences. For the next 2 days, mice were injected in both the morning (saline) and afternoon (drug) and then restricted to one chamber of the box for 30 min. During the post-test, mice were placed in the CPP box with free access to all chambers of the box for a 20 min test session. Data are represented as time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber during the posttest.

Viral reagents.
We utilized modified CRISPR constructs which we have described previously (19,22), and are described in more detail in the Supplement.

**RNAscope®.**

Tissue preparation is described in the Supplement. The following probes were used in this study: Zfp189 (Catalog #: 569561-C3), Drd1 (Catalog #: 461901), and Drd2 (Catalog #: 406501-C2). Slides were imaged at 40x magnification using a Zeiss (Oberkochen, Germany) LSM 900 confocal microscope and images were quantified using CellProfiler 4.2.1 (23).

**RNA isolation and qPCR.**

Total RNA was isolated from frozen dissected NAc tissue using the RNAeasy micro kit (Qiagen) according to manufacturer instructions. Following isolation, RNA was quantified by Nano Drop (Thermo Fisher) and converted to cDNA with iScript (Bio-Rad). qPCR samples were analyzed in triplicate using the standard ΔΔCT method. Hypoxanthine phosphoribosyltransferase 1 (Hprt1) was utilized as the reference gene for normalization in all experiments.

**Results:**

**NAc CREB-Zfp189 interactions regulate behaviors associated with chronic cocaine use.**

To explore the possibility that Zfp189 expression within the NAc is sensitive to cocaine exposure, we first treated mice with daily intraperitoneal (I.P.) injections of either saline or 20 mg/kg cocaine (Fig. 1a). Relative to saline-treated mice, mice treated with a single, acute cocaine injection exhibited a significant increase in Zfp189 mRNA expression within the NAc (Fig. 1b).
Mice treated with chronic cocaine did not display a significant increase in Zfp189 expression (Fig. 1b), revealing dynamic regulation of NAc Zfp189 expression over the time course of cocaine exposure.

We next explored the consequence of mouse cocaine intravenous self-administration (IVSA) on NAc Zfp189 expression levels. Mice were catheterized in their jugular vein and trained to respond on an active lever for either infusions of cocaine (0.5 mg/kg/infusion) or saline. As expected, cocaine IVSA mice self-administer significantly more infusions than saline IVSA mice (Fig. 1c). Further, there was a trend towards increased NAc Zfp189 expression in mice with a history of cocaine IVSA (Fig. 1d). These data suggest that both contingent and non-contingent cocaine exposure may similarly increase NAc Zfp189 mRNA expression.

Since the promoter region of Zfp189 possesses a CRE motif (12,19), and CREB has been demonstrated to bind the Zfp189 promoter in the NAc following cocaine treatment (7), we devised an approach to deliver active CREB specifically to the Zfp189 promoter in NAc neurons to query the role of the CREB-Zfp189 interaction in behavioral responses to cocaine. We applied a modified CRISPR/dCas9 approach wherein dCas9-CREB^{S133D} is targeted selectively to the promoter region of Zfp189 within the NAc via a sgRNA in close proximity to the consensus CRE motif in the Zfp189 promoter (Zfp189-sgRNA) (Fig. 2a). This approach has been validated and applied by our group to the Zfp189 locus in prefrontal cortex (PFC) neurons to study stress-related behaviors (19) and the Fosb locus in the NAc to study medium spiny neuron (MSN)-specific transcriptional responses (22).
We packaged dCas9-CREB$^{S133D}$, Zfp189-sgRNA, and the control non-targeting sgRNA (NT-sgRNA) into separate herpes simplex virus (HSV) vectors to enable stereotaxic delivery and expression in the NAc of awake and behaving mice. Upon delivery to the NAc, we observed that targeting CREB to the Zfp189 promoter increases Zfp189 expression (Fig. 2b). To interrogate the contribution of NAc CREB-Zfp189 interaction to cocaine-related behaviors, we first delivered our CRISPR tools intra-NAc and tested the consequence of CREB-mediated Zfp189 activation on cocaine reward associative learning (Fig. 2c-d). We found that animals with CREB-mediated Zfp189 activation throughout NAc neurons exhibited decreased preference for the 7.5 mg/kg cocaine paired side of the chamber relative to controls (Fig. 2d), which is consistent with a blunted response to the reinforcing effects of cocaine. This phenomenon of reduced cocaine CPP is also observed in female mice (Supp. Fig. 1), suggesting a similar role for CREB-Zfp189 interaction in governing cocaine-related behaviors in both sexes.

Interestingly, in repeating these experiments with morphine administration instead of cocaine, we observed no effect of CREB-Zfp189 interaction on morphine-induced locomotor activity or morphine CPP in male or female mice (Supp. Fig. 2), signifying that the capacity of NAc Zfp189 to affect drug-related behaviors occurs in response to cocaine, but may not extend to other classes of commonly used drugs such as opioids.

We also tested cocaine IVSA in mice to further explore whether NAc CREB-Zfp189 interaction drives changes in reward-related behaviors to cocaine beyond investigator-administered paradigms. Mice were trained to self-administer 0.5 mg/kg/infusion of cocaine on a fixed-ratio 1 schedule of reinforcement (Fig. 2e). We elected to bi-directionally regulate NAc Zfp189
expression by coupling the delivery of Zfp189-sgRNA with either dCas9-CREB^{S133D} or dCas9-G9a. The latter possesses the functional moiety of a transcriptionally repressive histone methyltransferase that we have demonstrated previously suppresses Zfp189 expression in brain (19). While there was a general reduction of IVSA rates immediately following HSV delivery to the NAc, mice with CREB-mediated induction of Zfp189 in the NAc self-administered more cocaine infusions than mice with G9a targeted to the Zfp189 promoter (Fig. 2f). These data further support the notion that NAc-localized Zfp189 expression mimics the hallmark of CREB activation itself (18): it diminishes the reinforcing effects of cocaine which is manifested here as heightened rates of cocaine self-administration. This effect of Zfp189 induction was maximal at the time of peak transgene expression of HSV vectors(24). These results suggest that Zfp189 is a CREB target gene whose activation is singularly sufficient to recapitulate the increased cocaine self-administration effect of general CREB overexpression.

**Cell-type-specific effects of the CREB-Zfp189 interaction in the NAc.**

Approximately 95% of the neurons within the NAc are MSNs, which are differentiated into two primary subtypes based on their predominant expression of dopamine receptor genes, Drd1 versus Drd2, which exist in roughly equal numbers in this brain region(25,26). Emerging evidence implicates D1 MSN function in promoting reward and motivated behaviors, whereas D2 MSNs are often associated with decreased reward and aversive behaviors(27–33). In particular, D1 and D2 MSNs have been shown to drive distinct and often opposing behavioral responses to drugs like cocaine. Therefore, we investigated MSN subtype-specific expression of Zfp189 in the NAc in response to either acute or chronic cocaine exposure (Fig. 3). In performing RNAscope® *in situ* hybridization for Zfp189, Drd1, and Drd2 in fixed NAc sections from mice
exposed to varying cocaine treatment regimens, we observed that, following acute cocaine exposure, solely the D1 MSN population exhibits a robust increase in the number of Zfp189+ cells (Fig. 3a-c). This observation is consistent with rodent NAc single cell RNAseq data demonstrating that acute cocaine upregulates Zfp189 specifically in the Drd1+ cell cluster (34).

After chronic cocaine, by contrast, we observe an increased number of Zfp189+ cells in both D1 and D2 MSNs (Fig. 3d-f). Thus, over the course of cocaine exposure, Zfp189 is activated within D1 MSNs acutely and in both D1 and D2 MSNs at chronic timepoints. This analysis does not quantify the expression level of Zfp189 within MSNs, but rather the percentage of Zfp189+ MSNs by subtype, which may explain the difference in results at the chronic cocaine timepoint between Figure 1 and Figure 3. Therefore, while acute cocaine induces a significant increase in total NAc Zfp189 expression levels (localized primarily within D1 MSNs), chronic cocaine increases the number of Zfp189-expressing D2 MSNs.

To explore the consequence of Zfp189 induction within NAc MSNs on cocaine-induced physiological function, we first virally overexpressed Zfp189 via a conventional overexpression vector in the NAc, which has been validated in brain previously (19). Mice were given an acute cocaine treatment regimen, and 12 hours after the final injection we performed whole-cell patch-clamp recording of virally-infected or uninfected MSNs. While we observed that neither cocaine treatment nor Zfp189 overexpression had any discernable effect on the spontaneous excitatory postsynaptic current (sEPSC) amplitudes (Fig. 4a), this acute cocaine procedure increased the frequency of sEPSCs in both non-transduced and GFP-expressing MSNs (Fig. 4b). HSV-Zfp189 delivery increased the sEPSC frequency of saline-treated mice to the level of cocaine-treated mice, with the effect of cocaine and Zfp189 overexpression being non-additive (Fig. 4b). These
results indicate that acute cocaine treatment specifically enhances sEPSC frequency in NAc MSNs and that \textit{Zfp189} overexpression is sufficient to induce this effect in saline-treated mice.

To understand the MSN subtypes responsible for this phenomenon, we adapted our viral delivery strategy to utilize Cre-dependent (loxP-STOP-loxP [LSL]) expression vectors to facilitate CRISPR-mediated CREB-\textit{Zfp189} interaction in an MSN-subtype-dependent manner. This approach has been previously applied by our group\cite{22}, and as in this earlier work, we see \textit{Zfp189} activation preferentially in NAc neurons that are conditionally expressing our CRISPR constructs (Supp. Fig. 3). In Drd1-Cre+ mice in the LSL-dCas9-CREB$^{S133D}$ + NT-sgRNA control condition, acute cocaine treatment potentiated sEPSC frequency (Fig. 4c), consistent with our observations in WT mice (Fig. 4b). Additionally, targeting CREB to the \textit{Zfp189} promoter within D1 MSNs is sufficient to potentiate sEPSC frequency, recapitulating the effect of acute cocaine (Fig. 4c). In Drd2-Cre+ mice, acute cocaine had no effect on sEPSC frequency in the control viral treatment condition (Fig. 4d). This result indicates that only the D1 MSN population is sensitive to acute cocaine treatment by this metric. However, targeting CREB binding to the \textit{Zfp189} promoter within D2 MSNs increases the sEPSC frequency in both the saline and cocaine treatment conditions (Fig. 4d). Collectively, these data reveal that only D1 MSNs are sensitive to an acute cocaine exposure, yet CRISPR-mediated delivery of CREB to the \textit{Zfp189} promoter is sufficient to potentiate the function of both D1 and D2 MSN subtypes. Furthermore, none of our manipulations had an effect on basal MSN electrophysiological metrics like membrane capacitance or membrane resistance (Supp. Fig. 4).
Catalyzing the CREB-Zfp189 interaction specifically within NAc Drd2+ neurons reduces conditioned place preference for cocaine.

We next tested the consequence of delivering the CREB-Zfp189 interaction within individual neuron subtypes on cocaine reward-related behaviors. In Drd1+ neurons, recruitment of CREB-Zfp189 interaction via CRISPR tools had no effect of cocaine CPP (Fig. 5b). By contrast, inducing the CREB-Zfp189 interaction in Drd2+ neurons significantly decreased cocaine CPP (Fig. 5c). This latter effect is consistent with the effect of CREB-Zfp189 interaction in all neurons in the NAc (Fig. 2d), consistent with the interpretation that this latter effect is driven by the CREB-mediated induction of Zfp189 in Drd2+ neurons selectively.

Discussion:

Here, we investigated a cell-type-specific molecular mechanism by which CREB regulates cocaine-induced neuroadaptations in NAc through the induction of Zfp189. Our findings show that, in response to a single dose of cocaine, Zfp189 is rapidly and selectively induced in D1 MSNs. This expression persists in D1 MSNs following repeated doses of cocaine. However, at these chronic time points, Zfp189 expression becomes induced in D2 MSNs as well. We go on to show that this increased Zfp189 expression in D2 MSNs promotes excitatory inputs upon this cell-type which drives heightened physiological activity and generates behaviors associated with chronic cocaine exposure, including behaviors indicative of a reduction in the reinforcing effects of cocaine. We summarize this proposed neurobiological mechanism as a graphic in Supplemental Figure 5. This work points to a NAc Drd2+ neuron-specific transcriptional cascade from CREB to Zfp189 as a novel mechanism that drives some of the damaging neuroadaptations associated with chronic cocaine use.
The CRISPR-mediated recruitment of CREB to the Zfp189 promoter, depicted in Figure 2, models a single molecular interaction that occurs within NAc neurons in response to cocaine, including both D1 and D2 MSNs. Given that Zfp189 is naturally activated by a single cocaine dose within D1 MSNs only (Fig. 3c), and each of our behavioral paradigms involves either pre-treatment of cocaine (CPP, IVSA), it is likely that the endogenous mechanism of activating Zfp189 within D1 MSNs occurs in parallel with our CRISPR manipulation. However, since we observed that Zfp189 is induced within Drd2+ NAc neurons only after chronic cocaine treatment (Fig. 3f), our NAc-wide CREB-Zfp189 interaction may preferentially be affecting behavioral responses to cocaine by regulating the function of Drd2+ neurons. Thus, we propose that the reduced reinforcing properties of cocaine observed in Figure 2 occur as a result of CREB-Zfp189 interaction within NAc Drd2+ neurons. This is corroborated by the selective delivery of CRISPR tools that drive CREB-Zfp189 interaction within Drd2+ neurons and the recapitulation of the behavioral effects on CPP (Fig. 5c). In sum, our findings support the hypothesis that CREB-Zfp189 interaction, particularly within NAc Drd2+ neurons, drives the animal into a chronic cocaine-exposed state, including a decreased sensitivity to the reinforcing effects of cocaine.

The cell-type-specific features of Zfp189 regulation are consistent with published reports that D1 and D2 MSNs display distinct patterns of activity and gene profiles (26,28). Further, considerable evidence points to the function of D1 MSNs regulating the function of D2 MSNs via the recruitment of cholinergic interneurons (35), to cholinergic interneurons contributing to cocaine self-administration behaviors (36), to cholinergic interneurons regulating glutamatergic
synaptic strength upon NAc MSNs (37), and to dramatic effects of cocaine exposure on gene expression within cholinergic interneurons (36). Importantly, for the experiments within this manuscript, we utilized Drd2-Cre+ mice, which drive the expression of Cre-recombinase in all Drd2+ brain cells. Given that there is a sparse population of cholinergic interneurons that are Drd2+, we cannot exclude the possibility that our conditional manipulations affect Zfp189 function within NAc Drd2+ cholinergic interneurons, which may contribute to our observed results, specifically in Figure 5c.

The exact brain mechanisms responsible for the diminished behavioral sensitivity to cocaine seen in response to Zfp189 induction in NAc must be further elucidated. It is possible that Zfp189 expression in NAc Drd2+ neurons singularly drives drug reward tolerance, which is a hallmark of chronic drug use and refers to decreased sensitivity to a drug following repeated exposures. Conversely, it is possible that the Zfp189-driven function of D1 MSNs opposes Zfp189-driven function of D2 MSNs in an opponent process which is differentially balanced over the course of cocaine experience, with increased weight dedicated to the D2 MSN-driven aversive properties as a function of chronic cocaine experience. Either of these possibilities could explain the behavioral results observed in this work and warrant future investigation.

Another major area for future research is investigating the molecular mechanisms responsible for the differential time course of CREB-mediated Zfp189 induction within D1 versus D2 MSNs. We presently possess an incomplete picture of what distinguishes the sensitivity of the Zfp189 locus in these two closely-related neuronal subtypes. It is possible that the expression of transcription factor regulatory co-factors, Zfp189 promoter chromatin state and CRE
accessibility, as well as transcript processing, among many other possibilities, are differentially sensitive to cocaine exposure and responsible for the time course of Zfp189 induction across MSN subtypes. This work also points to the possibility that CREB function is differentially engaged to regulate distinct gene targets over the course of drug use in a cell-type-specific manner. This possibility is supported by evidence that CRE motif accessibility and CREB-mediated gene regulation varies widely by cell type (38).

Despite the difference in time course, our findings suggest that CREB-mediated induction of Zfp189 might be a molecular mechanism through which both D1 and D2 MSNs respond to cocaine use to alter their physiological function. The application of our novel CRISPR approach for cell-type-specific recruitment of CREB-Zfp189 interaction initiates the endogenous, drug-course-dependent mechanism of CREB regulation, and precipitates the behavioral and physiological consequences of CREB-mediated Zfp189 induction in NAc. While our cocaine treatment regimen was acute in our electrophysiological experiments, our CRISPR-mediated induction of CREB-Zfp189 interaction within D2 MSNs modelled the transcriptional regulation that would occur upon chronic cocaine exposure. Shifting the population of NAc D2 MSNs to a chronic cocaine-like state would have circuit-wide consequences emanating from the cell types in which the CREB-Zfp189 interaction occurred. This may explain our observed increase in sEPSC frequency, which can be mediated by increases in presynaptic release probability, number of presynaptic inputs, number of synapses/release sites, or the general activity state of presynaptic terminals, all suggesting a strengthening of excitatory synaptic input on these cell types. Indeed, there is evidence that increased CREB activity enhances the intrinsic membrane excitability of NAc MSNs (39), cocaine exposure results in increased frequency of glutamate-
mediated EPSCs upon NAc MSNs (40), and altered function of MSNs can regulate synaptic transmission upon distinct MSN subtypes via multi-neuronal circuit regulation (35). Further, the fact that the increase in sEPSC frequency was not additive upon the combination of cocaine treatment and Zfp189 induction supports the notion of a conserved mechanism between cocaine treatment and Zfp189 induction, and not two independent mechanisms for potentiating MSN excitability. These data further indicate that even a relatively modest—yet physiologically relevant—increase in Zfp189 expression within MSNs, as is achieved via CREB-mediated activation, is sufficient to manifest a subset of the neuroadaptations associated with chronic cocaine exposure.

Despite the multiple lines of evidence presented herein that causally link the NAc CREB-Zfp189 interaction to regulating cocaine-induced behaviors, we found no evidence that CREB-mediated regulation of Zfp189 influences morphine-elicited behaviors (Supp. Fig. 2). While NAc CREB function is well documented in being sensitive to, and regulating the rewarding properties of, morphine (5,6), the observed lack of an effect supports the conclusion that CREB achieves these effects independently of its regulation at Zfp189. This points to a possibility that, while NAc CREB function regulates the rewarding properties of many drug classes (3, 41), CREB achieves this outcome by regulation of partly distinct downstream transcriptional networks. Here, CREB’s activity at Zfp189 appears causal in governing specifically cocaine-related outcomes. The degree to which other classes of stimulants, like amphetamines, share this CREB to Zfp189 mechanism is an interesting area for future research.
Given the possibility that CREB functions as a negative feedback mechanism to oppose the rewarding properties of classes of drugs via engagement of distinct transcriptional cascades, novel and highly specific targets for drug addiction pharmacotherapies may be identified within these transcriptional networks. Therefore, it is worthwhile to explore transcriptional regulation downstream of the CREB-Zfp189 interaction to identify possible interventions for cocaine use disorder. The Zfp189 gene product is a Krüppel-associated box (KRAB) domain containing zinc finger protein which suggests that ZFP189 acts as a transcription factor, but its function remains poorly understood (42). Zfp189 induction in PFC neurons has been demonstrated to regulate the expression of genes in response to chronic stress (19), but there is no evidence that this is the result of direct ZFP189-gene interactions. Moreover, it is probable that ZFP189 target genes vary by brain region and cell type. A thorough analysis of NAc MSN-specific ZFP189 gene targets, particularly in the context of cocaine exposure, would be an important future direction.

Collectively, this work points to the CREB-mediated induction of Zfp189 within NAc Drd2+ neurons as a key molecular event that drives the transition into phenotypes associated with chronic cocaine use and may be a promising molecular target for the development of interventions to combat the pathophysiology of cocaine addiction.

**Disclosures:**

The authors report no biomedical financial interests or potential conflicts of interest.

**Acknowledgements:**
This work was supported by the National Institute on Drug Abuse grants P50DA047233 and R37DA007359 to EJN; R00DA045795 and P30DA033934 to PJH. Microscopy was performed at the VCU Microscopy Facility, supported, in part, by funding from NIH-NCI Cancer Center Support Grant P30CA016059. Services in support of the research project were provided by the VCU Massey Cancer Center Transgenic/Knockout Mouse Core, supported, in part, with funding from NIH-NCI Cancer Center Support Grant P30CA016059.
References:


**Figure 1: Zfp189 expression in the nucleus accumbens is increased in response to cocaine.**

(a) The experimental timeline to determine the effect of acute or chronic intraperitoneal (I.P.) injections of cocaine on NAc Zfp189 mRNA expression. Each bubble represents a day. Light blue bubbles correspond to I.P. saline, whereas red bubbles correspond to I.P. 20 mg/kg cocaine. **(b)** Bilateral NAc Zfp189 mRNA levels quantified via qRT-PCR from each treatment condition. An acute cocaine injection significantly increased Zfp189 mRNA levels relative to saline-treated animals. One way ANOVA followed by Holm-Sidak’s multiple comparison test; * p-value < 0.05. n = 7 (Saline; Acute cocaine) or 9 (Chronic cocaine). (c) The rate of intravenous self-administration (IVSA) infusions for mice either self-administering cocaine (0.5 mg/kg/infusion) or saline on a FR1 schedule of reinforcement in 3 hour sessions. Cocaine IVSA mice self-administer more infusions relative to saline IVSA mice. Two-way repeated measures ANOVA drug effect; * p-value < 0.05. n = 5-7 mice per condition. **(d)** Bilateral NAc Zfp189 mRNA levels quantified via qRT-PCR from each treatment condition. A history of cocaine IVSA results in a trend towards increased NAc Zfp189 mRNA levels relative to saline IVSA animals. p-value = 0.066. Student’s t-test. n = 5 mice (saline IVSA), 7 mice (cocaine IVSA).
**Figure 2:** CREB-mediated Zfp189 induction within nucleus accumbens diminishes the rewarding effects of cocaine. (a) Cartoon of CRISPR-mediated localization of CREB$^{S133D}$ to the Zfp189 promoter within mouse NAc. (b) qRT-PCR quantification of NAc Zfp189 mRNA from mice virally manipulated with HSV-dCas9-CREB$^{S133D}$ and either non-targeting (NT) or Zfp189-targeting sgRNA. Localizing dCas9-CREB$^{S133D}$ to the Zfp189 promoter results in elevated NAc Zfp189 expression. Two-tailed Student’s t-test; * p-value < 0.05. n = 11 (NT-sgRNA) and 17 (Zfp189-sgRNA) mice. (c) Experimental timeline for cocaine conditioned place preference (CPP). (d) Mice in which the NAc CREB-Zfp189 interaction is induced spend less time on the 7.5 mg/kg I.P. cocaine-paired side of the CPP chamber. Two-tailed Student’s t-test; * p-value < 0.05. n = 18 (NT-sgRNA) and 22 (Zfp189-sgRNA) mice. (e) Experimental timeline for mouse cocaine intravenous self-administration. (f) Mice were virally delivered CRISPR tools to localize either the transcriptionally activating CREB$^{S133D}$ or the transcriptionally repressive histone methyltransferase G9a to the Zfp189 promoter within NAc. Mice in which NAc Zfp189 levels are elevated via the CREB-Zfp189 interaction elect to self-administer more cocaine infusions than mice in which G9a is targeted to the Zfp189 promoter. 0.5 mg/kg/infusion at a fixed ratio of one (FR1) for a 3-h session. Two-way repeated measures ANOVA viral treatment effect within sessions post viral delivery; $F_{(1,17)} = 6.686; *$ p-value < 0.05. n = 10 (dCas9-CREB$^{S133D}$) and 9 (dCas9-G9a) mice.
Figure 3: Acute cocaine exposure selectively induces Zfp189 expression within D1 MSNs, whereas chronic cocaine exposure induces Zfp189 expression within D1 and D2 MSNs. (a) The experimental timeline of acute 20 mg/kg I.P. cocaine injection. (b) Representative images for Drd1, Drd2, and Zfp189 mRNA probes in mouse NAc from animals acutely exposed to cocaine. Scale bar is 30 µm. Merged image is magnified 2x, labeled with DAPI nuclear labeling, and Zfp189+ regions are denoted with white arrows. (c) Quantification of the percentage of Drd1 or Drd2 positive medium spiny neurons (MSNs) that express Zfp189 in each treatment condition. Two-way ANOVA followed by Holm-Sidak’s multiple comparison test; **** p-value < 0.0001. n = 4-5 mice per condition. (d) The experimental timeline of chronic 20 mg/kg I.P. cocaine injection. (e) Representative images for Drd1, Drd2, and Zfp189 mRNA probes in mouse NAc from animals chronically exposed to cocaine. Scale bar is 30 µm. Merged image is magnified 2x, labeled with DAPI nuclear labeling, and Zfp189+ regions are denoted with white arrows. (f) Quantification of the percentage of Drd1 or Drd2 positive MSNs that express Zfp189 in each treatment condition. Two-way ANOVA followed by Holm-Sidak’s multiple comparison test; *** p-value < 0.001. * p-value < 0.05. n = 5 mice per condition.
Figure 4: Cocaine exposure and CRISPR-mediated CREB-Zfp189 interactions differentially regulate excitatory synaptic transmission to D1 and D2 MSNs. (a) Whole-cell patch-clamp recording of spontaneous excitatory postsynaptic current (sEPSC) amplitudes from NAc MSNs in acutely prepared brain slices from mice with intra-NAc injection of either HSV-GFP or HSV-Zfp189 immediately after acute injections of either saline or cocaine (10 mg/kg). Neither viral treatment nor drug experience affects MSN sEPSC amplitudes. Two-way ANOVA: viral effect $F_{(2,82)} = 0.89$; drug effect, $F_{(1,82)} = 0.01$; $n = 10-13$ recordings from 7-8 mice (non-transduced), 18-19 recordings from 5 mice (HSV-GFP) and 14 recordings from 4 mice (HSV-Zfp189). (b) Acute cocaine I.P. injection increases the sEPSC frequency in all treatment groups, and HSV-Zfp189 expression is sufficient to enhance sEPSC frequency in saline mice to levels observed in cocaine mice. Two-way ANOVA viral or drug treatment effect; $F_{(2,84)} = 6.504$ (viral effect); $F_{(1,84)} = 18.23$ (drug effect); Holm-Sidak’s multiple comparison test; * $p$-value < 0.05. $n = 12-13$ recordings from 7-8 mice (HSV-Zfp189), 18-19 recordings from 5 mice (HSV-GFP) and 14 recordings from 4 mice (non-transduced). (c) To assess the MSN-type specific consequences of CREB-mediated induction of Zfp189 on cocaine-induced physiological function, we combined viral delivery of Cre-dependent CRISPR expression vectors and transgenic mice which express Cre-recombinase under the Drd1 or Drd2 promoter. (top) Representative sEPSCs in transduced D1 and D2 MSNs. (bottom) In catalyzing CREB-Zfp189 interactions within D1 MSNs and subjecting mice to the acute cocaine regimen, either an acute cocaine injection or CRISPR-mediated CREB-Zfp189 interactions is sufficient to increase sEPSC frequency in D1 MSNs. Two-way ANOVA followed by Holm-Sidak’s multiple comparison test; **,*** $p$-value < 0.001, 0.0005. $n = 13-16$ recordings from 5-6 mice (NT-sgRNA) and 12 recordings from 4-5 mice (Zfp189-sgRNA). (d) The experiment is repeated in Drd2-Cre mice. (top) Representative
sEPSCs. (bottom) In delivering CREB-Zfp189 interactions within D2 MSNs and subjecting mice to the treatment regimen described above, we observe that only CRISPR-mediated CREB-Zfp189 interactions, and not an acute cocaine treatment, increases sEPSC frequency in D2 MSNs. Two-way ANOVA followed by Holm-Sidak’s multiple comparison test; * p-value < 0.05. n = 16-17 recordings from 4-5 mice (NT-sgRNA) and 15-18 recordings from 5 mice (Zfp189-sgRNA).
Figure 5: CREB-mediated Zfp189 induction within NAc Drd2+ neurons drives reduced sensitivity to the reinforcing properties of cocaine.

(a) Experimental timeline for cocaine conditioned place preference (CPP) in Drd1- or Drd2-Cre+ mice. (b) Drd1-Cre mice in which the NAc CREB-Zfp189 interaction is induced within Drd1+ neurons show no effect relative to non-targeting (NT)-sgRNA controls; cocaine 7.5 mg/kg. Two-tailed Student’s t-test; p-value > 0.05. n = 15 (NT-sgRNA) and 13 (Zfp189-sgRNA) mice. (c) Drd2-Cre mice in which the NAc CREB-Zfp189 interaction is induced within Drd2+ neurons show reduced cocaine CPP relative to non-targeting (NT)-sgRNA controls; cocaine 15 mg/kg. Two-tailed Student’s t-test; p-value < 0.05. n = 8 (NT-sgRNA) and 6 (Zfp189-sgRNA) mice.
A) Schematic of the RNA-guided Cas9 system for genome editing.

B) Graph showing the change in Zfp189 mRNA fold change normalized to non-targeting sgRNA.

C) Timeline for Cocaine Infusions (3 hr session) and Intra-NAc infusion.

D) Bar graph showing CFP Score (seconds) for different conditions.

E) Timeline for JVC surgery, Food training, Recovery, Cocaine IVSA, and Intra-NAc infusion.

F) Graph showing Cocaine Infusions (3 hr session) across different conditions.
**A**

![Graph showing sEPSC amplitude (pA)](image)

**C**

![Electrophysiological traces for D1 MSNs](image)

**D**

![Electrophysiological traces for D2 MSNs](image)
**A**

Intra-NAc infusion

<table>
<thead>
<tr>
<th>Day</th>
<th>Pretest</th>
<th>Posttest</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Saline AM

Drug PM

**B**

*Drd1+ neurons*

<table>
<thead>
<tr>
<th>CPP Score (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-NT-sgRNA</td>
</tr>
<tr>
<td>HSV-Zfp189-sgRNA</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>HSV-LSL-dCas9-CREB$^{S133D}$</td>
</tr>
</tbody>
</table>

**C**

*Drd2+ neurons*

<table>
<thead>
<tr>
<th>CPP Score (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-NT-sgRNA</td>
</tr>
<tr>
<td>HSV-Zfp189-sgRNA</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>HSV-LSL-dCas9-CREB$^{S133D}$</td>
</tr>
</tbody>
</table>