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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 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*

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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.

Keywords

Cocaine; transcription factor; CRISPR; addiction; nucleus accumbens; *Zfp189*

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^{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 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 *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-*Zfp189* interaction in an MSN-subtype-dependent manner. This approach has been previously applied by our group(22), and as in this earlier work, we see *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 *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 *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 *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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

1. Koob GF, Volkow ND (2016): Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry 3: 760–773. [PubMed: 27475769]
2. Nestler EJ, Aghajanian GK (1997): Molecular and cellular basis of addiction. Science 278: 58–63. [PubMed: 9311927]
3. Brunzell DH, Mineur YS, Neve RL, Picciotto MR (2009): Nucleus accumbens CREB activity is necessary for nicotine conditioned place preference. Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol 34: 1993–2001.
4. Pandey SC, Roy A, Zhang H, Xu T (2004): Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. J Neurosci Off J Soc Neurosci 24: 5022–5030.
5. Barrot M, Olivier JDA, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, et al. (2002): CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proc Natl Acad Sci U S A 99: 11435–11440. [PubMed: 12165570]
6. Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ (1991): A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 548: 100–110. [PubMed: 1651140]
7. Renthal W, Kumar A, Xiao G, Wilkinson M, Covington HE, Maze I, et al. (2009): Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. Neuron 62: 335–348. [PubMed: 19447090]
8. McClung CA, Nestler EJ (2003): Regulation of gene expression and cocaine reward by CREB and DeltaFosB. Nat Neurosci 6: 1208–1215. [PubMed: 14566342]
9. Teague CD, Nestler EJ (2021): Key transcription factors mediating cocaine-induced plasticity in the nucleus accumbens. Mol Psychiatry. 10.1038/s41380-021-01163-5

10. Carlezon WA, Duman RS, Nestler EJ (2005): The many faces of CREB. Trends Neurosci 28: 436–445. [PubMed: 15982754]
11. Hong EJ, West AE, Greenberg ME (2005): Transcriptional control of cognitive development. Curr Opin Neurobiol 15: 21–28. [PubMed: 15721740]
12. Zhang X, Odom DT, Koo S-H, Conkright MD, Canettieri G, Best J, et al. (2005): Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci U S A 102: 4459–4464. [PubMed: 15753290]
13. Mattson BJ, Bossert JM, Simmons DE, Nozaki N, Nagarkar D, Kreuter JD, Hope BT (2005): Cocaine-induced CREB phosphorylation in nucleus accumbens of cocaine-sensitized rats is enabled by enhanced activation of extracellular signal-related kinase, but not protein kinase A. J Neurochem 95: 1481–1494. [PubMed: 16219028]
14. Shaw-Lutchman TZ, Barrot M, Wallace T, Gilden L, Zachariou V, Impey S, et al. (2002): Regional and cellular mapping of cAMP response element-mediated transcription during naltrexone-precipitated morphine withdrawal. J Neurosci Off J Soc Neurosci 22: 3663–3672.
15. Shaw-Lutchman TZ, Impey S, Storm D, Nestler EJ (2003): Regulation of CRE-mediated transcription in mouse brain by amphetamine. Synap N Y N 48: 10–17.
16. Walters CL, Cleck JN, Kuo Y, Blendy JA (2005): Mu-opioid receptor and CREB activation are required for nicotine reward. Neuron 46: 933–943. [PubMed: 15953421]
17. Carlezon WA, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, et al. (1998): Regulation of cocaine reward by CREB. Science 282: 2272–2275. [PubMed: 9856954]
18. Larson EB, Graham DL, Arzaga RR, Buzin N, Webb J, Green TA, et al. (2011): Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats. J Neurosci Off J Soc Neurosci 31: 16447–16457.
19. Lorsch ZS, Hamilton PJ, Ramakrishnan A, Parise EM, Salery M, Wright WJ, et al. (2019): Stress resilience is promoted by a Zfp189-driven transcriptional network in prefrontal cortex. Nat Neurosci 22: 1413–1423. [PubMed: 31427770]
20. Hamilton PJ, Lim CJ, Nestler EJ, Heller EA (2018): Neuroepigenetic Editing. Methods Mol Biol Clifton NJ 1767: 113–136.
21. Yim YY, Teague CD, Nestler EJ (2020): In vivo locus-specific editing of the neuroepigenome [no. 9]. Nat Rev Neurosci 21: 471–484. [PubMed: 32704051]
22. Lardner CK, van der Zee Y, Estill MS, Kronman HG, Salery M, Cunningham AM, et al. (2021): Gene-Targeted, CREB-Mediated Induction of ΔFosB Controls Distinct Downstream Transcriptional Patterns Within D1 and D2 Medium Spiny Neurons. Biol Psychiatry 90: 540–549. [PubMed: 34425966]
23. McQuin C, Goodman A, Chernyshev V, Kamentsky L, Cimini BA, Karhohs KW, et al. (2018): CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol 16: e2005970. [PubMed: 29969450]
24. Neve RL, Neve KA, Nestler EJ, Carlezon WA (2005): Use of herpes virus amplicon vectors to study brain disorders. BioTechniques 39: 381–391. [PubMed: 16206910]
25. Lobo MK, Karsten SL, Gray M, Geschwind DH, Yang XW (2006): FACS-array profiling of striatal projection neuron subtypes in juvenile and adult mouse brains. Nat Neurosci 9: 443–452. [PubMed: 16491081]
26. Kronman H, Richter F, Labonté B, Chandra R, Zhao S, Hoffman G, et al. (2019): Biology and Bias in Cell Type-Specific RNAseq of Nucleus Accumbens Medium Spiny Neurons. Sci Rep 9: 8350. [PubMed: 31171808]
27. Tai L-H, Lee AM, Benavidez N, Bonci A, Wilbrecht L (2012): Transient stimulation of distinct subpopulations of striatal neurons mimics changes in action value. Nat Neurosci 15: 1281–1289. [PubMed: 22902719]
28. Calipari ES, Bagot RC, Purushothaman I, Davidson TJ, Yorgason JT, Peña CJ, et al. (2016): In vivo imaging identifies temporal signature of D1 and D2 medium spiny neurons in cocaine reward. Proc Natl Acad Sci U S A 113: 2726–2731. [PubMed: 26831103]
29. Heinsbroek JA, Neuhofer DN, Griffin WC, Siegel GS, Bobadilla A-C, Kupchik YM, Kalivas PW (2017): Loss of Plasticity in the D2-Accumbens Pallidal Pathway Promotes Cocaine Seeking. J Neurosci Off J Soc Neurosci 37: 757–767.

30. Cole SL, Robinson MJF, Berridge KC (2018): Optogenetic self-stimulation in the nucleus accumbens: D1 reward versus D2 ambivalence. PloS One 13: e0207694. [PubMed: 30496206]
31. Hikida T, Kimura K, Wada N, Funabiki K, Nakanishi S (2010): Distinct roles of synaptic transmission in direct and indirect striatal pathways to reward and aversive behavior. Neuron 66: 896–907. [PubMed: 20620875]
32. Lobo MK, Covington HE, Chaudhury D, Friedman AK, Sun H, Damez-Werno D, et al. (2010): Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. Science 330: 385–390. [PubMed: 20947769]
33. van Zessen R, Li Y, Marion-Poll L, Hulo N, Flakowski J, Lüscher C (2021): Dynamic dichotomy of accumbal population activity underlies cocaine sensitization. eLife 10: e66048. [PubMed: 34608866]
34. Savell KE, Tuscher JJ, Zipperly ME, Duke CG, Phillips RA, Bauman AJ, et al. (2020): A dopamine-induced gene expression signature regulates neuronal function and cocaine response. Sci Adv 6: eaba4221. [PubMed: 32637607]
35. Francis TC, Yano H, Demarest TG, Shen H, Bonci A (2019): High-Frequency Activation of Nucleus Accumbens D1-MSNs Drives Excitatory Potentiation on D2-MSNs. Neuron 103: 432–444.e3. [PubMed: 31221559]
36. Lee JH, Ribeiro EA, Kim J, Ko B, Kronman H, Jeong YH, et al. (2020): Dopaminergic Regulation of Nucleus Accumbens Cholinergic Interneurons Demarcates Susceptibility to Cocaine Addiction. Biol Psychiatry 88: 746–757. [PubMed: 32622465]
37. Lee J, Finkelstein J, Choi JY, Witten IB (2016): Linking Cholinergic Interneurons, Synaptic Plasticity, and Behavior during the Extinction of a Cocaine-Context Association. Neuron 90: 1071–1085. [PubMed: 27210555]
38. Cha-Molstad H, Keller DM, Yochum GS, Impey S, Goodman RH (2004): Cell-type-specific binding of the transcription factor CREB to the cAMP-response element. Proc Natl Acad Sci U S A 101: 13572–13577. [PubMed: 15342915]
39. Dong Y, Green T, Saal D, Marie H, Neve R, Nestler EJ, Malenka RC (2006): CREB modulates excitability of nucleus accumbens neurons. Nat Neurosci 9: 475–477. [PubMed: 16520736]
40. Dobi A, Seabold GK, Christensen CH, Bock R, Alvarez VA (2011): Cocaine-induced plasticity in the nucleus accumbens is cell specific and develops without prolonged withdrawal. J Neurosci Off J Soc Neurosci 31: 1895–1904.
41. Misra K, Pandey SC (2006): The decreased cyclic-AMP dependent-protein kinase A function in the nucleus accumbens: a role in alcohol drinking but not in anxiety-like behaviors in rats. Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol 31: 1406–1419.
42. Najafabadi HS, Mnaimneh S, Schmitges FW, Garton M, Lam KN, Yang A, et al. (2015): C2H2 zinc finger proteins greatly expand the human regulatory lexicon. Nat Biotechnol 33: 555–562. [PubMed: 25690854]

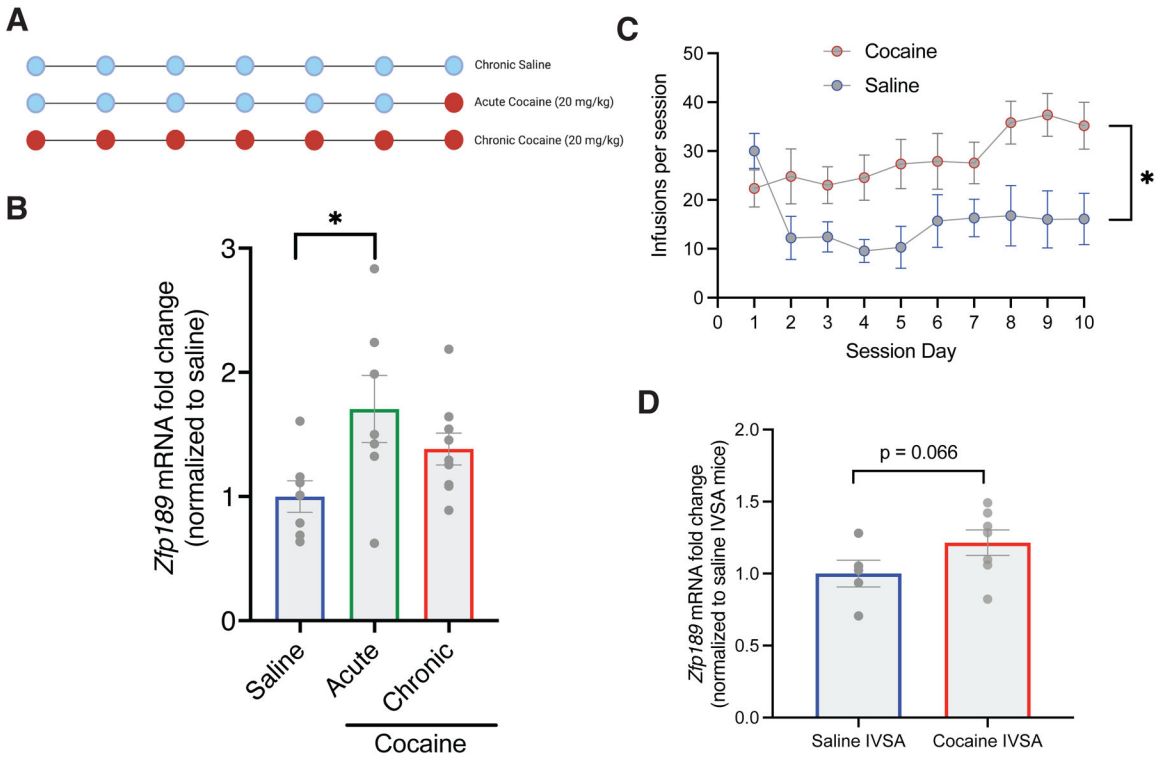

Figure 1: *Zfp189* expression in the nucleus accumbens is increased in response to cocaine.

(**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).

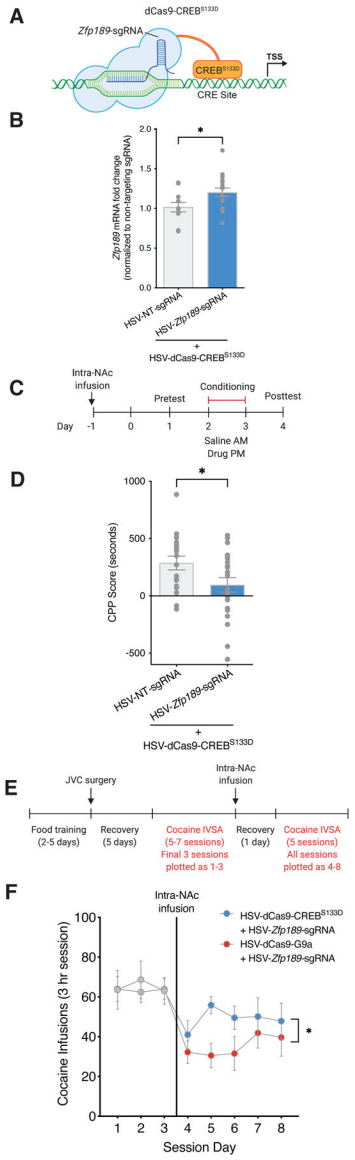

Figure 2: CREB-mediated *Zfp189* induction within nucleus accumbens diminishes the rewarding effects of cocaine.

(**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.

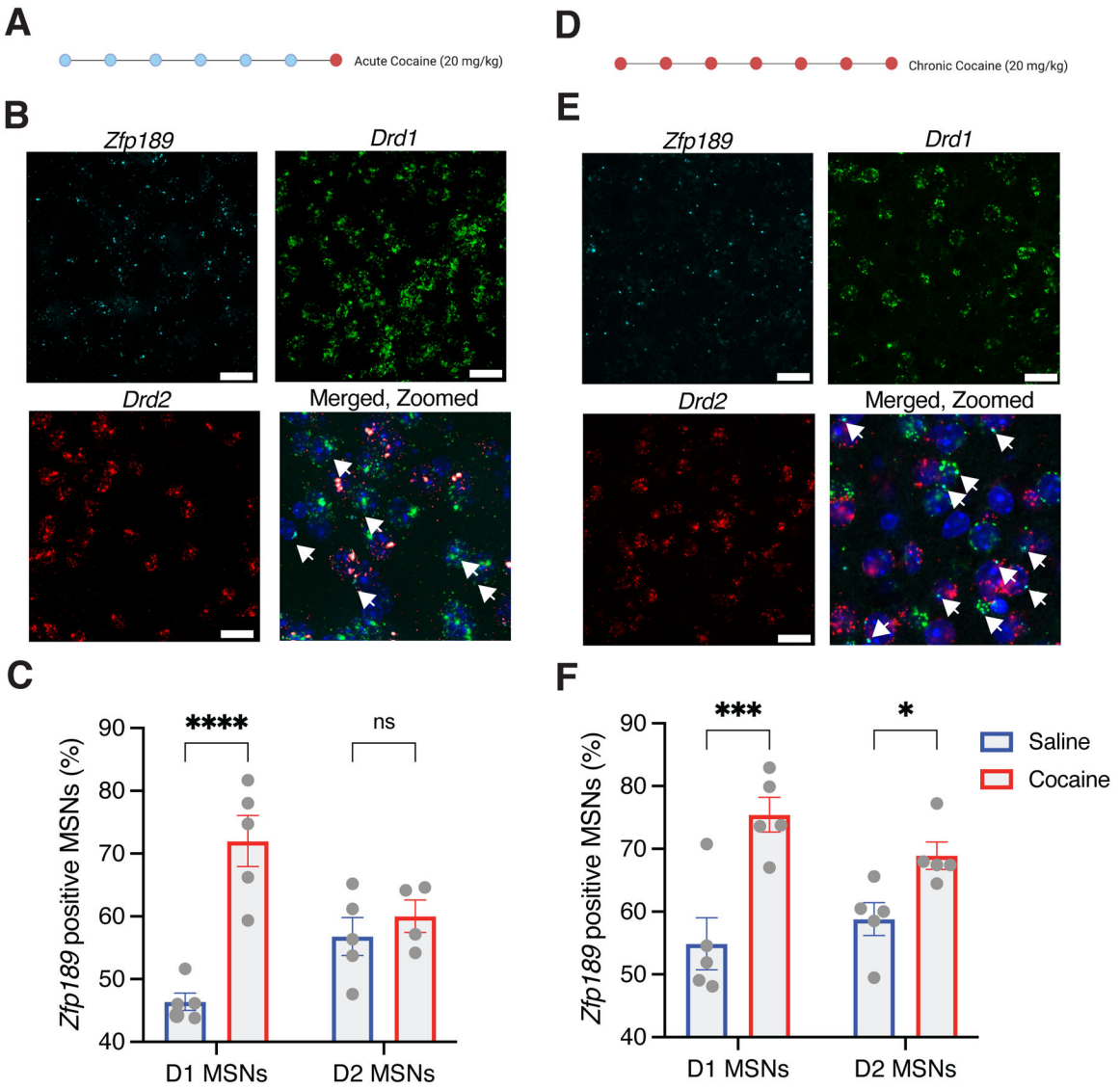

Figure 3: Acute cocaine exposure selectively induces *Zfp189* expression within D1 MSNs, whereas chronic cocaine exposure induces *Zfp189* expression within D1 and D2 MSNs.

(**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.

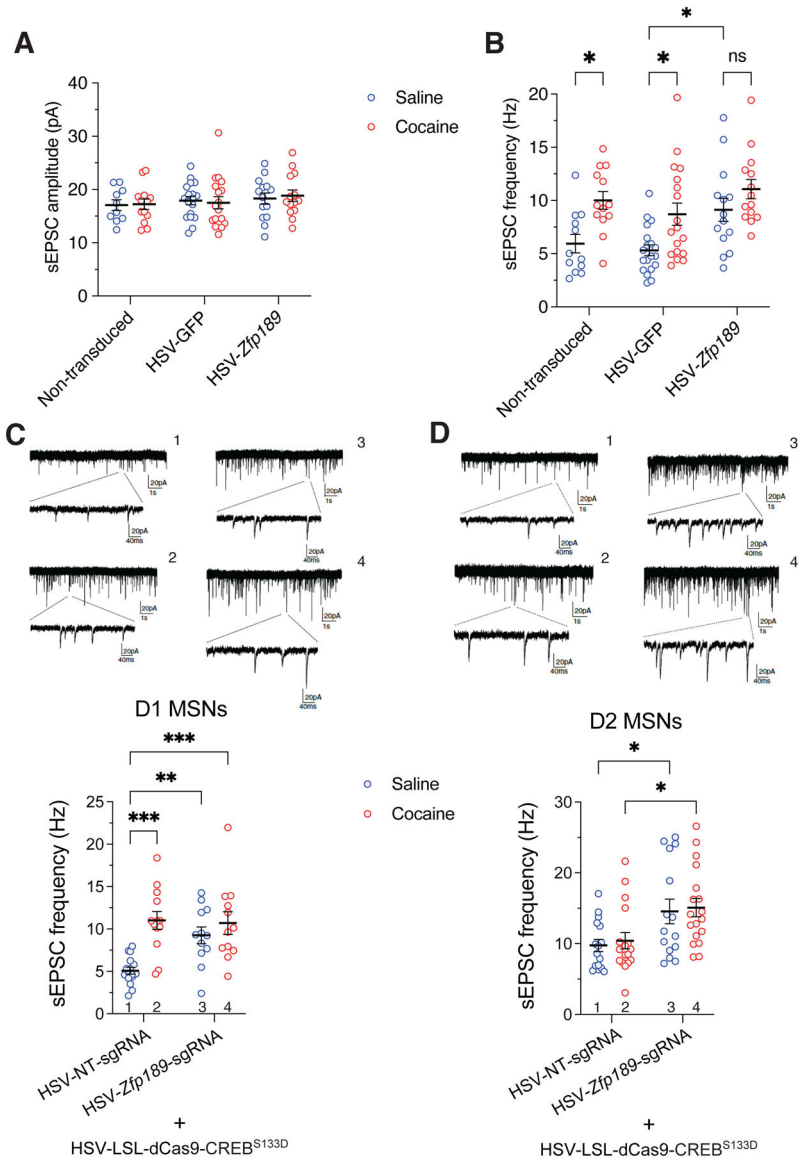

Figure 4: Cocaine exposure and CRISPR-mediated CREB-*Zfp189* interactions differentially regulate excitatory synaptic transmission to D1 and D2 MSNs.

(**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 tranduced 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).

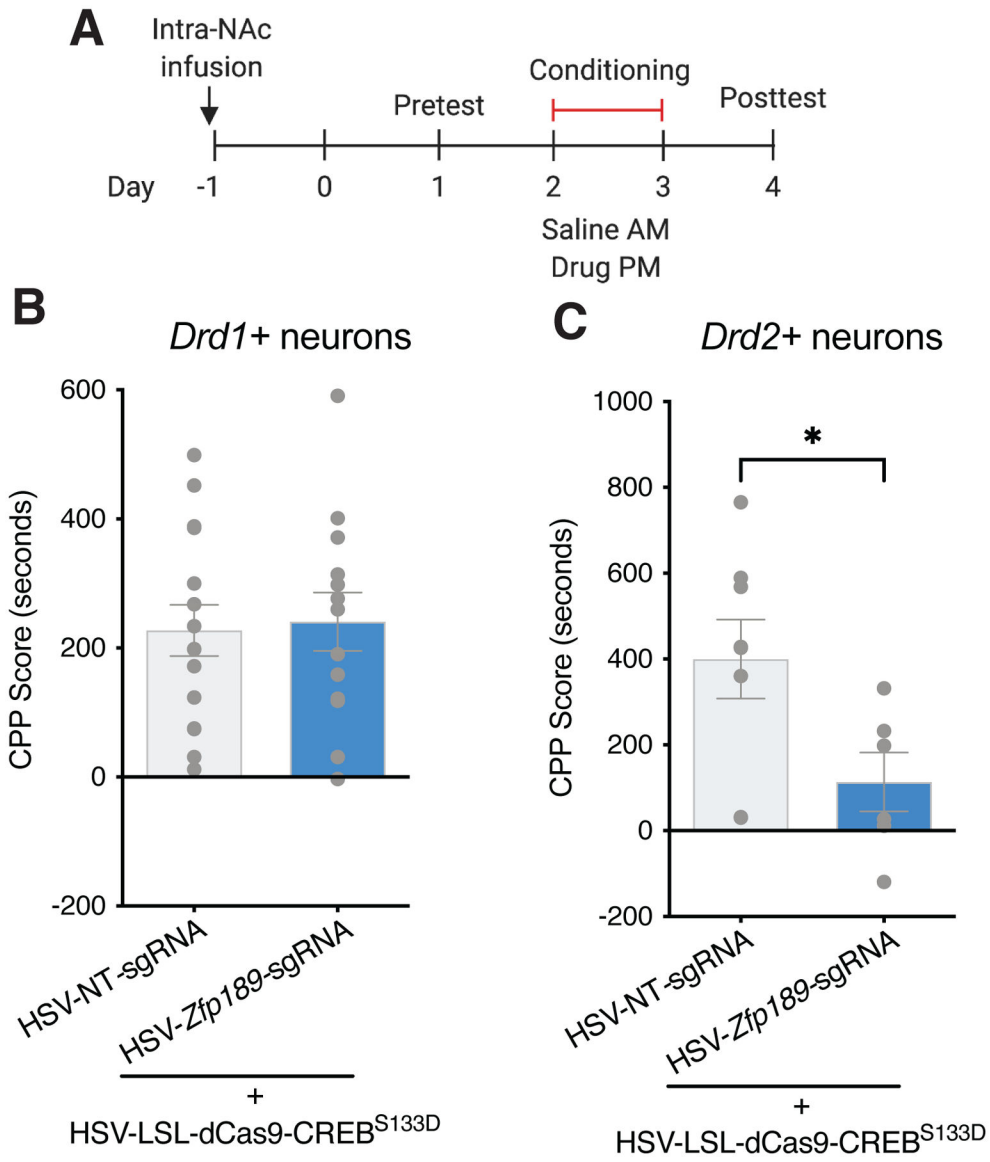

Figure 5: CREB-mediated *Zfp189* induction within NAc *Drd2*+ neurons drives reduced sensitivity to the reinforcing properties of cocaine.

(**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.