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Cocaine-induced chromatin modifications associate with increased expression and 3D looping of *Auts2*

Olivia Engmann[1], **Benoit Labonte**[1], **Amanda Mitchell**[2], **Pavel Bashtrykov**[3], **Erin S. Calipari**[1], **Chaggai Rosenbluh**[4], **Yong-Hwee E. Loh**[1], **Deena M. Walker**[1], **Dominika Burek**[1], **Peter J. Hamilton**[1], **Orna Issler**[1], **Rachael L. Neve**[5], **Gustavo Turecki**[6], **Yasmin Hurd**[2], **Andrew Chess**[4], **Li Shen**[1], **Isabelle Mansuy**[7], **Albert Jeltsch**[3], **Schahram Akbarian**[2], and **Eric J. Nestler**[1]

[1]Fishberg Department of Neuroscience and Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY USA [2]Departments of Psychiatry and Neuroscience, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA [3]Institute of Biochemistry, Stuttgart University, Stuttgart, Germany [4]Departments of Developmental and Regenerative Biology and of Genetics and Genomic Sciences and Icahn Institute for Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, New York, NY, USA [5]McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, MA, USA [6]Douglas Mental Health University Institute, Montreal, Canada [7]University of Zurich / ETH Zurich, Brain Research Institute, Zurich, Switzerland

Abstract

Background—Exposure to drugs of abuse alters the epigenetic landscape of the brain's reward regions such as the nucleus accumbens (NAc). We investigated how combinations of chromatin modifications affect genes that regulate responses to cocaine. We focused on autism-candidate 2 (*Auts2*), a gene linked to human evolution and cognitive disorders, which displays strong clustering of cocaine-induced chromatin modifications in this brain region.

Methods—We combined chromosome conformation capture (4C and 3C) and related approaches with behavioral paradigms relevant to cocaine phenotypes. Cell type-specific functions were assessed by FACS-sorting and viral-mediated overexpression in Cre-dependent mouse lines.

Results—We observed that *Auts2* gene expression is increased by repeated cocaine administration specifically in D2-type medium spiny neurons (MSNs) in NAc, an effect seen in male but not female mice. *Auts2* mRNA expression was also upregulated postmortem in NAc of male human cocaine addicts. We obtained evidence that chromosomal looping, bypassing 1,524 kb

Corresponding author's contact information Eric Nestler, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1065, New York, NY 10029-6574, phone: (212) 659-5656, fax: (212) 659-8510, eric.nestler@mssm.edu.

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of linear genome, connects *Auts2* to the calneuron 1 (*Caln1*) gene locus under baseline conditions. This looping was disrupted after repeated cocaine exposure, resulting in increased expression of both genes in D2 MSNs. Cocaine exposure reduces binding of CTCF, a chromosomal scaffolding protein, and increases histone and DNA methylation, at the *Auts-Caln1* loop base in NAc. Cell type-specific overexpression of *Auts2* or *Caln1* in D2 MSNs demonstrated that both genes promote cocaine reward.

Conclusions—These findings suggest that cocaine-induced alterations of neuronal 3D genome organization destabilize higher order chromatin at specific loci that regulate responses to the drug.

Keywords

Addiction; CTCF; histone methylation; DNA methylation; chromatin looping; nucleus accumbens

Introduction

Changes in chromatin architecture have been associated with gene expression since the early 20th century (1). The recent development of chromatin conformation capture techniques, in combination with state-of-the-art approaches in molecular biology, has allowed a conceptual link between genomic function and architecture (2; 3). These mechanisms are beginning to be examined in psychiatric conditions (4). However, the relationship between genomic architecture and drug addiction has not yet been explored.

Autism-candidate 2 (*Auts2*) is a rapidly evolving gene, which has been linked to evolution (5) as well as to autism (6), alcoholism (7), and heroin dependence (8). Recent work has shown that the Auts2 protein is part of the PRC2 polycomb complex (9) and alters Rac signaling. Consisting mostly of untranscribed regions, the large *Auts2* gene locus may have structural functions, since most disease-related SNPs occur in non-coding segments of the gene.

We identified *Auts2* as a key target for cocaine-induced chromatin modifications: analysis of published chromatin immunoprecipitation (ChIP)-sequencing data in NAc (10) revealed that this locus contains among the largest number of cocaine-induced chromatin changes genome-wide, yet its mRNA expression levels are not affected in this region based on RNA-sequencing data of whole NAc extracts. As a first step in investigating this paradox, we examined chromatin looping interactions formed by *Auts2* in NAc, based on the notion that the high degree of chromatin modifications might influence the 3D structure of this locus. Using chromatin conformation capture approaches, we show that *Auts2* indeed interacts with the calneuron 1 (*Caln1*) gene at baseline and that this interaction is disrupted by repeated cocaine administration. While cocaine-induced changes in *Auts2* and *Caln1* expression were not observed in whole NAc extracts (10), we demonstrate that increased *Auts2* and *Caln1* transcription occurs selectively in D2-type medium spiny neurons (MSNs) of NAc, with no effect seen in D1-type MSNs. We next show that the cocaine-induced change in interaction between the *Auts2* and *Caln1* genes is accompanied by changes in H3K4me3 (trimethylation of Lys4 of histone H3), in binding of CTCF (CCCTC binding factor), and in DNA methylation at these loci. By employing a novel CRISPR-epigenome editing approach, we show that targeting DNA methyltransferase (DNMT) 3a/3L to the

Auts2 gene controls *Caln1* expression in cell culture. Finally, viral-mediated overexpression of Auts2 or Caln1 selectively in D2 MSNs of NAc increases rewarding responses to cocaine. Together, these findings shed light on a novel mechanism by which cocaine-induced chromatin modifications underlie the complex regulation of an ensemble of genes in NAc to influence behavioral sensitivity to the drug.

Methods and Materials

Animals

Sprague-Dawley rats and C57BL/6J mice were purchased from Jackson, Bar Harbor, Maine. D1-tomato, D2-GFP, D1-Cre, and D2-Cre mice were obtained from N. Heintz (Rockefeller) and C. Gerfen (NIMH). Rats and mice were used for their unique strengths; see Results and supplementary material. All experiments were conducted on male animals, except where indicated.

Chromosome conformation capture (3C) and circularized chromosome-conformation capture (4C)

Protocols were modified from (11–13).

Cocaine self-administration

Rats containing chronic indwelling jugular catheters were trained for v. self-administration as described (14; 15).

Human brain tissue

NAc from cocaine-addicted or depressed male patients and matched male controls were acquired from McGill University. Psychological autopsies were performed as described (16).

FACS from D1-Tomato and D2-GFP mice

D1 and D2 MSNs were isolated from NAc punches by use of a BD FACS Aria II. RNA was extracted using the Direct-zol RNA miniprep kit (Zymo, Irvine, USA, #R2050).

Quantitative ChIP

qChIP was performed as described (17).

Bisulfite sequencing

Bisulfite conversation was conducted on purified DNA with the EZ DNA methylation kit (Zymo, Irvine, USA, #D5001). Libraries were prepared with a TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, USA, #FC-121-4003) according to manufacturers' instructions. Libraries were pooled and sequenced on an Illumina MiSeq sequencer, using a 600 cycle, V3-chemistry sequencing kit (Illumina, San Diego, USA, #MS-102-3003). Sequencing data were then analyzed using Bismark software (Reference: PMID: 21493656).

CRISPR epigenome-editing

A vector coding the dCas9-DNMT3ACD-DNMT3LCD-3xFLAG fusion gene was constructed by Gibson assembly. Guide RNAs were designed via a published protocol (https://www.addgene.org/crispr/church/).

miRNA vectors

were designed with the Thermo Fisher Scientific BLOCK-iT™ RNAi Designer tool. Constructs, purchased from IDT, were cloned into a pcDNA 6.2 GW/miR vector with the BLOCK- iT™ Pol II miR RNAi Expression Vector Kit (Thermo Fisher Scientific, Waltham, USA, #K493500).

Herpes simplex virus (HSV) vectors

Plasmids for *Auts2* and *Caln1* were purchased from Origene (Rockville, USA, # MR225787 and MR202484) and cloned into a p1005-LS1L-vector, then packaged.

Conditioned place preference

was carried out as described previously (18).

A complete description of methods is provided in supplementary material online.

Results

Auts2 and *Caln1* genes interact at the chromatin level

Repeated exposure to cocaine induces numerous epigenetic modifications in NAc as assessed from genome-wide ChIP-sequencing analyses (10). To identify genes that are highly regulated by cocaine, we analyzed changes in several chromatin marks that are altered in this brain region 24 h after 7 days of cocaine and sorted for genes with the highest number of modifications (Table S1), revealing *Auts2* as a top hit. In cultured cells, *Auts2* has been shown to form chromatin loops with other genes (19). Recently developed databases for the prediction of chromatin interactions suggest genomic interactions of the *Auts2* gene in mouse cortex as well (Yue Lab 3D Genome browser, http://promoter.bx.psu.edu). *Auts2* is a large gene (1,099,500 bp in rat), which consists mostly of intronic regions, and it is in those regions that most cocaine-induced chromatin modifications occur. We therefore hypothesized that *Auts2* might interact with other genes in NAc and that cocaine may alter this interaction profile.

To test this hypothesis, we collected punches of NAc shell and core from rats that had received 7 daily IP injections of saline or 20 mg/kg cocaine and performed 4C with an *Auts2* bait. 4C allows an unbiased approach to detect all chromatin interacting regions with a given locus of interest (see Supplementary methods for details). We targeted an un-transcribed region near the *Auts2* promoter that fulfilled the criteria necessary for 4C baits (Fig. S1). Because high cell numbers are required to study chromatin interactions, we conducted the 4C experiment in rats. As NAc shell and core have different roles in cocaine and other drug responses (20–22), separate 4C libraries were made for the two subregions. Paired end sequencing of the libraries yielded fragments that are in good agreement with the 4C

literature (23). Most 4C-interacting fragments were located on the same chromosome as *Auts2* (chr12) and the number of fragments correlated with vicinity to the 4C bait (Fig. 1a-c; (Table S2). On average, 8,735,380 reads/sample were obtained for NAc core and 9,198,209 for NAc shell from 1,486 and 1,615 fragments, respectively. 4C fragments that were differentially regulated by cocaine showed no overlap between NAc shell and core (Fig. 1c, Fig. S2).This is in agreement with the specific functions of these NAc subregions in cocaine-related behaviors, as noted above. The majority (58%) of interacting fragments were intergenic and 35% were intragenic (Fig. 1d, Fig. S3). In NAc core, only 0.8% of all detected fragments were significantly affected by cocaine, while 3.8% were altered in NAc shell (no cutoff applied). The majority of these fragments comprised the *Auts2* gene itself or proximal intergenic regions (data not shown). The next gene that interacted most with the *Auts2* bait in NAc shell was *Calnl*, which notably was also one of the 10 genes with most cocaine-induced chromatin modifications, even after adjusting for gene length (Table S1, Fig. S4). *Calnl* is a brain-specific calcium-binding protein that regulates Golgi-to-plasma membrane trafficking of vesicles (24; 25) and under certain conditions is reported to alter D2-A2AR heteromerization (26). The predicted *Calnl* -fragment is located 1,524 kb away from the *Auts2* bait. Between *Auts2* and *Caln17* lies the *Wbscr17* gene, which has been associated with chromatin-breakage in Williams syndrome (27). Both in mice and rats, *Caln1, Wbscr17* and *Auts2* are neighboring genes, however, the direction of the genes is inverted between the two species (Fig. 1e). 3C confirmed a cocaine-induced reduction in interactions between *Auts2* and *Caln1* in NAc shell of IP-injected rats ($F_{(1,48)}$=16.20, P<0.001, Fig. 1f). 3C on NAc core showed no signal. This is consistent with the fact that no interaction between *Auts2* and *Calnl* was detected by 4C in NAc core even in saline conditions.

Next, we assessed whether 3C changes occur after cocaine self-administration. Rats selfadministered cocaine for 10 days and were analyzed 24 h after the last session. We observed a reduction in 3C interactions in NAc core after self-administering cocaine vs. saline controls ($F_{(1,40)}$=45.92, P<0.0001, Fig. 1g). This agrees with previous studies showing that the NAc core is more active in cocaine seeking paradigms (28). We also confirmed the cocaine-induced reduction in *Auts2-Caln1* looping in mice (effect of drug: $F_{(1,48)}$=31.34, P<0.0001) (Fig. 1h). Because of the high amounts of DNA required, this experiment was conducted on whole NAc. Because there is a high homology between *Wbscr17* and the 4C-predicted *Caln1* -fragment in rats, but not in mice, this experiment was particularly important to confirm the specificity of *Caln1-Auts2* binding. In summary, we show that cocaine reduces chromatin interactions between *Auts2* and *Caln1*, although in different NAc subregions with investigator- vs. self-administered cocaine.

Increased cocaine-induced *Auts2* and *Caln1* gene expression in D2 MSNs

We next asked whether cocaine-induced changes in *Auts2-Caln1* chromatin looping are associated with altered gene expression. Both *Auts2* and *Caln1* mRNA were increased in rat NAc shell after cocaine injections (*Auts2:* $F_{(1,28)}$=6.71, P<0.05; *Caln1:* $F_{(1,44)}$=10.68, P<0.01) and in NAc core after self-administration (*Auts2:* $F_{(1,30)}$=9.76, P<0.01; *Caln1:* $F_{(1,32)}$=9.06, P<0.01, Fig. 2a-f, Fig. S5). *Auts2* mRNA was also increased in whole NAc of mice that had been injected for 7 days with cocaine and analyzed 24 h after the last dose

($F_{(2,59)}$=3.80, P<0.05, Fig. 2g,h). No effect of cocaine was seen at earlier withdrawal time points. *Caln1* expression showed a trend towards an increase at 24 h but this effect did not reach significance (P>0.05, Fig. 2h). The fact that increased *Auts2* mRNA levels were detected in whole NAc of mouse, despite no effect seen earlier by RNA-sequencing under identical experimental conditions, illustrates false negative errors when using high stringency statistical analyses of RNA-sequencing data (see Discussion). It may also relate to isoform-specific alterations (Table S3).

Roughly 95% of NAc neurons are MSNs, approximately half of which are D1-type vs. D2-type. Cells from NAc punches of D1-Tomato and D2-GFP mice with repeated cocaine or saline treatment were isolated by FACS. Both *Auts2* and *Caln1* mRNA levels were significantly increased in D2 MSNs of male mice (*Auts2:* $F_{(3,56)}$=6.97, P<0.05; *Caln1:* $F_{(1,84)}$=13.38, P<0.001, Fig. 2i-l), with no effect seen in D1 MSNs. The accuracy of FACS was confirmed by showing enrichment of *Pdyn* in D1 MSNs and *Penk* in D2 MSNs (Fig. 2i). This is in good agreement with a previous study, which observed a cocaine-induced D2-specific increase in *Auts2* expression (29). In contrast, female mice displayed no effect of cocaine for either gene in either cell type. To rule out possible confounds of the estrous cycle, we tested whether mRNA levels of *Auts2* or *Caln1* fluctuated with the cycle (Fig. 2m,n); this was not the case (P>0.05).

We next tested whether *Auts2* or *Caln1* mRNAs are altered in NAc of humans with cocaine addiction examined postmortem. *Auts2* and *Caln1* expression was augmented in NAc of male cocaine addicts, while there were no changes in NAc of male patients with depression examined for comparison (Auts2: $F_{(1,53)}$=5.36, P<0.05; Caln1 : $F_{(1,51)}$=5.36, P<0.05, Fig. 2o, p, Table S4).

In summary, we observed an inverse correlation between chromatin 3D interactions of *Auts2* and *Caln1* and expression of these genes in NAc, and the induction of both in D2 MSNs by cocaine. Expression of *Wbscr17*, the gene located between *Auts2* and *Caln1*, was not altered in all three species examined (rats, mice, and humans: P>0.05, data not shown).

Cocaine induces chromatin alterations on *Auts2* and *Calnl* genes

CTCF is a key mediator of chromatin interactions (30). To test the hypothesis that CTCF binding might be altered by cocaine in the vicinity of 4C interaction sites, qChIP was performed on NAc punches from IP-injected mice for CTCF. Levels of immunoprecipitated *Auts2*- and *Caln1*- fragments in the vicinity of the 4C sites were quantified by qPCR (Fig. 3a-d). CTCF binding was reduced at specific regions on both genes (Bonferroni posthoc after Two-way ANOVA: *Auts2+2:* P<0.05; *Caln1* 4C.5: P<0.01) in agreement with the reduced chromatin looping observed between *Auts2* and *Caln1* under these conditions. Next, we tested whether altered CTCF-binding is associated with altered histone marks. For instance, CTCF occupancy negatively correlates with the permissive histone mark H3K4me3 (31), and chromatin looping is favored at enhancers, which are enriched in H3K27 acetylation (H3K27ac). qChIP for H3K4me3 revealed a cocaine- induced increase in this mark at the same DNA regions that had displayed reduced CTCF-binding on both genes (Bonferroni posthoc after Two-way ANOVA: *Auts2+2:* P<0.001; *Caln1* 4C.5: P<0.01, Fig.

3e,f). qChIP against H3K27ac did not show cocaine-induced alterations at these regions (P>0.05 for all locations, Fig. 3g,h).

Another genomic mark that is linked to CTCF binding is DNA methylation, which generally inhibits CTCF binding (32). To assess whether cocaine alters DNA methylation at our genes of interest, we performed bisulfite sequencing spanning the vicinity of *Auts2-Caln1* interaction regions (Fig. S1). The *Caln1* region had much higher baseline CG methylation than *Auts2* (Fig. 3a,b). In both genes, we detected a small but significant increase in DNA methylation after cocaine-exposure (*Auts2:* P<0.05 for 11 out of 55 CGs; *Caln1:* P<0.05 or less for 5 out of 6 CGs) (Fig. 3i,j). In *Auts2*, the largest increase occurred at a putative CTCF site (CCGCGCCGGGGATC, Fig. S6) (33). In summary we observed reduced CTCF binding, increased H3K4me3 enrichment, and increased DNA methylation in the vicinity of 4C interaction sites between *Auts2* and *Caln1*.

Causality of DNA methylation and Caln1 expression regulation by Auts2

While data presented in the previous section demonstrated a correlation between DNA methylation and altered *Auts2-Caln1* interactions and expression, we wanted to test whether these alterations can be causally linked. A dCas9-DNMT3a-DNMT3L-3xFlag CRISPR was designed, which targets deposition of DNA methylation at a particular DNA region of interest. Targeting of DNA methylation has been shown previously (34). Guide RNAs were created to target DNMT activity to the regions of *Auts2* and *Caln1* that had shown increased DNA methylation after cocaine administration (Fig. S1), which were located in the proximal promoter and intronic gene body of the two genes, respectively. Non-targeting p1005-guide RNA was designed to bind nowhere in the mouse genome and served as a control. This experiment was performed in cell culture because it is not yet feasible to do so in brain. First, Neuro2A cells were transfected with the dCas9-DNMT3A/3L and guide RNAs targeting *Caln1* (gCaln1 1–5). We then extracted RNA and performed qPCR against both *Auts2* and *Caln1* (Fig. 4a-c). This targeted methylation increased gene expression of *Caln1* ($F_{(5,35)}$=5.50, P=0.001), without affecting *Auts2* mRNA levels, indicating that DNA methylation changes at the *Caln1* intronic region do not alter *Auts2* expression ($F_{(5,35)}$=0.59, P>0.05). Next, we co-transfected the dCas9-DNMT3A/3L with guide RNAs against *Auts2* (Fig. 4d-f). Again, the dCas9-DNMT3A/3L did not affect *Auts2* expression, suggesting that DNA methylation changes at the *Auts2* promoter region are not causal for regulation of *Auts2* expression ($F_{(4,29)}$=0.86, P>0.05). However, qPCR for *Caln1* revealed that CRISPR-dCas9 mediated epigenome-editing of *Auts2* increased the expression of *Caln1* ($F_{(4,29)}$=6.41, P=0.001). This is direct evidence that manipulation of one gene (*Auts2*) can alter the expression of a distant chromatin-interaction partner. Additionally, co-transfection of *Auts2* guide RNA with a dCas9-FLAG did not affect *Caln1* expression, indicating that the effect is not caused by steric artifacts of the dCAS9 bound to the gene (interaction of gene and gRNA: $F_{(1,8)}$=1 82, P>0.05; Fig S7). While guide RNAs + dCas9-DNMT3A/3L increased DNA methylation on the targeted genes (Fig. 4K,L, Fig. S8-10), selected guide RNAs also altered methylation on the looping partner (Fig. S8-10). The absence of DNA methylation changes in an unrelated control gene suggests that these effects were not due to lack of specificity of guide RNA binding (Fig. S11). Instead this molecular footprint

provides further evidence that looping between *Caln1* and *Auts2* can result in shared chromatin regulation.

As repeated cocaine administration increases *Auts2* expression (Fig. 2), we wanted to rule out that the effects on *Caln1* are not mediated indirectly by increased *Auts2* mRNA or protein. For instance, Auts2 regulates the PRC2-complex (9), which could conceivably control *Caln1* expression. To that end, we designed a miR-Auts2 construct and expressed it in Neuro2A cells (Fig. 4g,h). While *Auts2* expression was suppressed by *Auts2*-miR (t_{13}=5.82, P<0.0001***), *Caln1* expression was unaffected (t_{13}=0.90, P>0.05). Similarly, overexpression of *Auts2* with a Cre- dependent HSV vector in NAc of sterotaxically-injected D2-Cre mice increased *Auts2* expression levels (t_6=2.736, *P<0.05), but did not affect *Caln1* levels (t_6=0.4314, P>0.05, Fig. 4i,j).

Cell type-specific effects of *Auts2* and *Calnl* overexpression on cocaine place preference

After establishing a pathway by which chromatin looping affects *Caln1* expression via *Auts2* in NAc, we tested whether altered expression of either gene affects behavioral responses to cocaine. We used Cre-dependent HSV vectors to overexpress *Auts2* or *Caln1* in a cell type-specific manner (Fig. 4i,j; Fig. S12). Overexpression of either gene in D1-Cre mice did not affect cocaine place preference (CPP), which provides an indirect measure of cocaine reward, relative to the expression of a control vector ($F_{(5,71)}$=7.02, P<0.0001. Tukey's test: pretest vs. test: GFP *P<0.05; *Auts2:* *P<0.05; *Caln1:* **P<0.01; gene factor within test: P>0.05) (Fig. 5a,b; Fig. S13). However, overexpression of Caln1 or *Auts2* in D2-Cre mice increased cocaine CPP ($F_{(5,61)}$=9.36, P<0.0001. Tukey's test: pretest vs. test: GFP ***P<0.001; *Auts2:* ***P<0.0001; *Caln1:* **P<0.01; within test: GFP vs. *Auts2:* *P<0.05; GFP vs. *Caln1:* *P<0.05) (Fig. 5c). To avoid ceiling effects, CPP had been optimized on wild-type mice at 5 and 7.5 mg/kg doses. As CPP occurred at both doses, the lower dose was chosen for the HSV experiment (drug factor: $F_{(1,36)}$=36.18, P<0.001; Bonferroni: saline vs. cocaine: 5 mg/kg: *P<0.05; 7.5 mg/kg: ***P<0.001, data not shown). CPP in D1-Cre mice after viral overexpression was repeated at a 3 mg/kg dose—due to small differences in baseline preference seen in the two transgenic mouse lines despite both being on the same background (see supplementary methods)—and again neither *Auts2* nor *Caln1* overexpression affected CPP ($F_{(5,45)}$=0.54, P>0.05, data not shown). Together, this experiment suggests a permissive, cell type-specific role of *Auts2* and *Caln1* in cocaine-elicited behavior.

Discussion

Cocaine and other drugs of abuse have been shown by genome-wide approaches to induce numerous chromatin modifications in NAc and other brain reward regions (35). While some of these alterations correlate with drug-induced changes in expression of the specific target genes affected, most of them are not correlated with altered gene transcription (10). These observations have raised questions about the functional role played by such changes in chromatin regulation. Results of the present study provide new and important insight into this question.

We provide evidence that one function of cocaine-induced chromatin alterations is to regulate the expression of a gene—not only directly by altering that gene's promoter—but also by altering the 3D structure of chromatin around that gene which then alters its accessibility for transcription. Specifically, we demonstrate that repeated cocaine exposure induces several types of chromatin alterations at the *Auts2* and *Caln1* loci, including decreased CTCF binding and increased H3K4me3 deposition and DNA methylation, which are associated with reduced chromatin looping between these nearby genes and with their increased expression. By utilizing CRISPR-dCas9 mediated epigenome-editing, we demonstrate a causal link between altered DNA methylation on the *Auts2* gene and altered expression of the *Caln1* gene. This latter experiment was performed in cell culture due to current technical limitations; it will be important in future research to replicate these causal data in NAc as methodological improvements are made.

Our study, which focused on the *Auts2* and *Caln1* genes, provides a proof of principle for the ability of cocaine to control gene expression and behavior through the regulation of the 3D architecture of chromatin within a target brain area such as NAc. We propose that such regulation is likely far more widespread and involves numerous other genomic loci based on the relatively large number of genes that show strong chromatin modifications without an associated change in RNA expression (10). It would thus be interesting to study this question systematically in the drug addiction field to obtain a complete overview of drug-induced regulation of the 3D structure of the entire genome. HiC techniques provide such a genome-wide interrogation and are now being applied increasingly to numerous systems including brain (36). It is not yet feasible to apply them to small brain regions but recent advances suggest that the technique should become suitable for smaller amounts of tissue (37).

DNA methylation often correlates inversely with CTCF binding, which is implicated in controlling chromatin structure (32). Our data are in good agreement with this literature. DNA methylation has historically been associated with gene silencing, although more recent evidence has demonstrated a far more complex role based on the sequence and genomic region undergoing methylation and the other types of chromatin alterations that occur in concert (38). Our findings are in line with this latter view, as we found increased DNA methylation associated with reduced CTCF binding, increased H3K4me3 enrichment, and increased expression of *Auts2* and *Caln1*. In cell culture we found that CRISPR-dCas9-mediated targeting of DNMT3A/3L to the *Auts2* gene had no effect on that gene's expression levels, but nevertheless induced *Caln1*, consistent with an indirect mechanism such as reduced chromatin looping. In contrast, targeting DNMT3A/3L to *Caln1* induced that gene without affecting *Auts2*, suggesting a direct cis-effect of methylation at *Caln1*. The mechanism of looping between *Auts2* and *Caln1* is likely very complex, as DNA methylation is also known to prevent H3K4me3, and therefore other mechanisms in addition to weakening of the loop could play a role. Ultimately, further work is needed to define how increased methylation at these two loci induces gene expression albeit via different mechanisms. Further work is also needed to confirm the mechanism of action of dCas9-DNMT3A/3L, which could be achieved through other means such as recruiting DNMT3-binding proteins to the vicinity of the targeted sequences.

It is interesting that the ability of cocaine to control *Auts2* and *Caln1* expression in NAc occurs exclusively in male mice, with no effects seen in females. We do not know what mechanisms underlie these sex differences. Sex-specific effects in psychiatric models are becoming increasingly well known (39; 40). Hence, our observation deserves further investigation.

We identified *Caln1* as a key interacting gene of *Auts2* in NAc based on 3C and 4C data. However, in NAc shell, the *Auts2* bait displayed interactions with several intergenic regions as well, and many of these interactions were highly regulated by repeated cocaine exposure. These regions could represent distal enhancers of additional genes in the genome and should be studied further, not only in the context of cocaine exposure, but also with respect to other *Auts2*- related conditions, including autism, alcoholism, and heroin dependence.

The *Wbscr17* gene is located between *Auts2* and *Caln1*, although with different orientations in mouse vs. rat. *WBSCR17* is also sandwiched by *AUTS2* and *CALN1* in humans with an orientation more similar to rat. *WBSCR17* is associated with Williams syndrome, a neurodevelopmental condition caused by chromosome breakage at this locus. Our model suggests that *WBSCR17* may be exposed on a loop formed between *AUTS2* and *CALN1* under control conditions. Whether this contributes to an increased susceptibility for breakage remains to be studied.

To conclude, our data reveal a novel mechanism by which repeated cocaine administration affects the genome of NAc neurons, namely, through regulation of the 3D structure of chromatin at specific sites. Cocaine regulation of this chromatin looping occurs in concert with cocaine-induced alterations in several types of chromatin alterations and ultimately leads to expression changes of key genes, *Auts2* and *Caln1*, which we demonstrate control behavioral responses to cocaine. These findings establish a new line of research into the molecular basis of cocaine's effects on the genome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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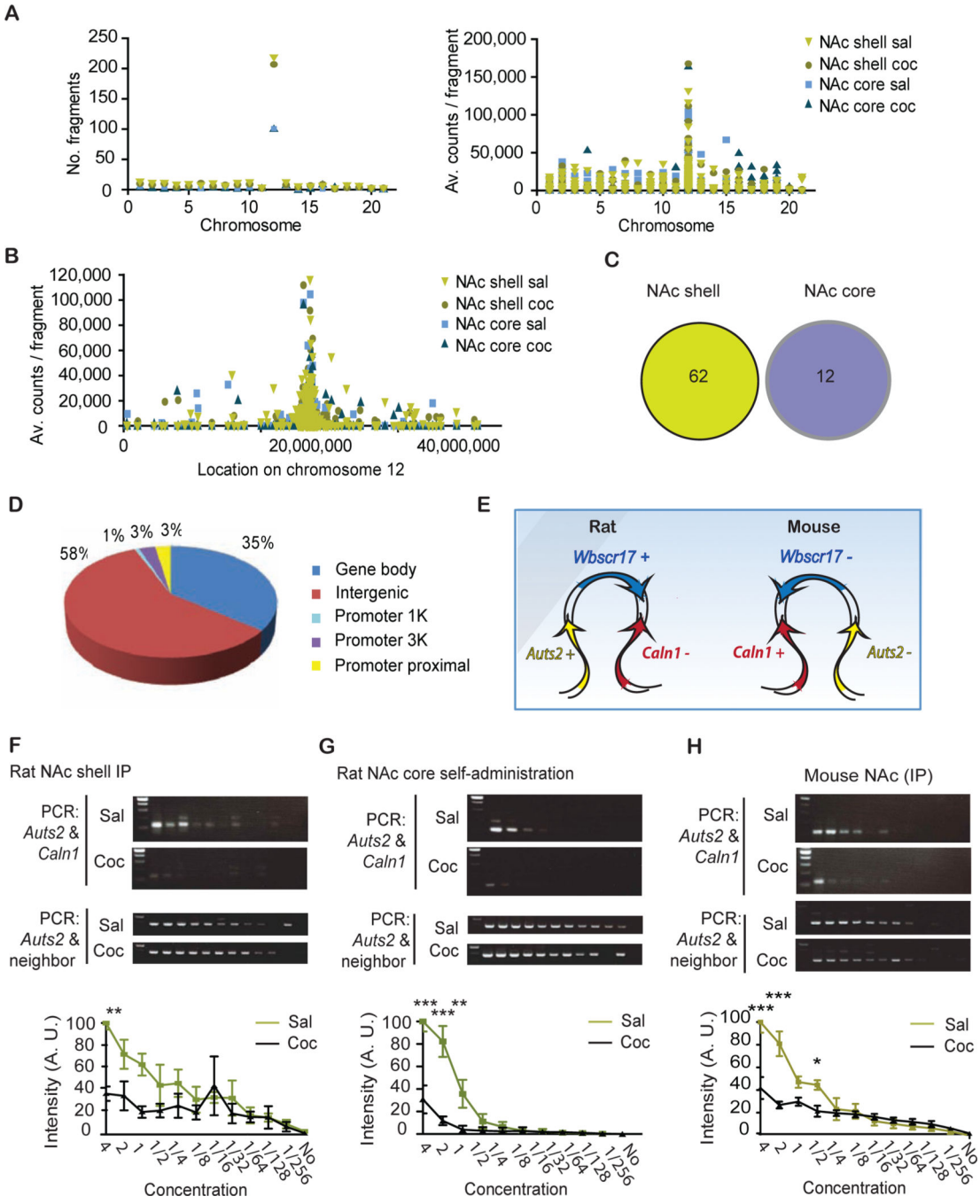

Figure 1.

Auts2 and *Caln1* gene interactions are reduced by repeated cocaine administration. ***a***, Number of detected fragments and number of counts per fragment plotted for chromosome location over the entire rat genome. *Auts2* is located on chr12. Sal, saline; Coc, cocaine. ***b***, Average counts per fragment on chr12. The 4C bait of *Auts2* is located at 27,155 kbp. ***c***, Pie chart of genomic locations of detected 4C fragments (shell and core combined). ***d***, Venn diagram of significantly changed fragments in NAc shell and core. ***e***, Overview over gene locations in mouse (chr5) and rat (chr12). ***f***, 3C libraries were prepared from NAc shell

punches of rats that had been I.P.-injected for 7 days with saline or 20 mg/kg cocaine and were analyzed 24 h later. N=3,3. Two-way ANOVA: interaction: $F_{(11,48)}$=2.10, P<0.05; drug factor: $F_{(1,48)}$=16.20, P<0.001; dilution factor: $F_{(11,48)}$=6.56, P<0.0001. Bonferroni: sal vs. coc: **P<0.01; all following: P>0.05. ***g***, 3C libraries were prepared from NAc core punches of rats that have acquired cocaine during self-administration for at least 7 days with 20 mg/kg cocaine. They were analyzed 24 h later. N=3,3. Two-way ANOVA: interaction: $F_{(9,40)}$=11.40, P<0.0001; drug factor: $F_{(1,40)}$=45.92, P<0.0001; dilution factor: $F_{(9,40)}$=28.43, P<0.0001. Bonferroni: sal vs. coc: ***P<0.001; ***P<0.001; **P<0.01; all following: P>0.05. ***h***, 3C libraries were prepared from NAc shell punches of rats that had been I.P.-injected for 7 days with saline or 20 mg/kg of cocaine and were analyzed 24 h later. N=3,3. Two-way ANOVA: interaction: $F_{(11,48)}$=9.834, P<0.0001; drug factor: $F_{(1,48)}$=31.34, P<0.0001; dilution factor: $F_{(11,48)}$=35.06, P<0.0001. Bonferroni: sal vs. coc: ***P<0.001; ***P<0.001; all following: P>0.05. ***f-h***, Average +/− SD shown.

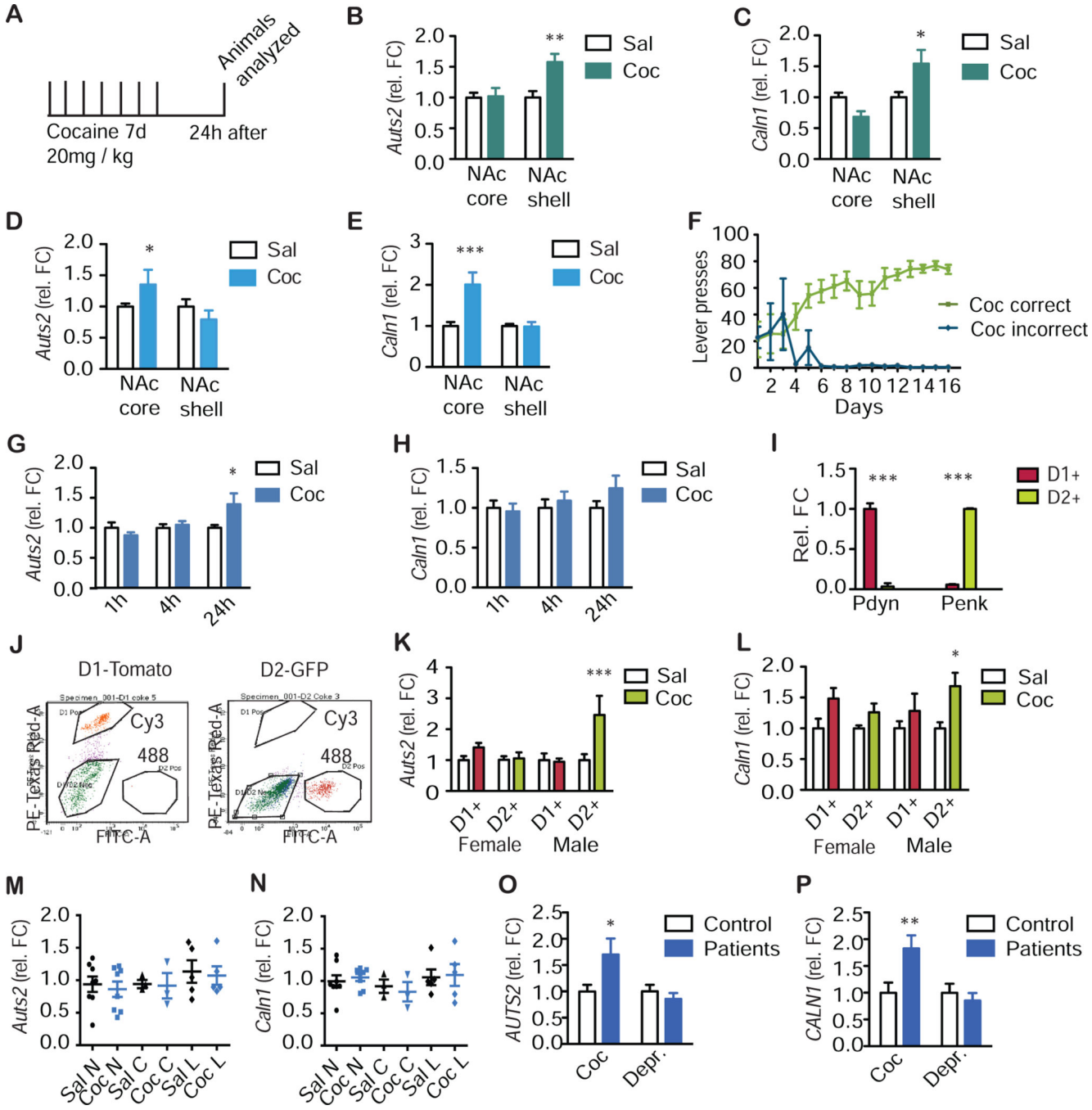

Figure 2.

Expression of *Auts2* and *Caln1* is increased by repeated cocaine administration. ***a***, Cocaine paradigm: Mice or rats were I.P.-injected for 7 days with saline (sal) or 20 mg/kg cocaine (coc) and analyzed 24 h later. ***b,c***, Separate punches were taken from rat NAc shell and core. ***b***, qPCR was conducted for *Auts2*. N=8,8,8,8. Two-way ANOVA: interaction: $F_{(1,28)}$=5.83, P<0.05; drug factor: $F_{(1,28)}$=6.71, P<0.05; brain region: $F_{(1,28)}$=5.83, P<0.05. Bonferroni: sal vs. coc: NAcc: P>0.05; NAcs: **P<0.01. *c*, qPCR was conducted for *Caln1*. N=12,12,12,12. Two-way ANOVA: interaction: $F_{(1,44)}$=10.68, P<0.01; drug factor: $F_{(1,44)}$=0.16, P>0.05;

brain region: $F_{(1,44)}$=2.22, P<0.01. Bonferroni: sal vs. coc: NAcc: P>0.05; NAcs: *P<0.05. ***d, e***, Rats self-administered cocaine for 10 days and were analyzed 24 h after the last session. Separate punches were taken from NAc shell and core. ***d***, qPCR was conducted for *Auts2*. N=9,8,9,8. Two-way ANOVA: interaction: $F_{(1,30)}$=9.76, P<0.01; drug factor: $F_{(1,30)}$=2.22, P>0.05; brain region: $F_{(1,30)}$=9.76, P<0.01. Bonferroni: sal vs. coc: P<0.05*; P>0.05. ***e***, qPCR was conducted for *Caln1*. N=9,9,9,9. Two-way ANOVA: interaction: $F_{(132)}$=9.57, P<0.01; drug factor: $F_{(1,32)}$=9.06, P<0.01; brain region: $F_{(1,32)}$=9.57, P<0.01**. Bonferroni: sal vs. coc: NAcc: ***P<0.001; NAcs: P>0.05. ***f***, Acquisition curve for cocaine self-administration. Correct and incorrect lever presses for the cocaine group are shown. N=8 per group. Two-way ANOVA: interaction: $F_{(121,182)}$=5.51, P<0.0001; drug factor: $F_{(1,182)}$=328.9, P<0.0001; time factor: $F_{(12,182)}$=4.36, P<0.0001. Bonferroni: sal vs. coc: Days −3: P>0.05; all other days: P<0.001. ***g,h***, Punches were taken of whole NAc of male mice. ***g***, qPCR was conducted for *Auts2*. N=10,11 ;11,11;11,11. Two-way ANOVA: interaction: $F_{(2,59)}$=3.80, P<0.05; drug factor: $F_{(1,59)}$=1.84, P>0.05; time factor: $F_{(2,59)}$=3.80, P<0.05. Bonferroni: sal vs. coc: P>0.05; P>0.05; *P<0.05. ***h***, qPCR was conducted for *Caln1*. N=10,11,11,11,11,11. 2 way ANOVA with Bonferroni: interaction: $F_{(2,59)}$=0.87, P>0.05; drug factor: $F_{(1,59)}$= 1.15, P>0.05; time factor: $F_{(2,59)}$=0.84, P>0.05. Bonferroni: sal vs. coc: all P>0.05. *i-l*, FACS sorting on NAc punches of D1-GFP and D2-Tomato mice. *i*, Specificity of D1/D2-MSN markers was assessed by qPCR for *Pdyn* and *Penk*. N=36,36;30,30. Two-way ANOVA: interaction: $F_{(1,128)}$=485.1, P<0.0001; gene factor: $F_{(1,128)}$=0.07, P>0.05; cell factor: $F_{(1,128)}$=0.07, P>0.05. Bonferroni: D1 vs. D2: ***P<0.001; ***P<0.001. *j*, FACS on Cy3 and Alexa488 lasers. ***k***, qPCR was conducted for *Auts2*. N=10,10:7,7;8,6;9,7. Two-way ANOVA: interaction: $F_{(3,56)}$=3.84, P<0.05; drug factor: $F_{(3,56)}$=6.97, P<0.05; factor of sex and cell type: $F_{(3,56)}$=3.79, P<0.05. Bonferroni: sal vs. coc: P>0.05; P>0.05; P>0.05; ***P<0.001. ***l***, qPCR was conducted for *Caln1*. N=10,10;7,7;9,7;9,7. Two-way ANOVA: interaction: $F_{(3,84)}$=0.72, P>0.05; drug factor: $F_{(1,84)}$=13.38, P<0.001; factor of sex and cell type: $F_{(3,84)}$=0.72, P>0.05. Bonferroni: sal vs. coc: P>0.05; P>0.05; P>0.05; *P<0.05. *m, n*, Punches were taken of whole NAc of female mice at nuclear (N), corneal (C), and lucidic (L) stages of their estrous cycle. ***m***, qPCR was conducted for *Auts2*. N=8,8;3,3;5,5. Two-way ANOVA: interaction: $F_{(2,26)}$=0.01, P>0.05; drug factor: $F_{(1,26)}$=1.01, P>0.05; estrous factor: $F_{(2,26)}$=0.19, P>0.05. Bonferroni: sal vs. coc: all P>0.05. ***n***, qPCR was conducted for *Caln1*. N=8,8;3,3;5,5. Two-way ANOVA: interaction: $F_{(2,26)}$=0.20, P>0.05; drug factor: $F_{(1,26)}$=1.47, P>0.05; estrous factor: $F_{(2,26)}$=0.01, P>0.05. Bonferroni: sal vs. coke: all P>0.05. ***o, p***, RNA was extracted from NAc postmortem samples from human cocaine addicts or patients that suffered from depression and their matched controls (ctrl). ***o***, qPCR was conducted for *Auts2*. N=15,14,14,14. Two-way ANOVA: interaction: $F_{(1,53)}$=5.36, P<0.05; factor of disease: $F_{(1,53)}$=2.74, P>0.05; factor of patients: $F_{(1,53)}$=5.36, *P<0.05. Bonferroni: ctrl vs. patients: *P<0.05; P>0.05. ***p***, qPCR was conducted for *Caln1*. N=14,13,14,14. Two-way ANOVA: interaction: $F_{(1,51)}$=5.36, P<0.05; factor of disease: $F_{(1,51)}$=5.47, P<0.05; factor of patients: $F_{(1,51)}$=5.36, P<0.05,. Bonferroni: ctrl vs. patients: **P<0.01 ; P>0.05. ***b-i, k, l, o, p***, Average +/− SD shown.

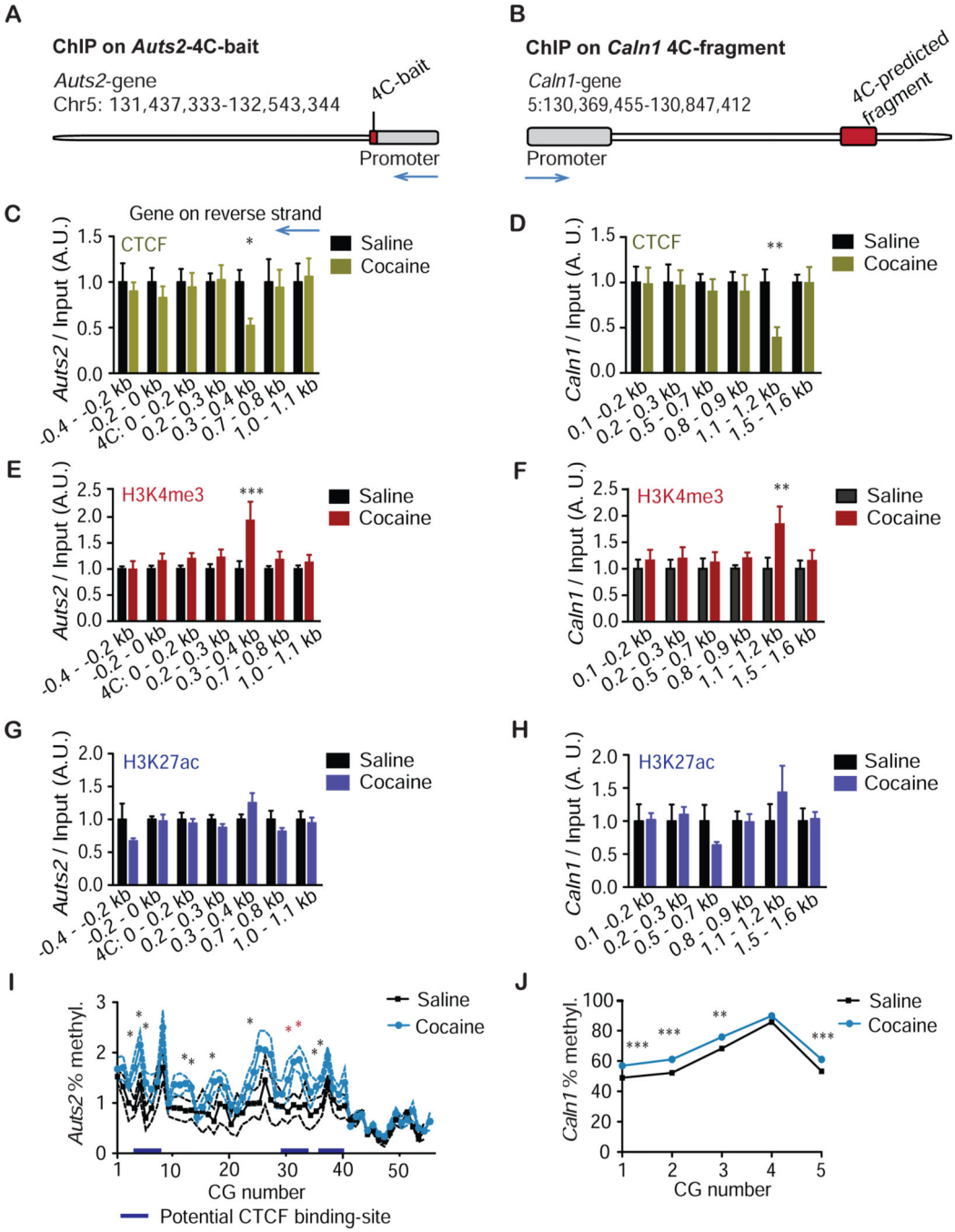

Figure 3.

Cocaine induces multiple epigenetic modifications on *Auts2* and *Caln1* genes. ***a***, Overview of location of the 4C bait relative to the *Auts2* gene (reverse strand) in mice. The mouse genomic area homologous to the 4C-bait from rat was identified using the UCSC genome browser BLAT tool. ***b***, Overview of the location of the Caln1 4C-predicted fragment in mice. The 4C fragment from rat was determined for the mouse genome via the UCSC genome browser BLAT tool. ***c, d***, Chromatin was purified from NAc of mice that had been injected for 7 days with saline (sal) or 20 mg/kg cocaine (coc) and analyzed 24 h later. Chromatin

was precipitated with a CTCF antibody. ***c***, qPCR was conducted for *Auts2*. N=4,4;4,4;10,9;8,7;15,16;4,4;4,4. Two-way ANOVA: interaction: $F_{(6,83)}$=0.52, P>0.05; drug factor: $F_{(1,83)}$=1.44, P>0.05; location factor: $F_{(6,83)}$=0.52, P>0.05. Bonferroni: sal vs. coc: P>0.05; P>0.05; P>0.05; P>0.05; *P<0.05; P>0.05; P>0.05. X-axis refers to primer locations up- and downstream of the 4C-bait. ***d***, qPCR was conducted for *Caln1*. N=4,4;4,4;8,7;8,8;9,8;8,7. Two-way ANOVA: interaction: $F_{(5,67)}$=1.13, P>0.05; drug factor: $F_{(1,67)}$=2.70, P>0.05; location factor: $F_{(5, 67)}$= 1.13, P>0.05. Bonferroni: sal vs. coc: P>0.05; P>0.05; P>0.05; P>0.05; **P<0.01; P>0.05. X-axis refers to primer locations within the 4C-predicted fragment. ***e,f***, like ***c,d***, but chromatin was precipitated with a H3K4me3 antibody. ***e***, qPCR was conducted for *Auts2*. N=8,8;14,10;26,26;8,8;14,10;8,8;8,8. Two-way ANOVA: interaction: $F_{(6,142)}$=1.81, P>0.05; drug factor: $F_{(1,142)}$=8.85, P<0.01; location factor: $F_{(6,142)}$=1 80, P>0.05. Bonferroni: sal vs. coc: P>0.05; P>0.05; P>0.05; P>0.05; ***P<0.001; P>0.05; P>0.05. X-axis refers to primer locations up- and downstream of the 4C bait. ***f***, qPCR was conducted for *Caln1*. N=4,4;10,9;8,8;17,17;4,4;4,4. Two-way ANOVA with Bonferroni: interaction: $F_{(5,130)}$=0.99, P>0.05; drug factor: $F_{(1,130)}$=5.80, P<0.05; dilution factor: $F_{(5,130)}$=0.99, P>0.05. Bonferroni: sal vs. coc: P>0.05; P>0.05; P>0.05; P>0.05; **P<0.01; P>0.05. X-axis refers to primer locations within the 4C predicted fragment. ***g,h***, like ***c,d***, but chromatin was precipitated with a H3K27ac antibody. ***g***, qPCR was conducted for *Auts2*. N=4,6;8,8;12,13;8,8;8,8;4,5;4,5. Two-way ANOVA: interaction: $F_{(6,87)}$=1.39, P>0.05; drug factor: $F_{(1,87)}$=1.72, P>0.05; location factor: $F_{(6,87)}$=1.39, P>0.05. Bonferroni: sal vs. coc: all P>0.05. X- axis refers to primer locations up- and downstream of the 4C bait. ***h***, qPCR was conducted for *Auts2*. N=4,6;4,6;4,6;8,8;8,8;4,6. Two-way ANOVA: interaction: $F_{(5,60)}$=0.58, P>0.05; drug factor: $F_{(1,60)}$=0.06, P<0.01; location factor: $F_{(5,60)}$=0.58, P>0.05. Bonferroni: sal vs. coc: all P>0.05. X- axis refers to primer locations within the 4C-predicted fragment. i, j: Bisulfite sequencing was conducted for regions of *Auts2* and *Caln1* that were altered by 4C / ChIP in a cocaine-dependent manner. One-way ANOVA was conducted for each CG. i, Bisulfite sequencing with *Auts2* primers: positions 4–6, 13–14, 18,26: *P<0.05; positions 32, 33: *P=0.01 (red, these CGs were targeted in later experiments by CRISPR-dCas9); all other positions: P>0.05. . **j**, Bisulfite sequencing with *Caln1* primers CG 1–3, 6: ***P<0.0001; CG 4: **P<0.01; CG 5: P>0.05. **c-h**, Average +/− SD shown.

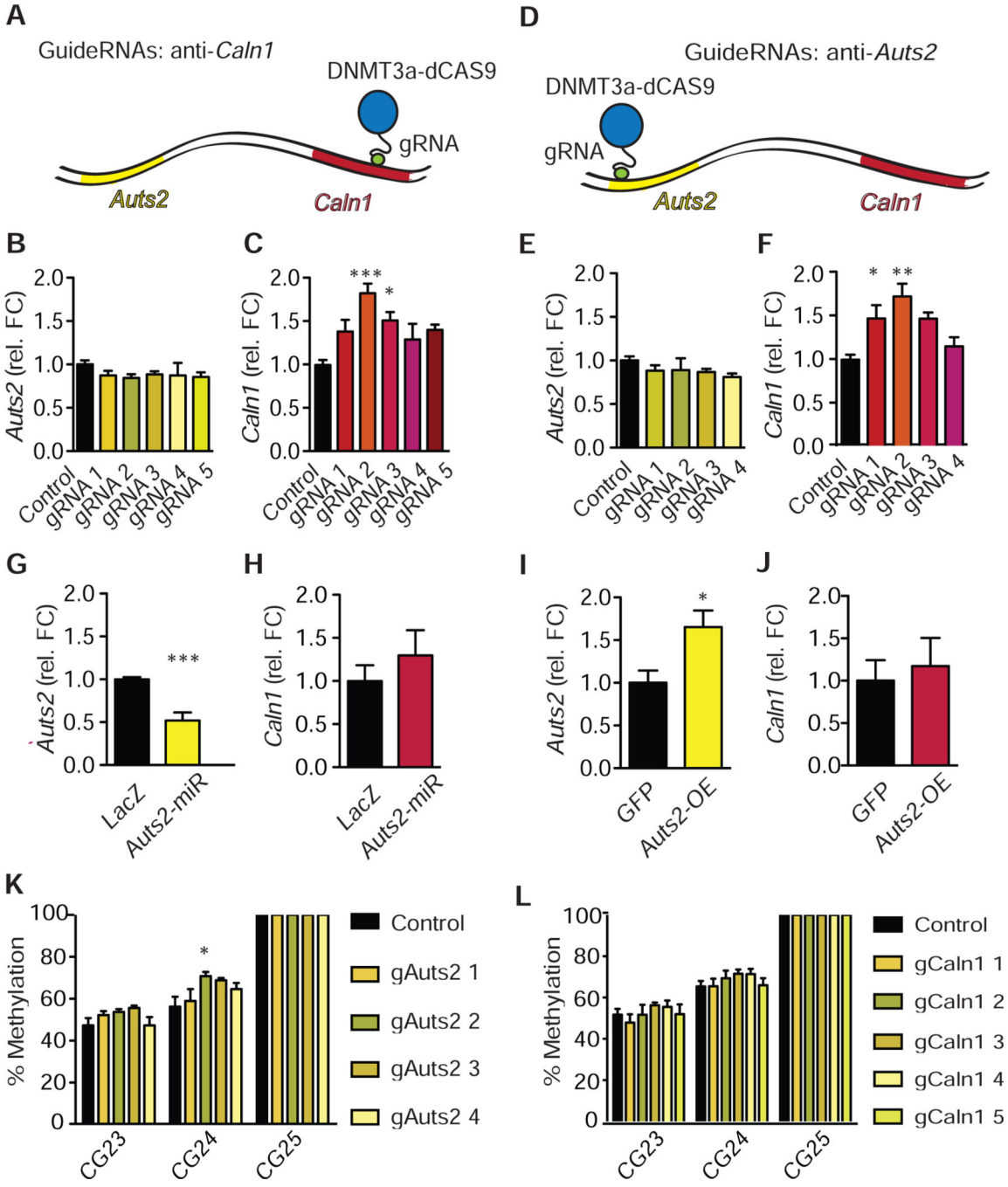

Figure 4.

Increased DNA methylation on Auts2 and Caln1 genes increase Caln1 expression. ***a***, Overview over CRISPR-binding on *Caln1*. ***b,c***, Neuro2A cells were co-transfected with dCas9-DNMT3ACD-DNMT3LCD-3xFLAG CRISPR and either sham (“p1005”) or *Caln1* guide RNA. ***b***, qPCR was conducted for *Auts2*. N=6 per group. One-way ANOVA: $F_{(5,35)}$=0.59, P>0.05; Tukey’s test vs. p1005-ctrl: all P>0.05. ***c***, qPCR was conducted for Caln1. N=6 per group. One-way ANOVA: $F_{(5,35)}$=5.50, P=0.001; Tukey’s test vs. p1005-ctrl: P>0.05; ***P<0.001; *P<0.05; P>0.05; P>0.05. ***d***, Overview over CRISPR-binding on

Auts2 ***e, f***, Neuro2A cells were co-transfected with dCas9-DNMT3ACD-DNMT3LCD-3xFLAG CRISPR and either sham ("p1005") or *Auts2* guide RNA. ***e***, qPCR was conducted for *Auts2*. N=6 per group. One-way ANOVA: $F_{(4,29)}$=0.86, P>0.05; Tukey's test vs. p1005-control: all P>0.05. *f*, qPCR was conducted for Caln1. N=6 per group. One-way ANOVA: $F_{(4,29)}$=6.41, P=0.001; Tukey's test vs. p1005-control: *P<0.05; **P<0.01; P>0.05; P>0.05. ***g, h***, Neuro2A cells were transfected with either Auts2-miR or LacZ control. ***g***, qPCR was conducted for *Auts2*. N=9,6. Student's t-test: t_{13}=5.82, P<0.0001***. ***h***, qPCR was conducted for *Caln1*. N=9,6. Student's t-test: t_{13}=0.90, P>0.05. ***i, j***, NAc punches were obtained from D2-Cre mice that had been injected 3 days prior with an *Auts2*-p1005-LSIL-HSV or p1005- LSIL-HSV, which express Auts2 or GFP, respectively, in a Cre-dependent manner. ***i***, qPCR was conducted for *Auts2*. N=5.3. Student's t-test: t_6=2.736, *P<0.05. ***j***, qPCR was conducted for *Caln1*. N=5,3. Student's t-test: t_6=0.4314, P>0.05. **k, l**, DNA methylation on a putative CTCF-site of *Auts2* is increased by dCas9-DNMT3A/3L combined with *Auts2* guide-RNAs (**k**) but not by guide-RNAs against *Caln1* (**l**). Statistics for **k** and **l** are given in Fig. S8 and S9. ***b, c, e-l***, Average +/− SD are shown.

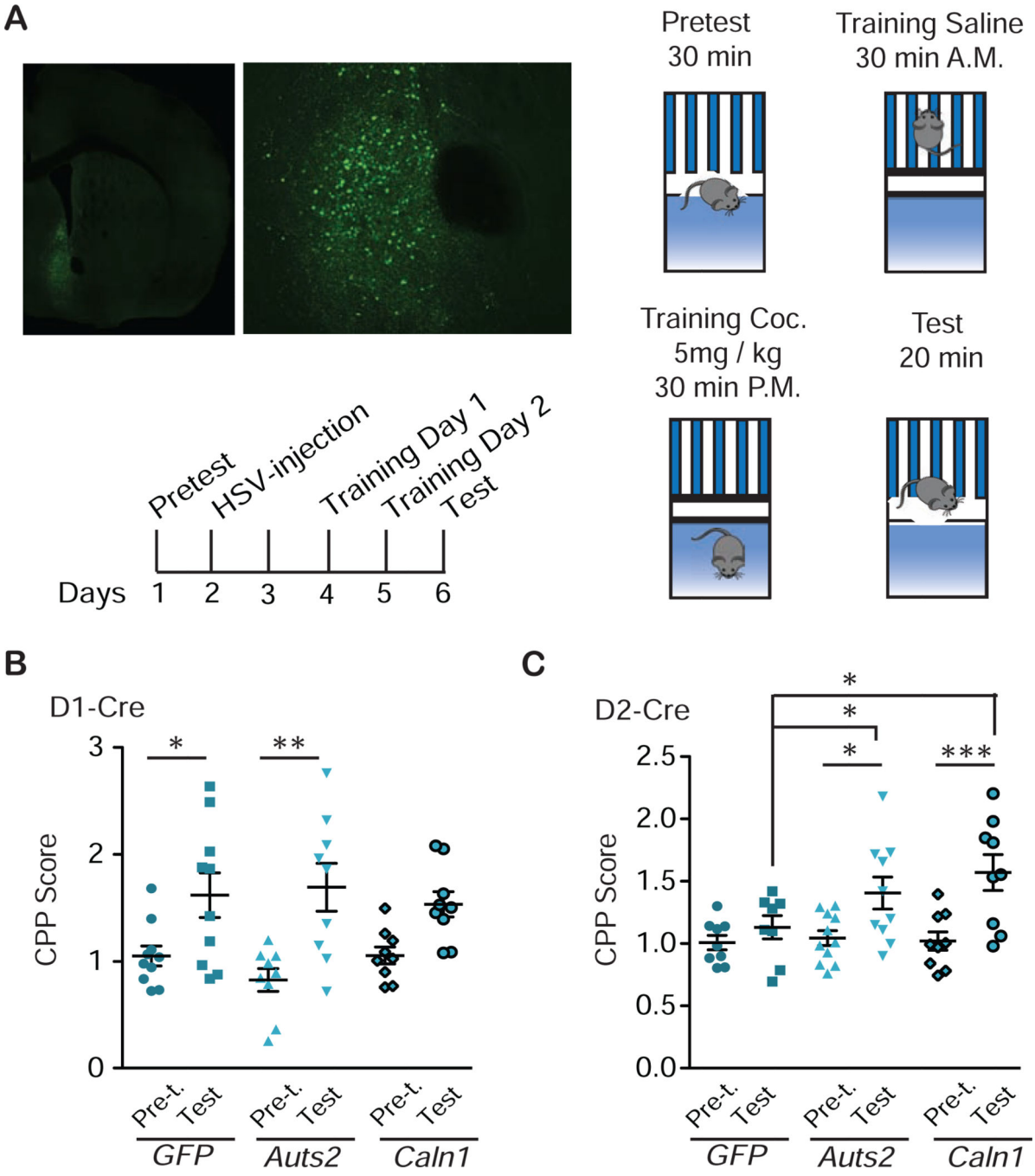

Figure 5.

D2-specific overexpression of Auts2 or *Caln1* in NAc increases cocaine place conditioning. ***a***, Procedure for cocaine conditioned place preference (CPP): On day 1, D1- or D2-Cre mice were subjected to 30 min pretest in the CPP chambers to balance for bias in preference. (On average, we use an unbiased procedure, where there is no overall preference for one side or the other.) On day 2, mice were stereotaxically injected into NAc with *Auts2*-p1005-LSIL, *Caln1*-p1005-LSIL or p1005-LSIL control HSV vectors (see photos - viral expression: green; DAPI: blue; magnifications clockwise: 10x, 20x, and 100x with 2x zoom). On days 4

and 5, mice were conditioned and on day 6, place preference was tested. ***b***, CPP of D1-Cre mice. N=12,9,15. One-way ANOVA: $F_{(5,71)}$=7.02, P<0.0001. Tukey's test: pretest vs. test: GFP *P<0.05; *Auts2:* *P<0.05; *Caln1:* **P<0.01; gene factor within test: P>0.05. *c*, CPP of D2-Cre mice. N=8,14,9. One-way ANOVA: $F_{(5,61)}$=9.36, P<0.0001. Tukey's test: pretest vs. test: GFP ***P<0.001; *Auts2:* ***P<0.0001 ; *Caln1:* **P<0.01 ; within test: GFP vs. *Auts2:* *P<0.05; GFP vs. *Caln1:* *P<0.05. B,C, Average +/− SD shown.

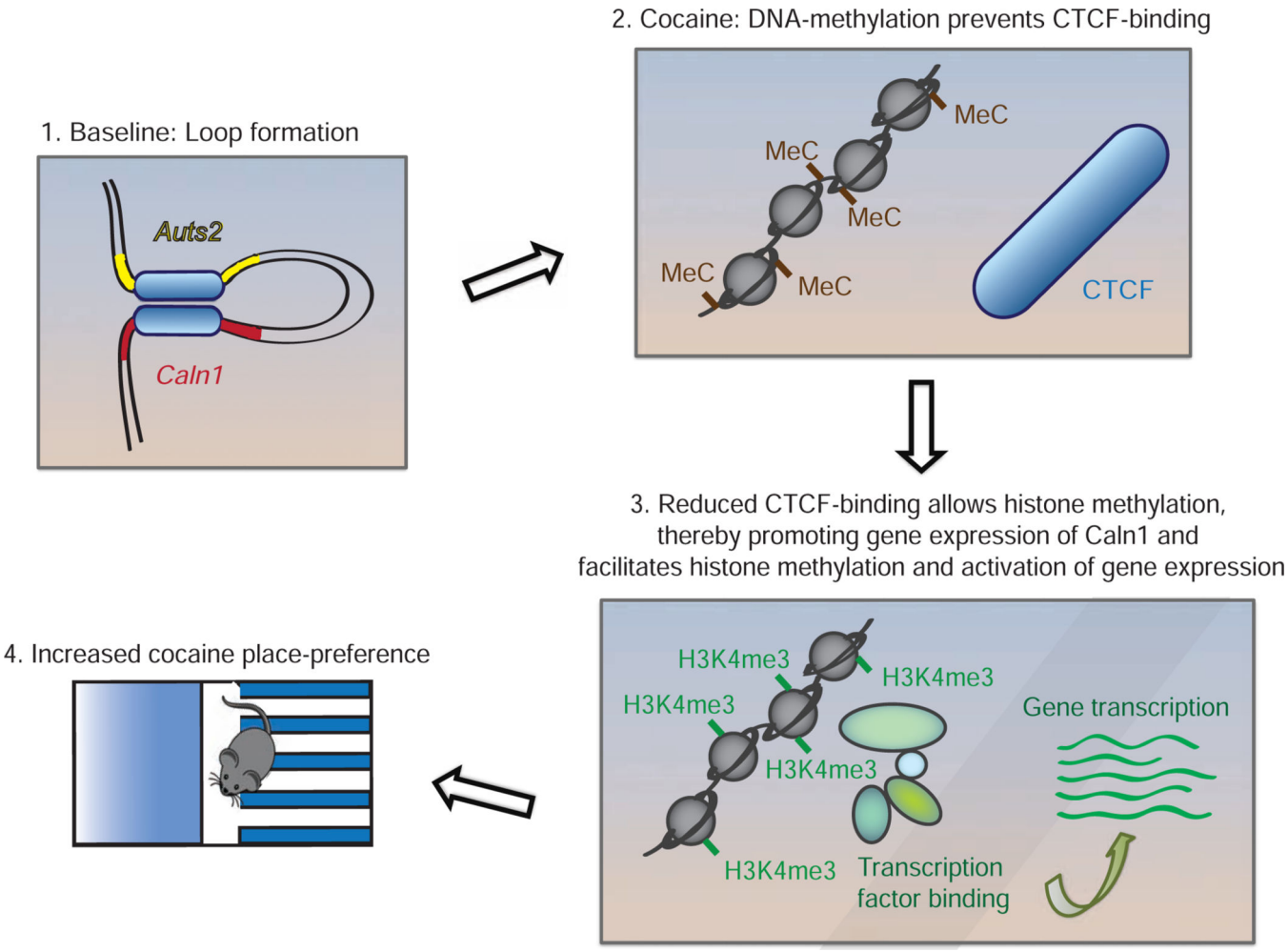

Figure 6.

Model for cocaine regulation of the 3D genome in NAc. 3C/4C demonstrate that *Auts2* and *Caln1* form a loop, which is disrupted by repeated cocaine. CTCF, a mediator of looping, binds less to *Auts2* and *Caln1* after cocaine administration, with coincident increases in the permissive chromatin mark, H3K4me3. We propose that these changes are mediated by increased DNA methylation at a CTCF site and lead to opening of the *Auts2-Caln1* loop, which mediates the cocaine-induced increase in their expression. Such induction of *Auts2* and *Caln1* then augments rewarding responses to cocaine. These mechanisms appear specific for NAc D2 MSNs.