Biological Psychiatry

Archival Report

CREB Binding at the *Zfp189* Promoter Within Medium Spiny Neuron Subtypes Differentially Regulates Behavioral and Physiological Adaptations Over the Course of Cocaine Use

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ABSTRACT

BACKGROUND: Over the course of chronic drug use, brain transcriptional neuroadaptation is thought to contribute to a change in drug use behavior over time. The function of the transcription factor CREB (cAMP response element binding protein) 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 the NAc of mice.

METHODS: To investigate the role of the CREB-*Zfp189* interaction in cocaine use, we virally delivered modified clustered regularly interspaced short palindromic repeats (CRISPR)/dCas9 constructs capable of selectively localizing CREB to the *Zfp189* gene promoter in the NAc of mice.

RESULTS: We observed that CREB binding to the *Zfp189* promoter increased *Zfp189* expression and diminished the reinforcing responses to cocaine. Furthermore, we showed that NAc *Zfp189* expression increased within D1 medium spiny neurons in response to acute cocaine but increased in both D1- and D2-expressing medium spiny neurons in response to chronic cocaine. CREB-mediated induction of *Zfp189* potentiated electrophysiological activity of D1- and D2-expressing medium spiny neurons, recapitulating the known effect of CREB on these neurons. Finally, 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 the NAc *Drd2*+ neurons as a molecular signature of chronic cocaine use that is causal in counteracting the reinforcing effects of cocaine.

https://doi.org/10.1016/j.biopsych.2022.07.022

Repeated drug use is correlated with persistent 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 CREB (cAMP response element binding protein) 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 through binding to CRE motifs within the promoter or enhancer regions of target genes and recruiting CREB binding protein, a histone acetyltransferase, and the basal transcription complex to these genes (8,12). CREB was implicated initially in drug addiction based on the empirical

ISSN: 0006-3223

observation that cocaine and morphine elevate adenylyl cyclase and cAMP-dependent protein kinase A levels in the NAc (2,6). Protein kinase A 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 paired with promoter microarrays identified numerous putative CREB target genes that may mediate its effects on drug tolerance, but most of these genes are yet to be studied (7,8). In addition, it remains unclear whether 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 CRISPR (clustered regularly interspaced short palindromic repeats)-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^{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^{S133D} specifically to the *Zfp189* promoter, initially throughout the 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 (RNAScope Multiplex Fluorescent V2 Assay; Advanced Cell Diagnostics).

Animals

C57BL/6J male and female mice ages 8–12 weeks were acquired from The Jackson Laboratory. D1-Cre and D2-Cre bacterial artificial chromosome transgenic mice (http://www.gensat.org/cre.jsp) were bred in-house. Animals were housed at 22–25 °C in a 12-hour 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 Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai or the Virginia Commonwealth University School of Medicine.

Behavioral Paradigms

For CPP, mice were placed in a 3-chambered CPP box for 20 minutes to assess pretest preferences. For the next 2 days, mice were injected in the morning (saline) and afternoon (drug) and then restricted to 1 chamber of the box for 30 minutes. During the posttest, mice were placed in the CPP box with free access to all chambers of the box for a 20-minute test session. Data are represented as the time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber during the posttest.

Viral Reagents

We used 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 40× magnification using a Zeiss Laser Scanning Microscope (LSM) 900 confocal microscope, and images were quantified using CellProfiler 4.2.1 (23).

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from frozen dissected NAc tissue using the RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. Following isolation, RNA was quantified by Nano Drop (Thermo Fisher) and converted to complementary DNA with iScript (Bio-Rad). Quantitative polymerase chain reaction samples were analyzed in triplicate using the standard ΔΔCT method. Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) was used 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 injections of either saline or 20 mg/kg cocaine (Figure 1A). Compared with saline-treated mice, mice

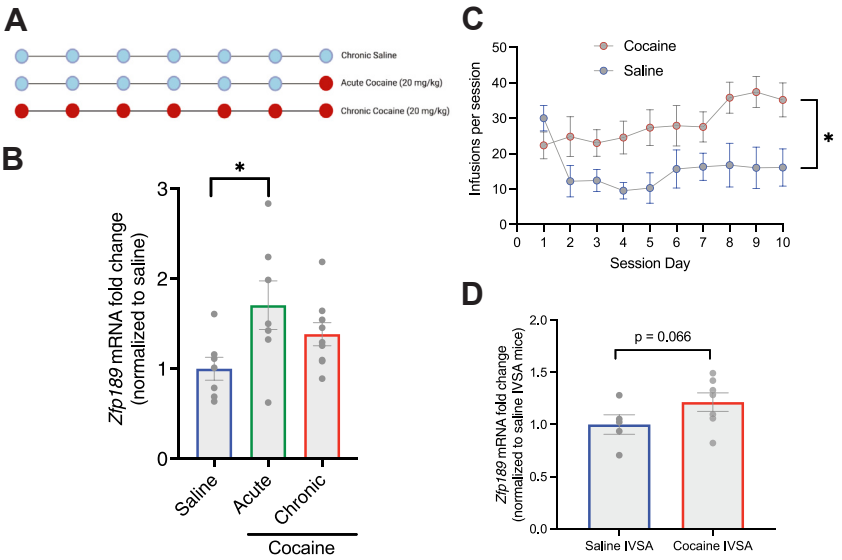

Figure 1. *Zfp189* expression in the NAc is increased in response to cocaine. **(A)** The experimental timeline to determine the effect of acute or chronic intraperitoneal injections of cocaine on NAc *Zfp189* mRNA expression. Each bubble represents a day. Light blue bubbles correspond to intraperitoneal saline, whereas red bubbles correspond to intraperitoneal 20-mg/kg cocaine. **(B)** Bilateral NAc *Zfp189* mRNA levels quantified via quantitative real time polymerase chain reaction from each treatment condition. An acute cocaine injection significantly increased *Zfp189* mRNA levels relative to saline-treated animals. One-way analysis of variance followed by Holm-Sidak's multiple comparison test; *p value $<$ .05. n = 7 (saline; acute cocaine) or 9 (chronic cocaine). **(C)** The rate of IVSA infusions for mice self-administering either cocaine (0.5 mg/kg/infusion) or saline on a fixed ratio of 1 schedule of reinforcement (i.e., reinforcement given after every response) in 3-hour sessions. Cocaine IVSA mice self-administered more infusions relative to saline IVSA mice. Two-way repeated-measures analysis of variance drug effect; *p value $<$ .05. n = 5–7 mice per condition. **(D)** Bilateral NAc *Zfp189* mRNA levels quantified via quantitative real-time polymerase chain reaction from each treatment condition. A history of cocaine IVSA resulted in a trend toward increased NAc *Zfp189* mRNA levels relative to saline IVSA animals. p = .066. Student t test. n = 5 mice (saline IVSA), 7 mice (cocaine IVSA). IVSA, intravenous self-administration; mRNA, messenger RNA; NAc, nucleus accumbens.

treated with a single, acute cocaine injection exhibited a significant increase in *Zfp189* messenger RNA expression within the NAc (Figure 1B). Mice treated with chronic cocaine did not display a significant increase in *Zfp189* expression (Figure 1B), revealing dynamic regulation of NAc *Zfp189* expression over the time course of cocaine exposure.

Next, we 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-administered significantly more infusions than saline IVSA mice (Figure 1C). Furthermore, there was a trend toward increased NAc *Zfp189* expression in mice with a history of cocaine IVSA (Figure 1D). These data suggest that contingent and noncontingent cocaine exposure may similarly increase NAc *Zfp189* messenger RNA expression.

Because the promoter region of *Zfp189* possesses a CRE motif (12,19) and CREB has been shown 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 determine 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 through an sgRNA in proximity to the consensus CRE motif in the *Zfp189* promoter (*Zfp189*-sgRNA) (Figure 2A). This approach has been validated and applied by our group to the *Zfp189* locus in prefrontal cortex neurons to study stress-related behaviors (19) and to the *Fosb* locus in the NAc to study MSN-specific transcriptional responses (22).

We packaged dCas9-CREB^{S133D}, *Zfp189*-sgRNA, and the control nontargeting 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 increased *Zfp189* expression (Figure 2B). To investigate 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 (Figure 2C, D). We found that animals with CREB-mediated *Zfp189* activation throughout the NAc neurons exhibited decreased preference for the 7.5 mg/kg cocaine-paired side of the chamber relative to control animals (Figure 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 (Figure S1 in Supplement 1), suggesting a similar role for CREB-*Zfp189* interaction in governing cocaine-related behaviors in both sexes.

Interestingly, on 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 (Figure S2 in Supplement 1), 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 (Figure 2E). We elected to bidirectionally 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, which we have demonstrated previously suppresses *Zfp189* expression in the brain (19). While there was a general reduction of IVSA rates

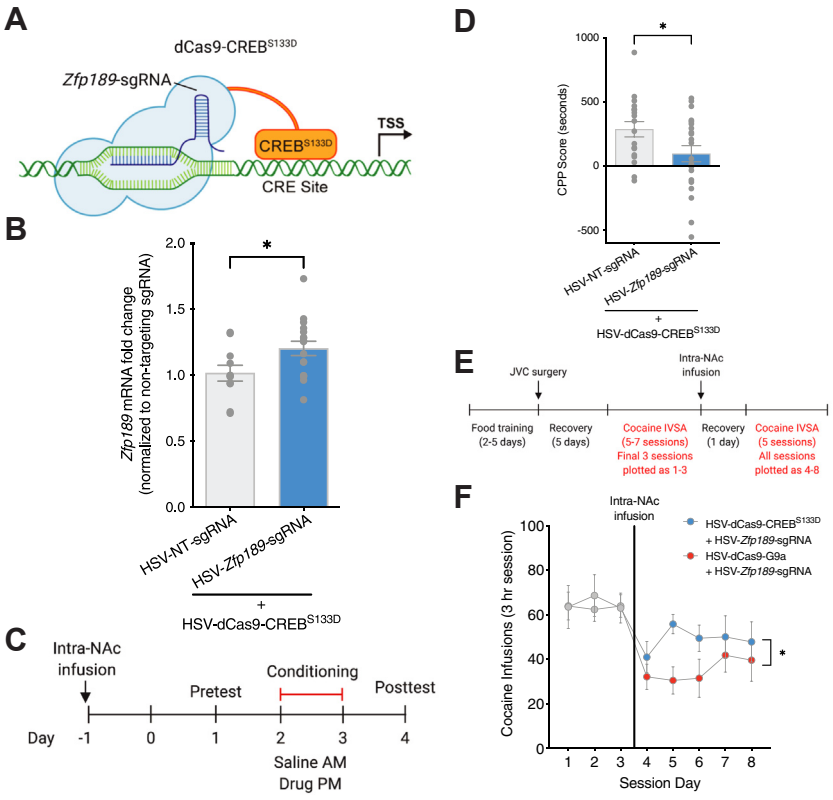

Figure 2. CREB-mediated *Zfp189* induction within the NAc diminishes the rewarding effects of cocaine. **(A)** Illustration of CRISPR-mediated localization of CREB^{S133D} to the *Zfp189* promoter within mouse NAc. **(B)** qRT-PCR quantification of NAc *Zfp189* mRNA from mice virally manipulated with HSV-dCas9-CREB^{S133D} and either NT or *Zfp189*-targeting sgRNA. Localizing dCas9-CREB^{S133D} to the *Zfp189* promoter resulted in elevated NAc *Zfp189* expression. Two-tailed Student *t* test; **p* value $<$ .05. n = 11 (NT-sgRNA) and 17 (*Zfp189*-sgRNA) mice. **(C)** Experimental timeline for cocaine CPP. **(D)** Mice with induced NAc CREB-*Zfp189* interaction spent less time on the 7.5 mg/kg intraperitoneal cocaine-paired side of the CPP chamber. Two-tailed Student *t* test; **p* value $<$ .05. n = 18 (NT-sgRNA) and 22 (*Zfp189*-sgRNA) mice. **(E)** Experimental timeline for mouse cocaine IVSA. **(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 the NAc. Mice with NAc *Zfp189* levels elevated via the CREB-*Zfp189* interaction elected to self-administer more cocaine infusions than mice in which G9a was targeted to the *Zfp189* promoter. 0.5 mg/kg/infusion at a FR1 for a 3-hour session. Two-way repeated-measures ANOVA viral treatment effect within sessions post viral delivery; $F_{1,17}$ = 6.686; **p* value $<$ .05. n = 10 (dCas9-CREB^{S133D}) and 9 (dCas9-G9a) mice. ANOVA, analysis of variance; CPP, conditioned place preference; CREB, cAMP response element binding protein; CRISPR, clustered regularly interspaced short palindromic repeats; FR, fixed ratio; HSV, herpes simplex virus; IVSA, intravenous self-administration; JVC, jugular vein cannulation; mRNA, messenger RNA; NAc, nucleus accumbens; NT, nontargeting; qRT-PCR, quantitative real-time polymerase chain reaction; sgRNA, single guide RNA; TSS, transcription start site.

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 (Figure 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 2 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 such as cocaine. Therefore, we investigated MSN subtype–specific expression of *Zfp189* in the NAc in response to either acute or chronic cocaine exposure (Figure 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 exhibited a robust increase in the number of *Zfp189*+ cells (Figure 3A–C). This observation is consistent with rodent NAc single-cell RNA sequencing data demonstrating that acute cocaine upregulates *Zfp189* specifically in the *Drd1*+ cell cluster (34). After chronic cocaine, by contrast, we observed an increased number of *Zfp189*+ cells in both D1- and D2-MSNs (Figure 3D–F). Thus, over the course of cocaine exposure, *Zfp189* was activated within D1-MSNs acutely and in both D1- and D2-MSNs at chronic time points. 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 time point between Figures 1 and 3. Therefore, while acute cocaine induced a significant increase in total NAc *Zfp189* expression levels (localized primarily within D1-MSNs), chronic cocaine increased 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* through a conventional

overexpression vector in the NAc, which has been validated in the 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 (Figure 4A), this acute cocaine procedure increased the frequency of sEPSCs in both nontransduced and green fluorescent protein–expressing MSNs (Figure 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 nonadditive (Figure 4B). These results indicate that acute cocaine treatment specifically enhances sEPSC frequency in NAc MSNs and that *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 use Cre-dependent (loxP-STOP-loxP) expression vectors to facilitate CRISPR-mediated CREB-*Zfp189* interaction in an MSN subtype–dependent manner. This approach has been applied by our group previously (22), and as in this earlier work, we saw *Zfp189* activation preferentially in the NAc neurons that are conditionally expressing our CRISPR constructs (Figure S3 in Supplement 1). In *Drd1*-Cre+ mice in the loxP-STOP-loxP-dCas9-CREB^{S133D}+NT-sgRNA control condition, acute cocaine treatment potentiated sEPSC frequency (Figure 4C), consistent with our observations in wild-type mice (Figure 4B). In addition, targeting CREB to the *Zfp189* promoter within D1-MSNs was sufficient to potentiate sEPSC frequency, recapitulating the effect of acute cocaine (Figure 4C). In *Drd2*-Cre+ mice, acute cocaine had no effect on sEPSC frequency in the control viral treatment condition (Figure 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 *Zfp189* promoter within D2-MSNs increased the sEPSC frequency in both the saline and cocaine treatment conditions (Figure 4D). Collectively, these data reveal that only D1-MSNs were sensitive to acute cocaine exposure, yet CRISPR-mediated delivery of CREB to the *Zfp189* promoter was sufficient to potentiate the function of both D1- and D2-MSN subtypes. Furthermore, none of our manipulations affected basal MSN electrophysiological metrics such as membrane capacitance or membrane resistance (Figure S4 in Supplement 1).

Catalyzing the CREB-*Zfp189* Interaction Specifically Within NAc *Drd2*+ Neurons Reduces CPP for Cocaine

Next, we 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 through CRISPR tools had no effect on cocaine CPP (Figure 5B). By contrast, inducing the CREB-*Zfp189* interaction in *Drd2*+ neurons significantly decreased cocaine CPP (Figure 5C). This latter effect is consistent with the effect of CREB-*Zfp189* interaction in all neurons in the NAc (Figure 2D), consistent with the interpretation that this latter effect is driven by the CREB-mediated induction of *Zfp189* in *Drd2*+ neurons selectively.

DISCUSSION

In this study, we investigated a cell type–specific molecular mechanism by which CREB regulates cocaine-induced neuroadaptations in the NAc through the induction of *Zfp189*. Our findings show that in response to a single dose of cocaine, *Zfp189* was rapidly and selectively induced in D1-MSNs. This expression persisted in D1-MSNs following repeated doses of cocaine. However, at these chronic time points, *Zfp189* expression became induced in D2-MSNs as well. We further found that this increased *Zfp189* expression in D2-MSNs promoted 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 have summarized this proposed neurobiological mechanism as a graphic in Figure S5 in Supplement 1. This work points to an 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 the 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 (Figure 3C) and that each of our behavioral paradigms involves pretreatment 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, because observed that *Zfp189* is induced within *Drd2*+ NAc neurons only after chronic cocaine treatment (Figure 3F), our NAc-wide CREB-*Zfp189* interaction may be preferentially 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 (Figure 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). Furthermore, considerable evidence points to the function of D1-MSNs regulating the function of D2-MSNs through 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 the dramatic effects of cocaine exposure on gene expression within cholinergic interneurons (36). Importantly, for the experiments reported within this article, we used *Drd2*-Cre+ mice, which drive the expression

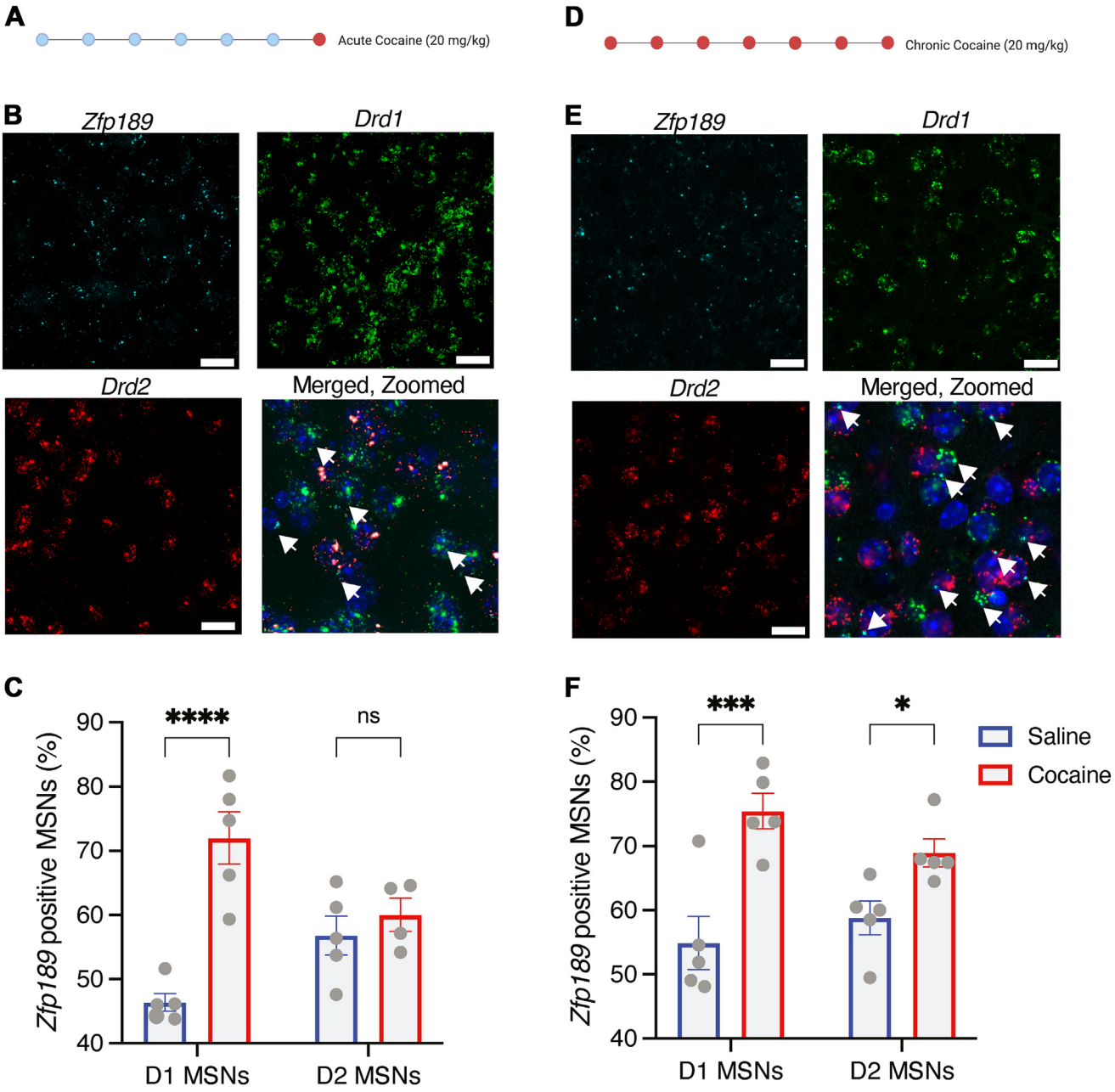

Figure 3. Acute cocaine exposure selectively induces *Zfp189* expression within D1-MSNs, whereas chronic cocaine exposure induces *Zfp189* expression within D1- and D2-MSNs. **(A)** The experimental timeline of acute 20 mg/kg IP cocaine injection. **(B)** Representative images for *Drd1, Drd2*, and *Zfp189* mRNA probes in mouse NAc from animals acutely exposed to cocaine. Scale bar = 30 μm. Merged image is magnified 2×, labeled with DAPI nuclear labeling, and *Zfp189*+ regions are denoted with white arrows. **(C)** Quantification of the percentage of *Drd1*- or *Drd2*-positive MSNs that expressed *Zfp189* in each treatment condition. Two-way analysis of variance followed by Holm-Sidak's multiple comparison test; ****p value $<$ .0001. n = 4–5 mice per condition. **(D)** The experimental timeline of chronic 20 mg/kg IP cocaine injection. **(E)** Representative images for *Drd1*, *Drd2*, and *Zfp189* mRNA probes in mouse NAc from animals chronically exposed to cocaine. Scale bar = 30 μm. Merged image is magnified 2×, 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 expressed *Zfp189* in each treatment condition. Two-way analysis of variance followed by Holm-Sidak's multiple comparison test; *p $<$ .05; ***p $<$ .001. n = 5 mice per condition. IP, intraperitoneal; MSN, medium spiny neuron; mRNA, messenger RNA; NAc, nucleus accumbens; ns, nonsignificant.

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 affected *Zfp189* function within NAc *Drd2*+ cholinergic interneurons, which might have contributed to our results, specifically those shown in Figure 5C.

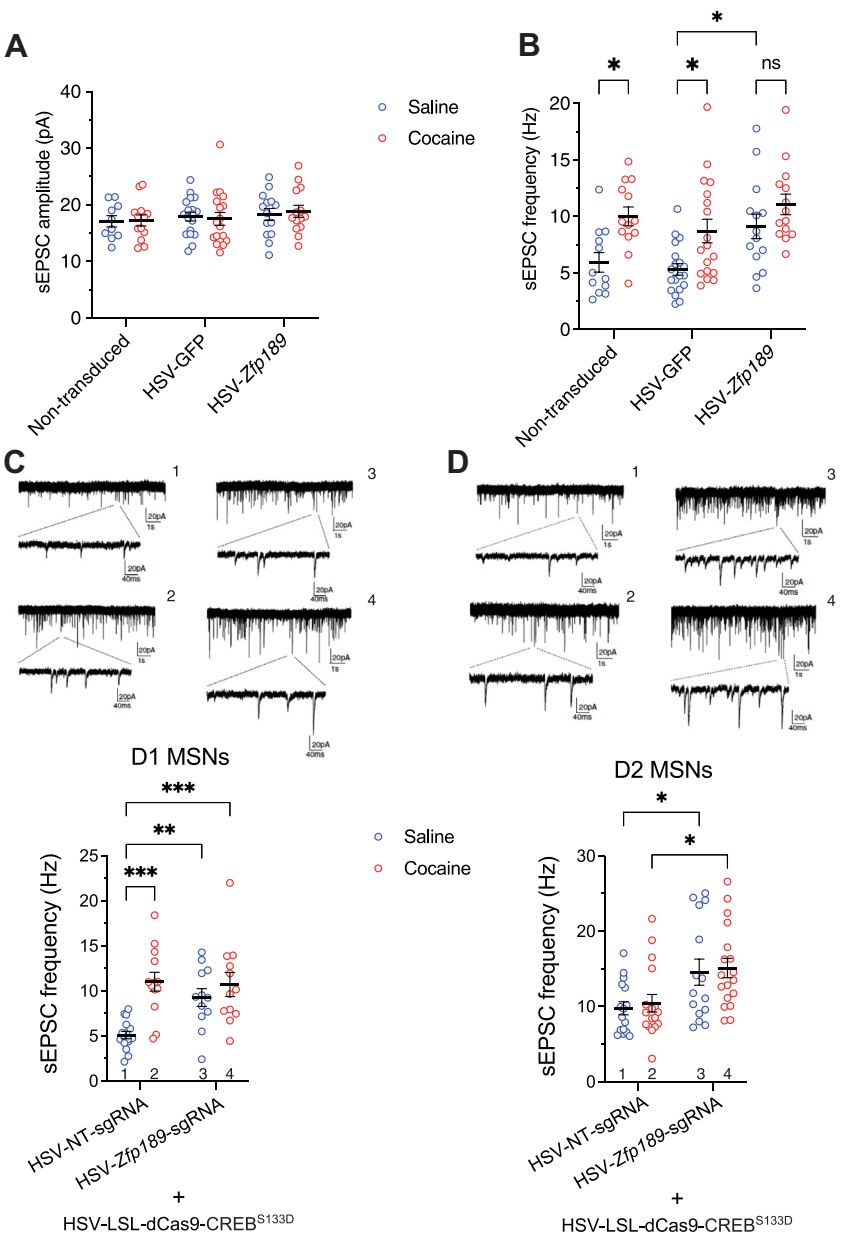

Figure 4. Cocaine exposure and CRISPR-mediated CREB-*Zfp189* interactions differentially regulate excitatory synaptic transmission to D1- and D2-MSNs. **(A)** Whole-cell patch-clamp recording of sEPSC amplitudes from NAc MSNs in acutely prepared brain slices from mice with intra-NAc injection of either HSV-GFP or HSV-*Zfp189* immediately after acute injections of either saline or cocaine (10 mg/kg). Neither viral treatment nor drug experience affected MSN sEPSC amplitudes. Two-way analysis of variance: viral effect $F_{2,82}$ = 0.89; drug effect, $F_{1,82}$ = 0.01; n = 10 to 13 recordings from 7 to 8 mice (nontransduced), 18 to 19 recordings from 5 mice (HSV-GFP), and 14 recordings from 4 mice (HSV-*Zfp189*). **(B)** Acute cocaine IP injection increased the sEPSC frequency in all treatment groups, and HSV-*Zfp189* expression was sufficient to enhance sEPSC frequency in saline mice to levels observed in cocaine mice. Two-way analysis of variance 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 $<$ .05. n = 12 to 13 recordings from 7 to 8 mice (HSV-*Zfp189*), 18 to 19 recordings from 5 mice (HSV-GFP), and 14 recordings from 4 mice (nontransduced). **(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 panel) Representative sEPSCs in transduced D1- and D2-MSNs. (Bottom panel) In catalyzing CREB-*Zfp189* interactions within D1-MSNs and exposing mice to the acute cocaine regimen, either an acute cocaine injection or CRISPR-mediated CREB-*Zfp189* interactions was sufficient to increase sEPSC frequency in D1-MSNs. Two-way analysis of variance followed by Holm-Sidak's multiple comparison test; **p $<$ .001, ***p $<$ .0005. n = 13 to 16 recordings from 5 or 6 mice (NT-sgRNA) and 12 recordings from 4 or 5 mice (*Zfp189*-sgRNA). **(D)** The experiment was repeated in *Drd2*-Cre mice. (Top panel) Representative sEPSCs. (Bottom panel) In delivering CREB-*Zfp189* interactions within D2-MSNs and exposing mice to the treatment regimen described above, we observed that only CRISPR-mediated CREB-*Zfp189* interactions, and not an acute cocaine treatment, increased sEPSC frequency in D2-MSNs. Two-way analysis of variance followed by Holm-Sidak's multiple comparison test; *p value $<$ .05. n = 16 to 17 recordings from 4 or 5 mice (NT-sgRNA) and 15 to 18 recordings from 5 mice (*Zfp189*-sgRNA). CREB, cAMP response element binding protein; CRISPR, clustered regularly interspaced short palindromic repeats; HSV-GFP, herpes simplex virus–green fluorescent protein; IP, intraperitoneal; MSN, medium spiny neuron; NAc, nucleus accumbens; ns, nonsignificant; NT, nontargeting; sEPSC, spontaneous excitatory postsynaptic current; sgRNA, single guide RNA.

The exact brain mechanisms responsible for the diminished behavioral sensitivity to cocaine seen in response to *Zfp189* induction in the 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 a decreased sensitivity to a drug following repeated exposures. Conversely, it is possible that the *Zfp189*-driven function of D1-MSNs opposes the *Zfp189*-driven function of D2-MSNs in an opponent process that 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 exposure. 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 currently 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

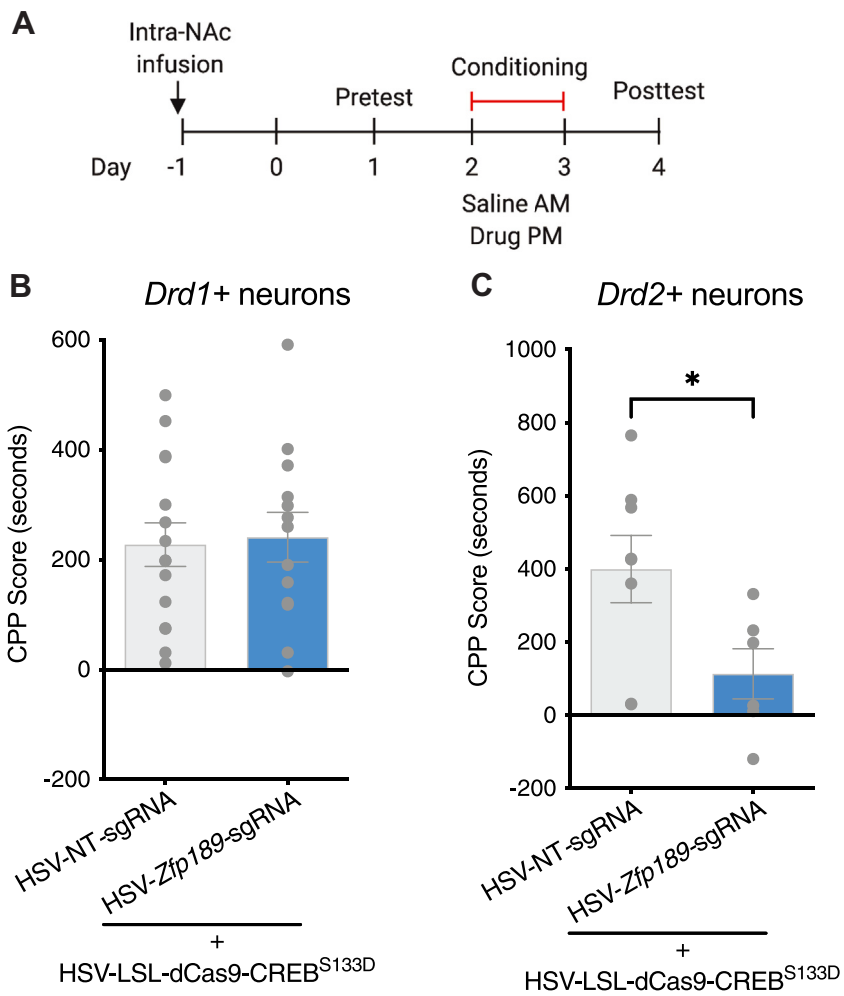

Figure 5. CREB-mediated *Zfp189* induction within NAc *Drd2*+ neurons drives reduced sensitivity to the reinforcing properties of cocaine. **(A)** Experimental timeline for cocaine conditioned place preference in *Drd1*- or *Drd2*-Cre+ mice. **(B)** *Drd1*-Cre mice in which the NAc CREB-*Zfp189* interaction was induced within *Drd1*+ neurons show no effect relative to NT-sgRNA controls; cocaine 7.5 mg/kg. Two-tailed Student *t* test; $p > .05$. $n = 15$ (NT-sgRNA) and 13 (*Zfp189*-sgRNA) mice. **(C)** *Drd2*-Cre mice in which the NAc CREB-*Zfp189* interaction is induced within *Drd2*+ neurons showed reduced cocaine conditioned place preference relative to NT-sgRNA controls; cocaine 15 mg/kg. Two-tailed Student *t* test; $^*p < .05$. $n = 8$ (NT-sgRNA) and 6 (*Zfp189*-sgRNA) mice. CREB, cAMP response element binding protein; NAc, nucleus accumbens; NT, nontargeting; sgRNA, single guide RNA.

expression of transcription factor regulatory cofactors, *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 vary 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 initiated the endogenous, drug-course–dependent mechanism of CREB regulation and precipitated the behavioral and physiological consequences of CREB-mediated *Zfp189* induction in the NAc. While our cocaine treatment regimen was acute in our electrophysiological experiments, our CRISPR-mediated induction of CREB-*Zfp189* interaction within D2-MSNs modeled the transcriptional regulation that would occur with 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 or 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), that cocaine exposure results in increased frequency of glutamate-mediated evoked excitatory postsynaptic currents upon NAc MSNs (40), and that altered function of MSNs can regulate synaptic transmission upon distinct MSN subtypes through multineuronal circuit regulation (35). Furthermore, 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 rather than 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 through 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 (Figure S2 in Supplement 1). 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 the 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 cocaine-related outcomes specifically. The degree to which other classes of stimulants, such as 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 domain containing zinc finger protein, which suggests that ZFP189 acts as a transcription factor, but its function remains poorly understood (42). *Zfp189* induction in prefrontal cortex 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 the 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.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by the National Institute on Drug Abuse grants (Grant Nos. P50DA047233 and R37DA007359 [to EJN]; R00DA045795 and P30DA033934 [to PJH]). Microscopy was performed at the Virginia Commonwealth University Microscopy Facility, supported, in part, by funding from National Institutes of Health-National Cancer Institute (NIH-NCI) Cancer Center Support grant (Grant No. P30CA016059 [to RAW]). Services in support of the research project were provided by the VCU Massey Cancer Center Transgenic or Knockout Mouse Core, supported, in part, with funding from NIH-NCI Cancer Center Support grant (Grant No. P30CA016059 [to RAW]).

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

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Received Jan 12, 2022; revised Jun 6, 2022; accepted Jul 5, 2022.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.biopsych.2022.07.022.

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