

Chronic Cocaine-Induced H3 Acetylation and Transcriptional Activation of *CaMKII α* in the Nucleus Accumbens Is Critical for Motivation for Drug Reinforcement

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The regulation of gene expression in the brain reward regions is known to contribute to the pathogenesis and persistence of drug addiction. Increasing evidence suggests that the regulation of gene transcription is mediated by epigenetic mechanisms that alter the chromatin structure at specific gene promoters. To better understand the involvement of epigenetic regulation in drug reinforcement properties, rats were subjected to cocaine self-administration paradigm. Daily histone deacetylase (HDAC) inhibitor infusions in the shell of the nucleus accumbens (NAc) caused an upward shift in the dose-response curve under fixed-ratio schedule and increased the break point under progressive-ratio schedule, indicating enhanced motivation for self-administered drug. The effect of the HDAC inhibitor is attributed to the increased elevation of histone acetylation induced by chronic, but not acute, cocaine experience. In contrast, neutralizing the chronic cocaine-induced increase in histone modification by the bilateral overexpression of HDAC4 in the NAc shell reduced drug motivation. The association between the motivation for cocaine and the transcriptional activation of addiction-related genes by H3 acetylation in the NAc shell was analyzed. Among the genes activated by chronic cocaine experiences, the expression of *CaMKII α* , but not *CaMKII β* , correlated positively with motivation for the drug. Lentivirus-mediated shRNA knockdown experiments showed that *CaMKII α* , but not *CaMKII β* , in the NAc shell is essential for the maintenance of motivation to self-administered cocaine. These findings suggest that chronic drug-use-induced transcriptional activation of genes, such as *CaMKII α* , modulated by H3 acetylation in the NAc is a critical regulatory mechanism underlying motivation for drug reinforcement.

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INTRODUCTION

Drug addiction is a debilitating psychiatric disorder characterized by (1) a compulsion to seek and take the drug, (2) a loss of control in limiting intake, and (3) the emergence of a negative emotional state when access to the drug is prevented (Hyman *et al*, 2006; Kalivas *et al*, 2005; Koob and Kreek, 2007). The complex behavioral changes leading to drug addiction are thought to result from molecular and cellular adaptation in specific brain regions (Brami-Cherrier *et al*, 2005). One area of intensive research to clarify neuroadaptation addresses the regulation of gene expression induced by the drug (Nestler, 2001). The drug induces changes in gene expression in key brain reward regions, such as the nucleus accumbens (NAc),

prefrontal cortex (PFC), and ventral tegmental area (VTA). For instance, the expression of the immediate early gene *c-Fos* and several other Fos family proteins is upregulated in the nucleus accumbens, striatum (Courtin *et al*, 2006) and frontal cortex (Thiriet *et al*, 2000) after acute cocaine treatment. The expression of genes, such as *AGS3*, *FosB*, and *BDNF*, is induced after chronic cocaine administration (Bowers *et al*, 2004; Grimm *et al*, 2003; Nestler *et al*, 1999; Pich *et al*, 1997).

Chromatin remodeling epigenetically controls gene transcription, indicating that complex mechanisms regulate the accessibility of genes to the transcriptional machinery (Colvis *et al*, 2005). In general, increased histone acetylation is associated with DNA relaxation and elevated transcriptional activity, whereas decreased acetylation, brought about by histone deacetylases (HDACs) and methyl-CpG-binding proteins, results in tighter DNA coiling and gene silencing. Histone acetylation/deacetylation is increasingly recognized as a crucial mechanism in several important phenomena in the brain, including neuronal differentiation (Hsieh and Gage, 2004; Lunyak *et al*, 2002), neurodegeneration (Hockley *et al*, 2003; Hoshino *et al*, 2003; Steffan *et al*,

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2001), circadian rhythm (Etcheagaray *et al*, 2003), seizure (Huang *et al*, 2002; Tsankova *et al*, 2004), memory formation (Guan *et al*, 2009; Levenson *et al*, 2004; Levenson and Sweatt, 2005), and drug addiction (Kumar *et al*, 2005; Levine *et al*, 2005).

Intravenous drug self-administration, which better models the human syndrome especially the addicted-state changes such as increased motivation for the drug (Renthal and Nestler, 2008), is a useful tool for exploring the neurobiology of drug positive reinforcement in experimental animals (Koob *et al*, 1989). It has been shown that the vertical upward shift in the dose-response curve obtained under a fixed-ratio schedule of reinforcement reflects the escalation in the hedonic set-point (Ahmed and Koob, 1998), which may be accompanied by increased motivation to take the drug (Paterson and Markou, 2003). A downward shift in the dose-response curve is opposite to changes associated with the development of cocaine addiction (Graham *et al*, 2007; Robledo and Koob, 1993). The progressive-ratio schedule of reinforcement is considered to reflect the motivation to self-administer a drug with less influence from factors such as drug satiation or tolerance than that observed in fixed-ratio schedule of reinforcement (Paterson and Markou, 2003). However, research is scarce into the epigenetic mechanism of drug self-administration and better models of the human syndrome, especially the addicted-state changes such as the increased incentive to drug reinforcement.

The NAc is well known to mediate the primary reinforcing and psychomotor stimulation of drugs of abuse (Everitt and Robbins, 2005; Ito *et al*, 2004). The NAc is a heterogenous structure with at least two distinct regions, the shell and core, which may contribute in different ways to drug self-administration. There is strong evidence that the mesolimbic dopamine projection to the NAc shell mediates the response rate-increasing or psychomotor-stimulatory effects of the drug. In contrast, the NAc core mediates the effects of the conditioned reinforcer through its afferent inputs from the limbic cortical structures (Belin and Everitt, 2008; Di Ciano *et al*, 2008; Everitt and Robbins, 2005; Olmstead *et al*, 2001; Parkinson *et al*, 1999).

In this study, the role of histone acetylation in the shell and core of the NAc during the incentive motivation for cocaine after a chronic phase of drug self-administration was examined in rats. Chromatin immunoprecipitation (ChIP) assays were performed to analyze the epigenetic mechanism by which gene transcriptional activity is regulated during chronic cocaine use. Our results establish an important role for the chronic cocaine-induced increase in histone H3 acetylation and the transcriptional activation of the gene encoding Ca^{2+} /calmodulin-dependent protein kinase (*CaMK*) II- α in the shell of the NAc in the motivation for cocaine reinforcement and suggest a new therapeutic mechanism for anti-addiction treatment.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Shanghai Center of Experimental Animal, Chinese Academy of Sciences) weighing 275–325 g were housed and maintained on a 12:12-h

reverse day/light cycle with free access to food and water. All experiments were conducted in strict accordance with the National Institute of Health Guide for the care and use of laboratory animals.

Drugs

Cocaine hydrochloride (Qinghai Pharmaceutical Firm) was dissolved in 0.9% saline to make 4 mg/ml solution. Trichostatin A (TSA, Sigma) and suberoylanilide hydroxamic acid (SAHA, Alexis) were dissolved in 10% dimethyl sulfoxide (DMSO, Sigma).

Viral Expression

Expression plasmids for HDAC4 (kindly provided by Dr Ilona S Skerjanc at University of Western Ontario), Δ HDAC4, and beta-galactosidase were subcloned into adenovirus; and plasmid-based CaMKII α shRNA and CaMKII β shRNA were subcloned into lentivirus. The details of adenovirus and lentivirus cloning and packaging are given in the Supplementary Materials and Methods.

Sucrose Training and Surgery

To facilitate subsequent acquisition of cocaine self-administration, animals were initially maintained at 85% original body weight and trained to press a lever for a 45 mg sucrose pellet in operant chambers (Med Associates, Georgia, VT) on fixed-ratio 1 schedule until acquisition criteria were achieved (100 pellets self-administered for 3 consecutive days). Animals were then fed *ad libitum* for at least 1 day before surgical intravenous catheterization and/or brain cannulation as described previously (Choi *et al*, 2006; Sun *et al*, 2008). Rats were anaesthetized with 10% chloral hydrate. A silastic catheter (0.04 cm, interior diameter) was inserted 3 cm into the right jugular vein and the other end was attached to a stainless steel pedestal. Bilateral 27-gauge guide cannulae were implanted in the NAc shell (+1.7 mm A/P, \pm 0.8 mm M/L, and -6.5 or 5.5 mm D/V), NAc core (1.7 mm A/P, \pm 2.2 mm M/L, and -6.0 or 5.0 mm D/V), or medial prefrontal cortex (mPFC; 3.2 mm A/P, \pm 0.7 mm M/L, and -2.2 mm D/V) with level skull according to Paxinos and Watson. Chemical or virus perturbations were delivered through 33-gauge (Plastics One) bilateral infusion cannulae cut to extend 1 or 2 mm beyond guide cannulae. The stainless steel pedestal was then mounted to the rat's skull followed by dental cement. Catheters were flushed every day with 0.2 ml of saline solution containing heparin (25 U/ml) and benzylpenicillin (6400 U/ml). After surgery, animals were subjected to standard postoperative care and were allowed to recover for 7 days before the start of any experiment. The positions of cannulae or the infusion of chemicals were verified by Nissl's staining or tracer diffusion, respectively, as described in the Supplementary Materials and Methods, and Supplementary Figure S1.

Cocaine Self-Administration Procedures

After 1 week of recovery period, animals were trained to self-administer intravenous injections of cocaine delivered

over 4 s (fixed-ratio 1) in 4-h session daily (Sun *et al.*, 2008). Each injection was accompanied by extinction of the house light and presence of combined conditioned cues (CS), including illumination of the stimulus light and simultaneous presence of an audible tone for 20 s. Depression of the inactive lever had no programmed consequences. For behavioral experiments, the response requirement was gradually increased to five lever presses (LPs) per injection (fixed-ratio 5 (FR5)) and training continued until cocaine intake was stabilized (number of total infusions varied <10% from the mean of three consecutive sessions). Catheter patency was verified after testing by brief anesthesia with sodium methohexital (0.1 mg in 0.1 ml). In some experiments, the reinforcement contingency was changed to a progressive-ratio schedule similar to that described by McGregor and Roberts (1995). Under this schedule, response requirements for each successive injection increased by progressive increments of the following series (Richardson and Roberts, 1996): 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, and so on according to response requirement = $(5e^{(\text{injection times} \times 0.2)}) - 5$. The number of cocaine infusions/number of sessions earned or the ratio achieved before a 1-h period of non-reinforcement was scored as the break point. Progressive-ratio schedule training was completed when 3 consecutive days of stable responding (± 2 break points) were obtained.

Microinjection Procedures

The obturators were removed from the guide cannulae and 33-gauge stainless steel microinjectors were inserted. These microinjectors were cut to a length that extended 1 mm (HDAC inhibitor infusions) or 2 mm (virus overexpression) below the guide cannulae and into the NAc shell, NAc core, and mPFC. Microinjections into the lateral septum were made through the same guide cannulae aimed at the shell. Animals received bilateral microinjections of 1.0 μl of 165 μM TSA at a rate of 0.1 $\mu\text{l}/\text{min}$ for 10 min, and a total volume of 2.0 μl SAHA (100 μM) was constantly delivered at a rate of 0.0083 $\mu\text{l}/\text{min}$ for 4 h by mini-pump. For virus-mediated gene transfer, adenovirus or lentivirus was microinjected intracerebral at the rate of 0.1 $\mu\text{l}/\text{min}$ for 10 min in 1.0 μl . After the microinjections, the stainless steel microinjectors were left in place for 5 min to allow the solution to diffuse away from the tips of the cannulae.

Western Blotting

Histone for immunoblotting was prepared as previously described (Levenson *et al.*, 2004). In western blotting analysis, the blots were incubated with IRDye 700CW or 800CW-conjugated secondary antibody, the infrared fluorescence image was obtained using Odyssey infrared imaging system (Li-Cor Bioscience), the optical density of the bands were quantified using the Image-Pro Plus 5.1 (Media Cybernetics), and the optical density of each AcH3, AcH4, or CaMKII α and CaMKII β bands were normalized to the optical densities of the corresponding H3, H4, or β -actin bands, respectively. Data were obtained from at least three independent experiments.

Immunohistochemistry

Brain sections were incubated overnight at 4 °C with AcH3 (rabbit polyclonal, Lys-9/Lys-14, 1 $\mu\text{g}/\text{ml}$, Upstate Biotechnology) or AcH4 (rabbit polyclonal, Lys-5/Lys-8/Lys-12/Lys-16, 2 $\mu\text{g}/\text{ml}$, Upstate Biotechnology) antibodies. Slices were then incubated for 1 h at room temperature with biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories) and processed with avidin-biotinylated horseradish peroxidase complex (Elite ABC kit, Vector Laboratories) for 45 min at room temperature. The reaction was visualized using diaminobenzidine (DAB, Sigma). Pictures of the regions of interest (the NAc shell, core, or mPFC) were taken with a microscopic CDD camera in $\times 40$ object lens (0.066 mm^2/graph). Quantification of immunoreactive cells was performed with Image-Pro Plus 5.1. In brief, taking into account that the positive cells were defined with nuclear staining above basal background, counts above threshold were taken in a standard frame sample area from the two consecutive sections across both hemispheres per animal, with at least eight animals per group, and these counts were averaged to produce a mean.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Upstate Biotechnology ChIP kit and following a modified protocol from the manufacturer. In brief, brain tissue was fixed in 1% formaldehyde and cell lysates were sheared by sonication in 1% SDS lysis buffer to generate chromatin fragments with an average length of 200–1000 bp. The chromatin was then immunoprecipitated overnight at 4 °C with antibodies specific to AcH3, AcH4, or an equivalent amount of control IgG. Protein–DNA–antibody complexes were precipitated with protein A-agarose beads for 2 h at 4 °C. Input or DNA in the complex was subjected to quantitative PCR. Primer pairs for specific promoter regions are shown in the Supplementary Table S3.

Reverse Transcription and Quantitative PCR

Total RNA was extracted from rat NAc shell with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of purified RNA (100 ng of total RNA) was performed using superscript III reverse transcriptase (Invitrogen). The quantification of DNA was performed using real-time PCR, using SYBR^R Premix Ex Taq (Takara) and ROTOR-GENE RG-3000A (Corbett Research). Ct values from each sample were obtained using the operating software of RG-3000A. Relative quantification of amplified template was performed as described by Chakrabarti *et al.* (2002). Each PCR reaction, run in triplicate for each brain sample, was repeated at least twice independently. ΔCt was obtained by normalizing to corresponding control or actin internal control.

Statistical Analysis

Histone acetylation data were analyzed using one-factor ANOVA, followed by Student–Newman–Keuls *post hoc* comparisons. Behavioral data were analyzed using two-factor ANOVA with repeated measures on dose or test

session. Significant interactions were followed by univariate ANOVA at each dose or test session, which was also used for sucrose self-administration data, and followed by Student–Newman–Keuls *post hoc* tests. All correlations were analyzed with Pearson's correlation test.

RESULTS

Intra-NAC HDAC Inhibitor Infusions Increase the Motivation for Cocaine Self-Administration

The effect of daily bilateral NAC shell infusions of the HDAC inhibitor TSA into the NAC shell on cocaine self-administration was examined in rats under a fixed-ratio schedule in which five LPs produced a cocaine injection. The animals were trained to self-administer cocaine 750 µg/kg per injection in daily 4 h sessions for 3–4 weeks until their cocaine intake had stabilized. The animals then received an intra-NAC-shell infusion of TSA (165 pmol) or vehicle (10% DMSO), before training session each day for 5 days. Cocaine self-administration session was carried out for 15 min after the completion of daily TSA or vehicle infusion. As shown in the left panel of Figure 1a, TSA infusions into the NAC shell had no significant effect on the self-administration of cocaine at 750 µg/kg/injection.

The animals were then tested in a dose-response procedure with descending doses of cocaine (1000, 500, 300, 100, and 0 µg/kg/injection) under the FR5 schedule, and intracerebral infusions of TSA or vehicle were given as described above. As shown in Figure 1a, cocaine was self-administered with a bell-shaped dose-response curve, in which the descending limb indicates a prolonged duration of the cocaine reward at higher injection doses. Notably, infusions of TSA into the shell of NAC led to an upward shift in the dose-response curve (treatment by dose interaction, $F_{4,101} = 3.263$, $P = 0.015$). Higher self-administration rates (at the doses of 300 and 500 µg/kg/injection) of TSA-treated animals were observed in the TSA-treated animals compared with those in the vehicle-infused control. TSA also had a significant effect when these dose-response data were transformed into cocaine intake over the testing session (number of injections \times dose per injection; Figure 1a, middle panels). An upward vertical shift in the self-administration dose-response curve is considered to reflect a transition to a more addicted state, including an increased motivation for drug in animal models (Ahmed and Koob, 1998; Koob *et al*, 1989; Paterson and Markou, 2003; Richardson and Roberts, 1996). Therefore, these data suggest that TSA infusions may facilitate this transition. Neither of these changes was observed when TSA was infused into the core of NAC (Figure 1b) or the mPFC (Supplementary Figure S2), indicating a region-specific interaction in the mesolimbic system.

To confirm that treatment with TSA results in a stronger motivation for cocaine reinforcement, we studied this effect on cocaine self-administration under a progressive-ratio schedule. During progressive-ratio testing, each cocaine injection requires a progressively greater number of LP responses, and the highest ratio of LPs per cocaine injection performed before the animal quit self-administration altogether reflects the incentive strength of cocaine (Graham *et al*, 2007). The final ratio of responses

successfully completed is defined as the 'break point'. After a stable break point baseline had been established on the progressive-ratio schedule at 750 µg/kg/injection, TSA or vehicle was administered by intra-NAC-shell infusions for 5 days. The rats were subsequently allowed to self-administer cocaine at 500 and 1000 µg/kg/injection under a progressive-ratio schedule, and the same daily intracerebral infusions were administered before each session. As shown in Figure 1c, the control animals receiving vehicle infusions showed a slight extinction-like responding (Richardson and Roberts, 1996) during training for 5 consecutive days (750 µg/kg/injection), and worked harder for the higher injection dose of cocaine (1000 vs 500 µg/kg), indicating that the higher dose of cocaine was more reinforcing. In contrast, compared with vehicle-infused control, intra-NAC-shell TSA infusions led to a gradually increasing effort to obtain cocaine, achieving higher response/injection ratios at 750 µg/kg/injection (treatment by test day interaction, $F_{4,90} = 7.126$, $P < 0.001$; Figure 1c, left panel). On the fifth test day, treatment with TSA increased the break point to 22.8 self-injections compared with a break point of 17.8 in rats treated with vehicle control. The effect of TSA treatment on drug intake is clearer when the data are expressed as the number of LPs the rat performed to obtain the last injection. During the last session, TSA-treated animals performed 479 ± 65 LPs on the active lever compared with 207 ± 29 LPs by the control animals ($F_{1,17} = 12.789$, $P = 0.002$; Figure 1c, left panel). The effect can also be illustrated by expressing the cumulative number of LPs performed by the rats during the entire session ($F_{1,17} = 17.831$, $P < 0.001$; data not shown). Similarly, the effect of TSA on the break point was also observed in rats treated at cocaine doses of 500 and 1000 µg/kg/injection (treatment by dose interaction, $F_{1,32} = 10.146$, $P = 0.003$; Figure 1c, middle panel). In contrast, neither of these changes was observed when TSA was infused into the NAC core (Figure 1d) or mPFC (Supplementary Figure S2). TSA infused in the core slightly and increased the cumulative LPs (750 µg/kg/injection) on the fifth test day ($F_{1,14} = 5.575$, $P = 0.033$; Supplementary Figure S7a left), whereas it had no significant effect on the break point. However, the effect of infusion of TSA into the shell of NAC on the motivation for cocaine was much stronger than the effect of TSA infusion into the core.

The delivery of SAHA, another highly specific inhibitor of class I and class II HDACs, into the NAC shell before the cocaine self-administration test produced similar results (FR5, $F_{4,96} = 2.941$, $P = 0.025$; progressive ratio, $F_{4,86} = 6.245$, $P < 0.001$; Figure 1e). These results showed that intra-NAC-shell infusions of HDAC inhibitors enhance motivation for rat to obtain cocaine, which may be attributable to the potentiation of histone acetylation induced by sustained HDAC inhibition.

Chronic Cocaine Experience Increases Histone Acetylation in the NAC

We next evaluated whether histone acetylation levels change after acute and chronic administration of cocaine. As shown by western blotting results in Figure 2a and b, the acetylation levels of H3 and H4 were elevated specifically in the NAC shell ($F_{2,27} = 6.327$, $P = 0.006$ and $F_{2,27} = 5.874$,

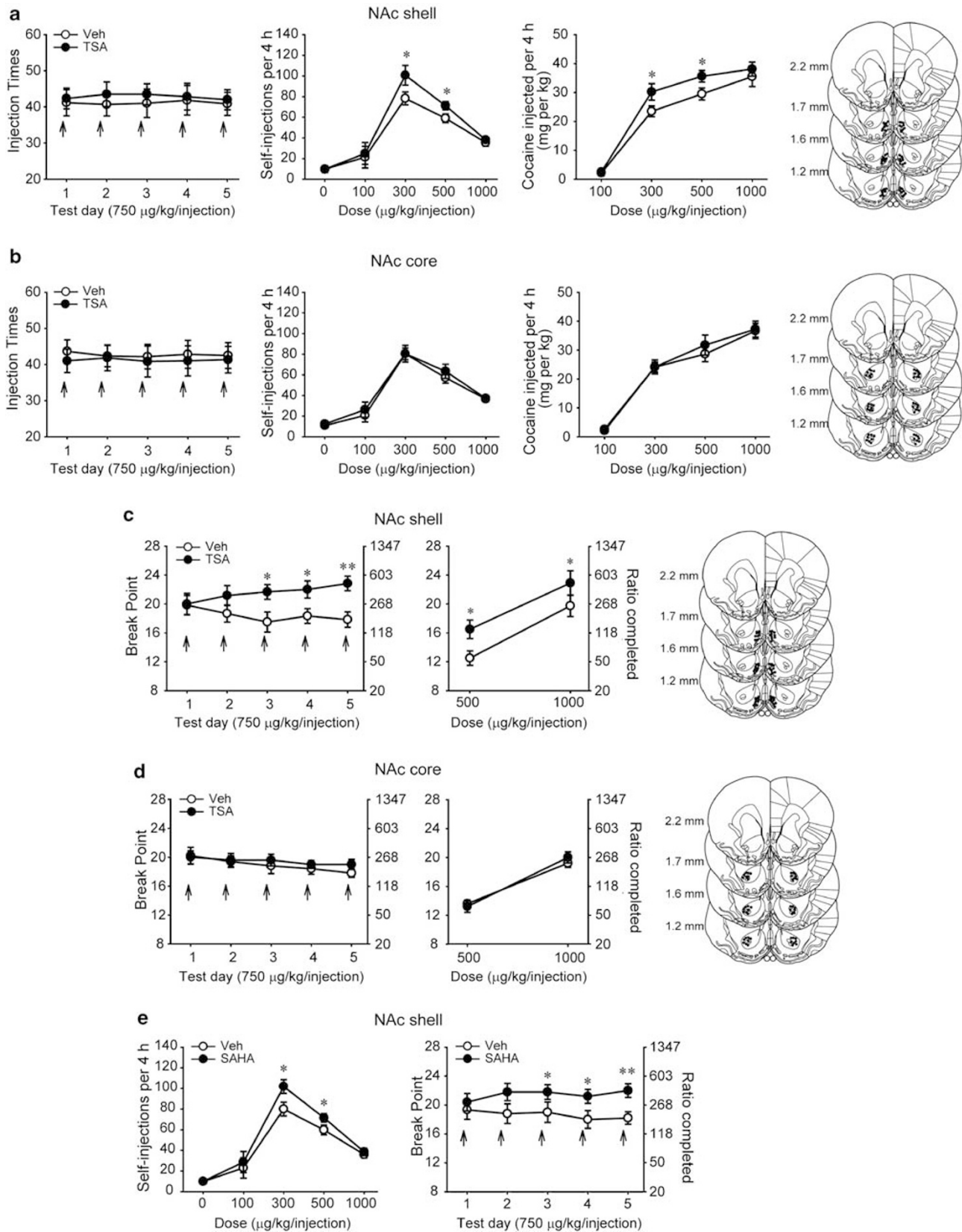


Figure 1 HDAC inhibitor infusions into the NAc shell increase the motivation for cocaine self-administration. (a, b) Daily infusions of TSA into the shell (a), but not the core (b) of NAc induced an upward shift in dose-response and dose-intake curves under fixed-ratio 5 (FR5) schedule of cocaine self-administration. (c, d) Daily infusions of TSA into the shell (c), but not the core (d) of NAc increased break point under progressive-ratio schedule. Injection sites in the shell or core are illustrated on the right panel. Daily infusions of SAHA (e) into the shell of NAc increased responding in dose-response curve and break point ($N = 8-12$ per group). Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, compared with vehicle-chronic cocaine control.

$P = 0.008$; Figure 2a and b, left), but not in the NAc core or the mPFC (data not shown) after chronic intravenous cocaine self-administration experience (4 h per day for 18 days). These results were further verified using immunohistochemistry analysis (ACh3, $F_{2,26} = 4.392$, $P = 0.023$; ACh4, $F_{2,26} = 3.914$, $P = 0.033$; Figure 2c and d and Supplementary Figure S3). These sustained histone modifications in the NAc shell could be detected at 3, 12, and even 24 h after chronic drug use (Supplementary Figure S4a). Cocaine injections delivered to the animals through self-administration (contingent upon LP behavior) or yoked animals (passively administered equal number of injections at the same temporal pattern) resulted in comparable increases in histone acetylation (Figure 2a–c). Moreover, histone acetylation levels in the shell or core of NAc did not change

after acute or chronic sucrose self-administration sessions (data not shown), indicating that the increase in histone acetylation in the NAc was unlikely a result from instrumental learning or generalized exposure to natural rewards. Moreover, histone acetylation levels in the shell or core of NAc or mPFC in rats receiving acute cocaine through either yoked- or self-administration remained unchanged. Therefore, the elevation of histone acetylation in the NAc shell seems to reflect a generalized pharmacological response to chronic cocaine use.

The effect of HDAC inhibitor infusions on histone acetylation in different encephalic regions was assessed using western analysis after chronic cocaine self-administration. As shown in Figure 2e and f, the TSA (165 pmol) or SAHA (200 pmol) infusions increased the basal levels of H3

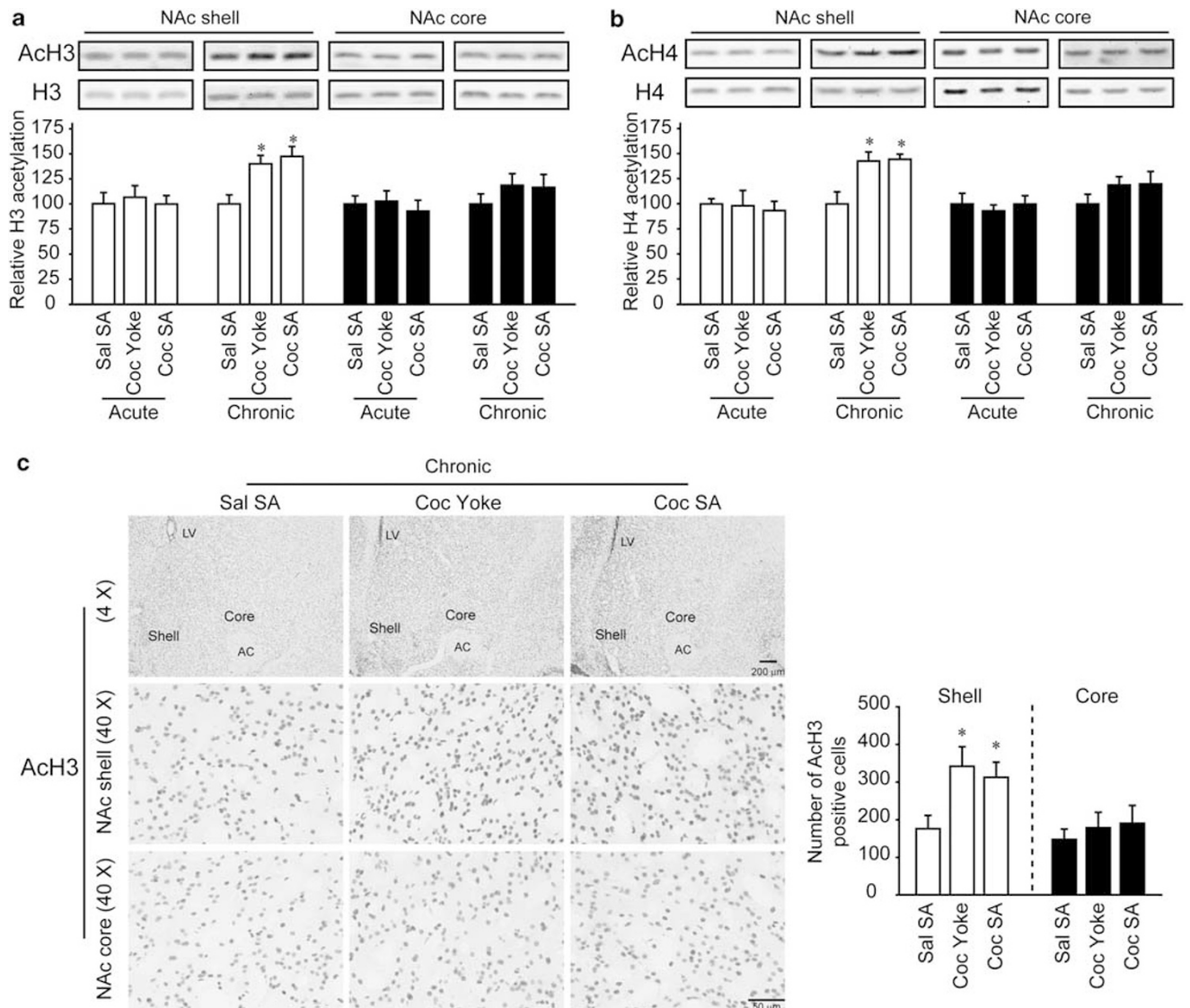


Figure 2 HDAC inhibitor augments histone acetylation elevation induced by chronic cocaine experience in the NAc shell. (a, b) Immunoblots of histone acetylation of H3 (ACh3) (a) and H4 (ACh4) (b) in the shell or core of NAc 30 min after 1 day (acute) or 18 days (chronic) cocaine yoked (Coc Yoke) or self-administration (Coc SA). (c, d) Immunohistochemistry data of ACh3 (c) and ACh4 (d) in the shell or core of NAc after chronic Coc Yoke or Coc SA. AC, anterior commissure; LV, lateral ventricle. (e, f) ACh3 (e) and ACh4 (f) levels after daily HDAC inhibitor infusions in the NAc shell or core. Immunoblot and immunohistochemistry data from cocaine-treated groups ($N = 8-12$ per group) are expressed as mean \pm SEM percentage of saline self-administration control ($N = 8-12$ per group). * $P < 0.05$, ** $P < 0.01$, compared with saline self-administration control or vehicle-chronic saline control; # $P < 0.05$, compared with vehicle-chronic cocaine control.

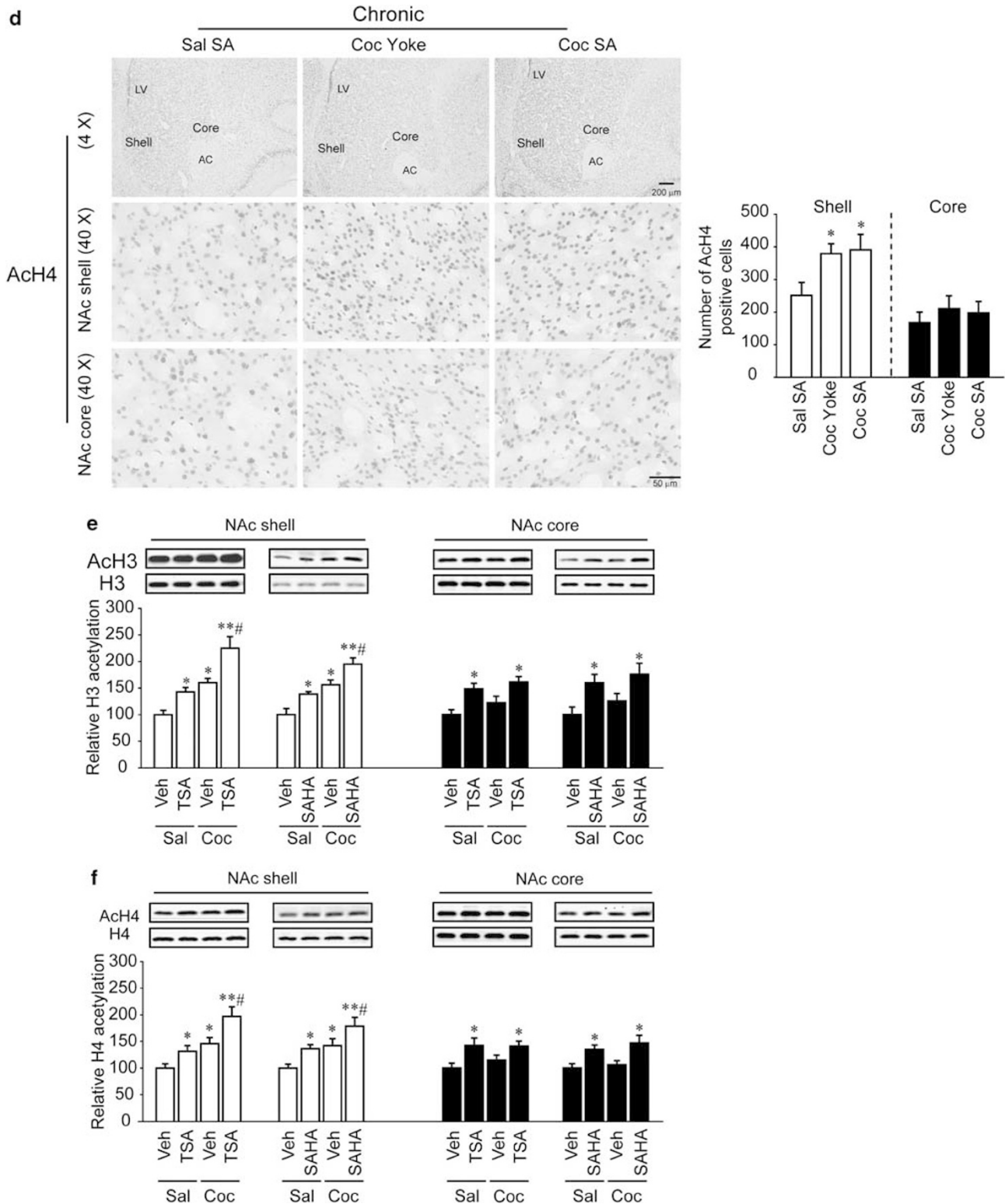


Figure 2 Continued.

and H4 acetylation in the shell and core of NAc, and in mPFC (data not shown). Chronic cocaine-stimulated increases in histone acetylation in the NAc shell were notably augmented by HDAC inhibitor pretreatment (Figure 2e and f). Moreover, Supplementary Figure S1 shows that HDAC inhibitor administered into the shell of

NAc did not diffuse to the core, or *vice versa*, and that the infusion of the HDAC inhibitor into the shell or core did not influence histone acetylation in the other site. These data, combined with the observation that the infusion of the HDAC inhibitor into the NAc core had no obvious effect on the dose-response curve or break point, support the notion

that increased histone acetylation in the NAc shell induced by chronic cocaine self-administration is associated with motivation for drug reinforcement.

Intra-NAc HDAC4 Overexpression Reduces the Motivation for Cocaine Self-Administration

To gain further insight into the relationship between histone acetylation and the motivation for cocaine self-administration, we analyzed the effect of neutralizing cocaine-stimulated increase in histone acetylation by overexpressing of HDAC4, a subtype of class II histone deacetylases expressed in the NAc (Renthal *et al*, 2007). We chose HDAC4 because it is recruited by *MEF*, *SMRT*, *BCOR*, and *CTBP* for transcriptional repression (Bertos *et al*, 2001). Recombinant adenovirus encoding flag-tagged HDAC4 (AV-HDAC4), truncation mutant of HDAC4 lacking the catalytic domain (AV- Δ HDAC4), or β -galactosidase (AV-gal) was infused into the NAc shell, NAc core, or mPFC (Figure 3a), and a highly localized overexpression of HDAC4, Δ HDAC4, or β -galactosidase was detected in the injected region (Figure 3b). Neurons infected with recombinant adenovirus were localized in the NAc shell (Figure 3b, left panel), NAc core (Figure 3b, right panel), or mPFC without gliosis or scarring (data not shown). As shown in Figure 3c–e and Supplementary Figure S5, the overexpression of full-length HDAC4, but not its truncated form, which lacks deacetylase activity, strongly inhibited the cocaine-induced increase in histone acetylation locally in the NAc shell. The effect of overexpression of HDAC4 in the NAc on self-administration of cocaine was examined 2 weeks after adenoviral infection, during which period the expression of the target protein reached its peak.

The expression of HDAC4 in the NAc did not alter cocaine self-administration at a dose of 750 μ g/kg/injection (Figure 3f and 3g, left panels). On the FR5 schedule, the dose-response curve shifted downward in the HDAC4-overexpressing rats compared with β -galactosidase (gal) control (treatment by dose interaction, $F_{8,132} = 3.293$, $P = 0.002$; Figure 3f, middle panel), and the overall cocaine intake was reduced in these animals across injection doses from 300 to 500 μ g/kg/injection (Figure 3f, right panel). In progressive-ratio testing, intra-NAc-shell HDAC4 overexpression led to a gradually decreased motivation for cocaine, achieving lower response/injection ratios at 750 μ g/kg/injection (treatment by test day interaction, $F_{8,121} = 8.149$, $P < 0.001$; Figure 3h, left panel). On the fifth test day, the overexpression of HDAC4 in the NAc shell reduced the break point to 18.8 self-injections compared with 15.2 in rats treated with gal control. During the last session, HDAC4-overexpressing animals performed 103 ± 12 LPs on the active lever, whereas the control group performed 248 ± 25 LPs ($F_{2,21} = 9.124$, $P = 0.007$; Figure 3h, left panel). This effect can also be expressed as the total number of LPs performed by the rats during an entire session ($F_{2,21} = 12.938$, $P = 0.002$, data not shown). The results were similar for animals receiving doses of 500 and 1000 μ g/kg/injection on the progressive-ratio schedule (treatment by dose interaction, $F_{2,48} = 12.74$, $P < 0.001$; Figure 3h, right panel). However, overexpression of Δ HDAC4 had no significant effect on the instrumental responding under the FR5 or progressive-ratio schedule,

which confirmed that the effect of HDAC4 expression on the motivation for cocaine self-administration is dependent on its deacetylation activity. Neither of these changes was observed when HDAC4 overexpression was restricted to the NAc core (Figure 3g and i) or mPFC (Supplementary Figure S6). On the fifth test day under progressive-ratio schedule (750 μ g/kg/injection), only the reduction in the cumulative LPs, but not that in the break point, approached statistical significance in animals transfected with HDAC4-recombinant virus in the NAc core ($F_{2,21} = 3.315$, $P = 0.056$; Supplementary Figure S7b). Taken together, these results confirm that the elevation of histone acetylation in the NAc shell is critical for drug motivation.

To analyze whether the response to natural rewards is also regulated by histone acetylation, rats were trained to self-administer sucrose pellets on a FR5 or progressive-ratio schedule, and the effects of HDAC inhibitor treatment and HDAC4 overexpression in the NAc shell were tested using procedures similar to those for cocaine self-administration. As shown in Supplementary Figure S7c–f, HDAC inhibition and HDAC4 overexpression had no effect on the latency of sucrose pellet self-administration or the break point on the progressive-ratio schedule. These data indicate that the altered response to cocaine induced by HDAC inhibition or HDAC4 overexpression involves motivation-related rather than performance-related effects, and suggest that histone acetylation in the NAc shell does not have a prominent role in maintaining natural reward-seeking behaviors.

Motivation for Cocaine is Associated with H3 Acetylation in the NAc

To further clarify the different roles of H3 and H4 acetylation in the motivation for cocaine reinforcement, the NAc shell was dissected from rats that had acquired a stable break point under the progressive-ratio schedule in chronic cocaine self-administration. The correlation between the break point and histone acetylation was analyzed. The break point correlated positively with the global acetylation level of H3, but not H4 (Figure 4a and b). However, this correlation was not observed in the NAc core (data not shown). These findings support the hypothesis that gene expression regulated by the acetylation of H3 in the NAc shell may have an important role in the motivation for cocaine.

It has been shown that chronic drug use induces many gene changes in *c-Fos*, *Egr1*, *AGS3*, *Creb*, *Cbp*, *BDNF*, *FosB*, *Cdk5*, *CaMKII α* , *CaMKII β* , *GluR2*, *GluR3*, *NR2A*, *NR2B*, and *Psd95* (Bowers *et al*, 2004; Bozon *et al*, 2003; Kumar *et al*, 2005; Li *et al*, 2008; McClung and Nestler, 2003; Thiriet *et al*, 2000; Tsankova *et al*, 2007). Therefore, the levels of H3 and H4 acetylation at the promoters of these genes were analyzed in the shell and core of the NAc using ChIP after chronic saline or cocaine self-administration, or cocaine yoked administration. As shown in Figure 4c and d, in the NAc shell, chronic cocaine use caused a robust (more than threefold) increase in H3 acetylation at promoters of *Cbp*, *BDNF-P2*, *BDNF-P3*, *FosB*, *Cdk5*, *CaMKII α* , *GluR2*, *NR2A*, *NR2B*, and *Psd95*. In contrast, the hyperacetylation of H4 was only detected at the promoters of *Egr1* and *Psd95*. In the NAc core, a slight (approximately twofold) increase in H3 acetylation was detected at the *BDNF-P2*, *FosB*, *Cdk5*,

GluR2, *NR2A*, *NR2B*, and *Psd95* promoters (Supplementary Table S1). Similar upregulation of H4 acetylation was observed at the *Egr1* and *Psd95* promoters (Supplementary

Table S2). These data are consistent with the correlation analysis results and indicate that chronic cocaine-induced gene transcriptional activation occurs primarily in the NAc

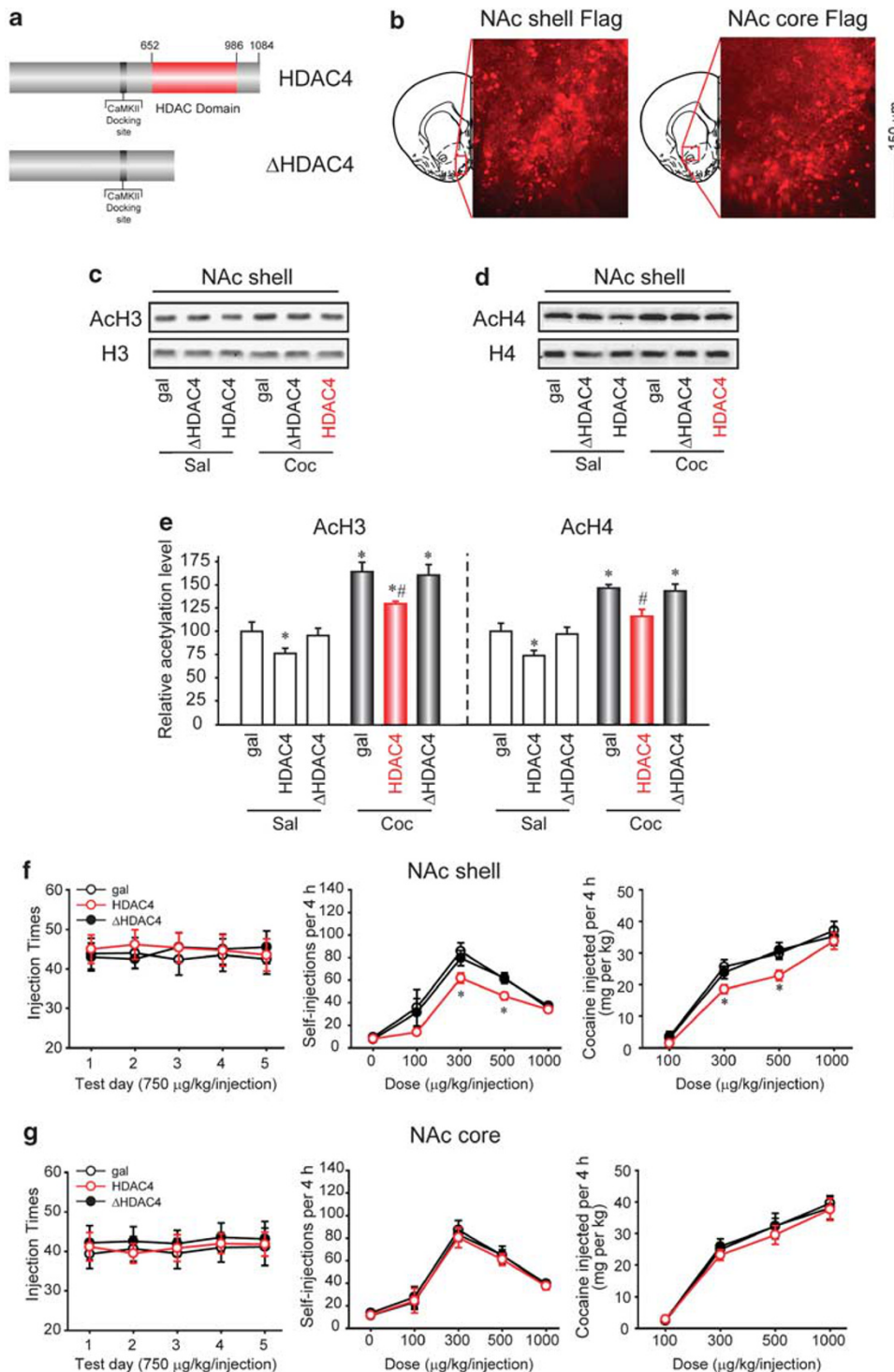


Figure 3 HDAC4 overexpression in the NAc shell downregulates histone acetylation elevation and reduces the motivation for cocaine self-administration. (a) HDAC4 domain structure, depicting the CaMKII docking site and the catalytic HDAC domain. The HDAC4 truncation (Δ HDAC) without the catalytic HDAC domain used for the behavioral experiments is also shown. (b) Representative staining of AV-HDAC4 in the NAc shell (left) and core (right). Neurons infected with HDAC4 were labeled using Flag immunofluorescence. (c–e) Immunoblots of AcH3 (c) and AcH4 (d) in the NAc shell of rats that underwent HDAC4 overexpression. Densitometric quantification of immunoblot in c, d (e) of NAc shell ($N = 8–12$ per group). Data are presented as mean \pm SEM. * $P < 0.05$, compared with gal-chronic saline control, # $P < 0.05$, compared with gal-chronic cocaine control. (f–i) HDAC4 overexpression in the NAc shell induced a downward shift in dose-response, dose-intake curves (f) and decreased break point (h), but not in the NAc core (g, i). $N = 8–12$ per group. Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, compared with gal-chronic cocaine control.

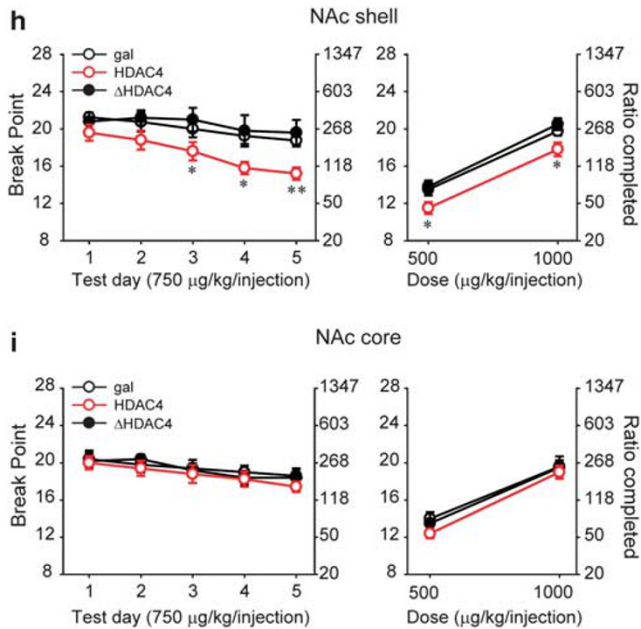


Figure 3 Continued.

shell and is predominantly modulated by the acetylation of H3.

To analyze the association between the motivation for cocaine and the gene transcriptional activation modulated by H3 acetylation, the effects of HDAC inhibitor infusions and overexpression of HDAC4 on chronic cocaine-induced histone acetylation at these promoters were further examined using ChIP assays. Daily intra-NAc-shell TSA infusions strongly augmented the increase in chronic cocaine-induced H3 acetylation at the promoters of *Cbp*, *BDNF-P2*, *BDNF-P3*, *FosB*, *Cdk5*, *CaMKII α* , *GluR2*, *NR2A*, and *NR2B* (1.8- to 4.2-fold upregulation; Figure 4e), accompanied by an increase in the motivation for cocaine (Figure 1a and c). The overexpression of HDAC4, but not Δ HDAC4, in the NAc shell significantly inhibited the chronic cocaine-stimulated elevation of H3 acetylation at these gene promoters (1.2- to 3.0-fold downregulation; Figure 4f), accompanied by a reduction in motivation (Figure 3f and h). However, neither chronic infusions of HDAC inhibitor nor HDAC overexpression affected H3 acetylation at these gene promoters in the saline self-administration group (Figure 4e and f). Moreover, only mild changes in H3 acetylation were detected at the *BDNF-P2*, *FosB*, *Cdk5*, *NR2A*, and *NR2B* promoters in the NAc core (0.8- to 2.0-fold upregulation and 0.5- to 1.2-fold downregulation; Supplementary Figure S8a and 8b). No significant change in histone acetylation in the mPFC was induced under similar treatment regimens (Supplementary Figure S8c and 8d). These results suggest that drug-induced gene transcriptional activation in the NAc shell, modulated by H3 acetylation, is a potential mechanism underlying the motivation for cocaine.

Knockdown Expression of *CaMKII α* in the NAc Decreases the Motivation for Cocaine Self-Administration

Recently, Anderson *et al* (2008) showed that CaMKII may be an essential link between the dopamine and glutamate

systems of the NAc shell, which are involved in the neuronal plasticity underlying cocaine relapse. However, the relationship between CaMKII and the motivation for cocaine self-administration is not clear. Therefore, the effect of cocaine administration on the transcript levels of the α and β isoforms of *CaMKII* in the NAc shell were examined using reverse transcription and quantitative PCR. An approximately fourfold upregulation of *CaMKII α* transcript expression, but no change in *CaMKII β* mRNA level, was observed at 30 min after the chronic yoked- or self-administration of cocaine (Figure 5a). Furthermore, the mRNA level of *CaMKII α* , but not *CaMKII β* , correlated positively with the break point (Figure 5b and c) under progressive-ratio schedule of cocaine self-administration. To confirm the effect of *CaMKII α* on the motivation for cocaine, we constructed short hairpin RNA (shRNA) expression plasmids α shRNA and β shRNA, which specifically and effectively knocked down >70% of *CaMKII α* and *CaMKII β* proteins, respectively (Figure 5d). To locally knockdown the expression of *CaMKII α* and *CaMKII β* , animals received bilateral NAc shell infusions of recombinant lentivirus encoding *CaMKII α* shRNA (Lenti- α shRNA), *CaMKII β* shRNA (Lenti- β shRNA), or green fluorescent protein (Lenti-GFP). Lentivirus expression reached its peak after seven daily training sessions. As shown in Figure 5e-g, *CaMKII α* and *CaMKII β* expression in the NAc shell of animals locally infected with lentivirus encoding the corresponding shRNA was reduced by approximately 39 and 50%, respectively, compared with that in the Lenti-GFP-transfected control (Figure 5f and g). However, the localized knockdown of *CaMKII α* or *CaMKII β* expression in the NAc shell did not affect cocaine self-administration at 750 μ g/kg/injection (data not shown).

The effects of the different isoforms of CaMKII on the motivation for cocaine self-administration on behavior were analyzed under progressive-ratio schedule. As shown in Figure 5h, intra-NAc-shell Lenti- α shRNA infusions led to a gradual reduction in the motivation for cocaine and the effect was statistically significant on the fifth test day at a dose of 750 μ g/kg/injection compared with the effect of the Lenti-GFP control ($F_{2,26} = 5.084$, $P = 0.014$, Figure 5h, left panel). A similar effect was observed at doses of 500 and 1000 μ g/kg/injection on progressive-ratio schedule ($F_{2,43} = 4.342$, $P = 0.019$; Figure 5h, right panel). Interestingly, the knockdown of *CaMKII β* expression by Lenti- β shRNA in the NAc shell had no significant effect under the same conditions, indicating a specific interaction between the motivation for cocaine and the different isoforms of CaMKII. However, the knockdown of *CaMKII α* expression in the NAc shell had no significant effect on motivation for sucrose (data not shown). Collectively, these data indicate that *CaMKII α* expression in the NAc shell, regulated by the cocaine-induced elevation of H3 acetylation, is involved in the motivation for cocaine self-administration.

DISCUSSION

Drug-induced changes in gene expression in key brain reward regions are thought to contribute to long-lasting behavioral abnormalities and neuroadaptation (Hyman *et al*, 2006; Koob and Kreek, 2007; Kumar *et al*, 2005).

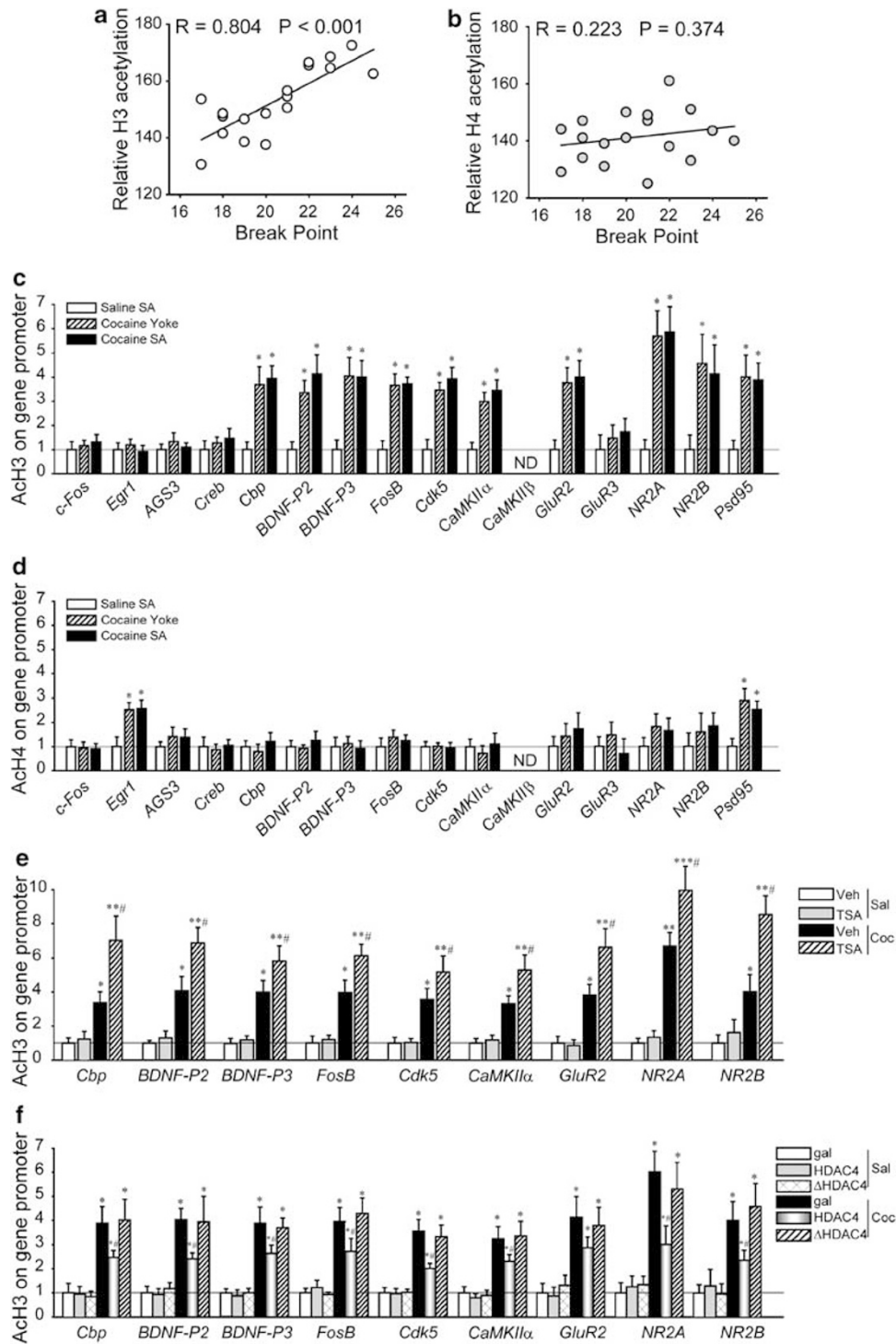


Figure 4 Motivation for cocaine is associated with H3 acetylation in the NAc shell. (a, b) The break point was positively correlated with global level of AcH3 (a), but not AcH4 (b) in the NAc shell. Diagonal solid lines indicate linear regression fit of data points ($N = 18$). (c, d) Changes of histone acetylation in specific gene promoter regions in the NAc shell under progressive-ratio schedule ($N = 6-8$ per group). Data are presented as mean \pm SEM. * $P < 0.05$, compared with saline self-administration control, ND, acetylation not detected. (e, f) The effect of HDAC inhibitor (e) or HDAC4 overexpression (f) on AcH3 at the indicated gene promoters in the NAc shell under progressive-ratio schedule ($N = 8-10$ per group). Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with vehicle- or gal-chronic saline control, # $P < 0.05$, compared with vehicle- or gal-chronic cocaine control.

Genome-wide mRNA analysis has identified many potential gene targets for drugs of abuse in distinct brain reward regions (Freeman *et al*, 2001; McClung and Nestler, 2003;

McClung *et al*, 2005; Winstanley *et al*, 2007; Yao *et al*, 2004). Therefore, it is of great interest to identify the underlying mechanisms by which chronic cocaine experience promote

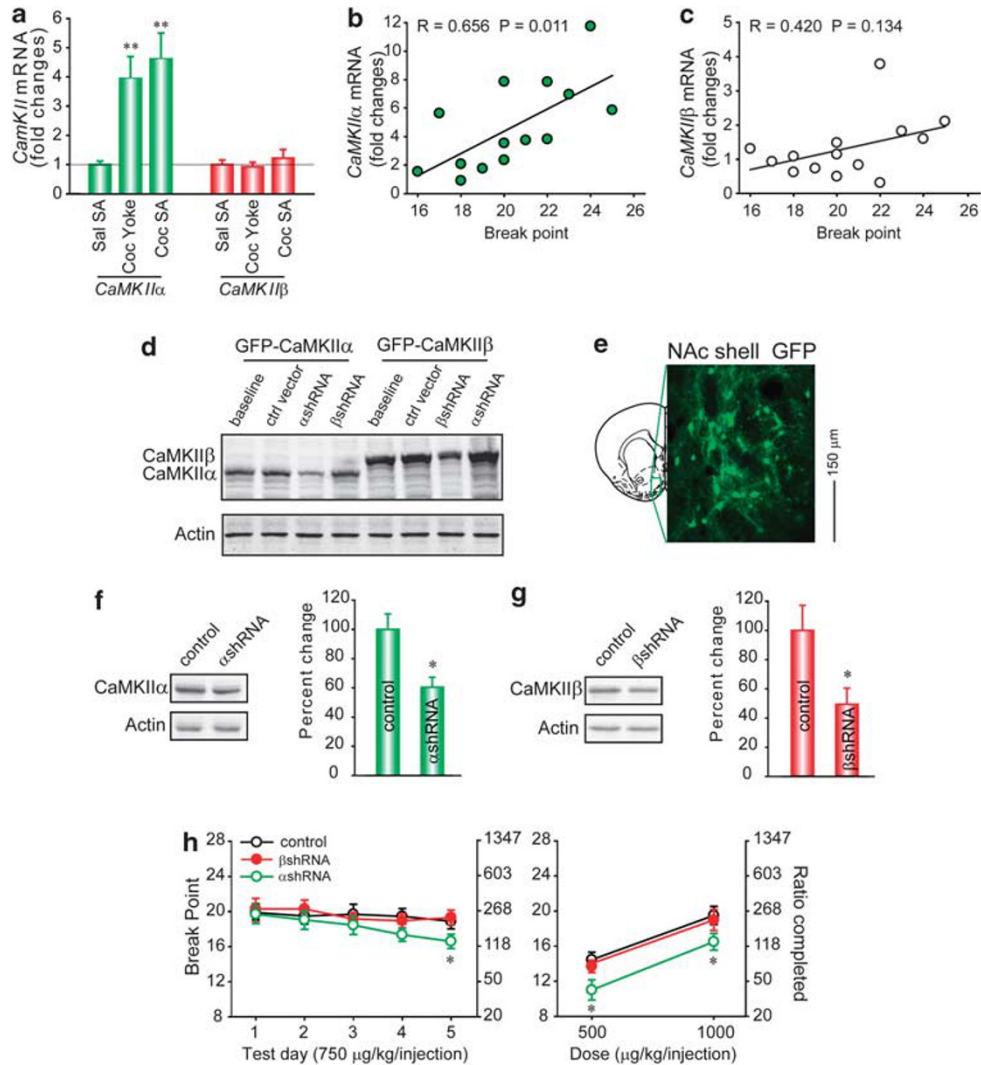


Figure 5 Localized *CaMKIIα* knockdown in the NAc shell decreases the motivation for cocaine. (a) *CaMKIIα* but not *CaMKIIβ* mRNA levels were increased in the NAc shell 30 min after chronic cocaine yoked or self-administration ($N = 8-12$ per group). Data are expressed as mean \pm SEM. $**P < 0.01$, compared with chronic saline self-administration control. (b, c) The break point was correlated with level of *CaMKIIα* mRNA (b), but not *CaMKIIβ* mRNA (c) in the NAc shell. Diagonal solid lines indicate linear regression fit of data points ($N = 14$). (d) GFP-tagged *CaMKIIα* or *CaMKIIβ* were cotransfected into HEK293T cells with or without plasmid encodes shRNA for *CaMKIIα* (α shRNA) or *CaMKIIβ* (β shRNA) expression and the cell lysate was immunoblotted with anti-GFP antibody. pBS-hU6 (vector) was used as a control. (e) Localized Lenti-GFP expression in the NAc shell. (f, g) Immunoblot from the NAc shell infected with Lenti- α shRNA or Lenti- β shRNA showed a reduction in protein level of *CaMKIIα* (f) or *CaMKIIβ* (g), compared with Lenti-GFP control ($N = 8-10$ / group). Data are expressed as mean \pm SEM. $*P < 0.05$, compared with Lenti-GFP control. (h) Intra-NAc-shell Lenti- α shRNA infusions decreased the motivation for cocaine ($N = 8-12$ per group). Data are expressed as mean \pm SEM. $*P < 0.05$, compared with Lenti-GFP control.

sustained changes in gene expression. Recent evidence has prompted the notion that epigenetic mechanisms exert lasting effects on gene expression through the regulation of chromatin structure. Increasing evidence indicates that hyperacetylation in promoter regions is strongly associated with gene transcriptional activation, whereas hypoacetylation correlates with reduced gene expression (Kurdistani *et al*, 2004; Pokholok *et al*, 2005). This association also exists in the brain *in vivo* in response to drug abuse. For example, chronic exposure to cocaine is known to activate a set of genes (eg, *Cdk5* and *BDNF*) in the NAc, and the expression of some of these genes remains elevated for days to weeks (Bowers *et al*, 2004; Grimm *et al*, 2003; Yao *et al*, 2004). Consistent with these changes in gene expression, increased H3 acetylation at the gene promoters of *Cdk5* and

BDNF, which last at least a week, has also been observed after chronic cocaine administration (Kumar *et al*, 2005). Genome-wide ChIP-chip analysis has shown that a large number of genes are H3 acetylated in the NAc in response to chronic cocaine exposure, whereas a significant set of previously unrecognized genes are hyperacetylated only on H4 (Renthal *et al*, 2009; Renthal and Nestler, 2008). In our experiment, the shell and core of the NAc were examined as two distinct regions. Our data show that chronic self- and experimenter-administered cocaine induced long-term increases in global H3 and H4 acetylation in the NAc shell, but showed only an increasing trend in the NAc core. This indicates that chronic cocaine may activate more genes and induce stronger transcriptional activation in the shell of the NAc than in the core.

The present hypothesis is that an escalation in cocaine intake predicts an increase in the hedonic set-point accompanied by an increased motivation for the drug, rather than the development of tolerance, which is generally indicated by a rightward shift in the curve (Ahmed *et al.*, 2000; Emmett-Oglesby *et al.*, 1993; Li *et al.*, 1994). In this context, escalating vertical shifts in the dose-response curve may reflect changes in the self-injection of the drug reward with an increase in the motivation to take the drug (increased peak or maximum rate of behavior). In contrast, mere tolerance to the pharmacological effects of the drug would only shift the curve to the right, without changing the maximum rate of behavior (Ahmed and Koob, 2004). This theory is consistent with our current results, which showed that daily HDAC inhibitor infusions into the NAc shell led to enduring increased responding in the dose-response curve on a fixed-ratio schedule, with higher peak self-administration rates or break point on a progressive-ratio schedule of cocaine self-administration.

To analyze the role of histone acetylation in the NAc shell in the motivation for cocaine, two specific HDAC inhibitors, TSA and SAHA, were used to augment the increase in histone acetylation. These treatments enhanced the motivation for cocaine, as reflected by the upward shift in dose-response curve and the increase of break point. The findings were similar when a third HDAC inhibitor, valproic acid (50 nmol per side), was infused into the NAc shell (data not shown). The neutralization of these changes by the intra-NAc-shell adenovirus-mediated overexpression of HDAC reduced the motivation for cocaine. Taken together, these effects suggest that the elevation of histone acetylation in the NAc shell during chronic cocaine use is critical in regulating the motivation for cocaine.

Two key properties of drug addiction are 'increased intake over time' and 'increased time and energy devoted to acquiring the drug'. The motivational property for drug reinforcement can be analyzed using a progressive-ratio schedule, which has been shown to be very useful in evaluating not only the reinforcing efficacy of the drug of abuse, but also the effect of a variety of pretreatments (Richardson and Roberts, 1996). Roberts and his colleagues have shown that the break point, which is derived from the progressive-ratio schedule, reflects the maximal effort (the increased time and energy devoted to acquire cocaine; Oleson and Roberts, 2009) made to receive a drug infusion and is sensitive to pharmacological challenge (Richardson and Roberts, 1996). Our present data show that chronic cocaine use induced an increase in histone acetylation of ~50%, and augmenting this increase in histone acetylation with HDAC inhibitors resulted in an increase in the break point of 20–27%. Although these changes are moderate, they reflect physiologically significant biochemical and behavioral alterations during the development of addiction. These results imply that the chronic cocaine-induced hyperacetylation of histone is associated with the enhanced motivation for the drugs.

A series of studies have identified an interesting phenomenon, 'incubation', which is a time-dependent increase in cocaine seeking induced by cocaine abstinence (Grimm *et al.*, 2001; Lu *et al.*, 2005). Persistent changes in gene expression, including that of immediate early genes, have been observed during incubation. Freeman *et al.* (2008)

reported the altered expression of *EGR1* and *NPY* after the cessation of drug administration, which was regulated by H3 acetylation. Recently, Romieu *et al.* (2008) found that the daily systemic administration of TSA during the initial phase of cocaine self-administration reduced the instrumental responding including break point, which may have important implications for the effects of epigenetic mechanisms in the early stage of addiction (acquisition of cocaine self-administration). In our experiments, we analyzed the role of histone acetylation in specific brain regions during the chronic phase of cocaine self-administration (long-term reinforcement of cocaine self-administration for more than 30 days). Our data show that chronic drug experience induce H3-acetylation-regulated gene transcriptional activation, which is a critical molecular mechanism underlying the increased motivation for drug reinforcement. Although different neurobiological mechanisms may be involved, gene transcriptional activation in the mesolimbic system, modulated by histone modification, underlies the development of drug addiction throughout its different phases, including the initial, reinforcement, and incubation stages.

A ChIP assay was performed to analyze the gene transcriptional activation modulated by histone acetylation after chronic cocaine use, especially chronic self-administration. We found that chronic cocaine use caused a robust induction of the acetylation of H3, but not H4, at promoters of *Cbp*, *BDNF-P2*, *BDNF-P3*, *FosB*, *Cdk5*, *CaMKII α* , *GluR2*, *NR2A*, and *NR2B* in the NAc shell. Furthermore, the fact that the inhibition or expression of HDAC induced changes in the motivation for cocaine self-administration is consistent with changes in histone acetylation at these gene promoters. These results strongly suggest that gene transcriptional activation induced by H3 acetylation in the NAc shell may have an important role in modulating the motivation for cocaine reinforcement. Recent studies have shown that although the hyperacetylation of H4 was detected at the promoters of a significant number of genes after chronic drug treatment, a larger number of genes are regulated by H3 acetylation rather than by H4 acetylation (783 vs 471) (Renthal *et al.*, 2009; Renthal and Nestler, 2008). It will be interesting to analyze the molecular mechanisms by which chronic cocaine use preferentially induces the hyperacetylation of H3 at gene promoters, and to extend our study of the role of H4 acetylation in drug addiction.

CaMKII, a member of the Ca²⁺/calmodulin-dependent protein kinase family, is a ubiquitous serine/threonine protein kinase consisting of four isozymes (α , β , γ , and δ). It is highly enriched in brain tissue. The brain-specific α and β isoforms constitute up to 2% of the total protein in the hippocampus of rodents and up to 1% of the total protein in the forebrain itself, whereas the other two isozymes (γ and δ) are present at ~0.02% of the level of the brain-specific forms (Erondy and Kennedy, 1985; Tobimatsu and Fujisawa, 1989). Neuronal CaMKII regulates important neuronal functions, including neurotransmitter synthesis and release, the modulation of ion channel activity, synaptic plasticity, and gene expression (Mima *et al.*, 2001). A previous study showed that CaMKII phosphorylates NR2B and Psd95 to regulate signal transduction downstream from the *N*-methyl-D-aspartic acid (NMDA) receptor (Gardoni

et al, 2006; Liu et al, 2006). Recent work showed that the activation of CaMKII has a key role in the development and maintenance of the addiction state, and that fast and slow positive feedback loops are interlinked through CaMKII underlying drug addiction (Li et al, 2008). Recent work by Wang and colleagues showed that CaMKII α could relieve the D3R-mediated inhibition on sensitized behavior to foster drug-seeking behavior, implicating CaMKII α as an important negative regulator of D3R (Liu et al, 2009). In our experiments, we analyzed the effects of different isoforms of CaMKII on drug reinforcement responses. We found that CaMKII α , but not CaMKII β , was robustly induced by chronic cocaine use and that this correlated with the motivation for cocaine. Furthermore, using the localized knockdown of CaMKII α expression, we found that CaMKII α is required for the maintenance of the motivation. Considering these data collectively, we propose that CaMKII α may be a key molecule regulating the motivation for cocaine, similar to other well-established proteins such as BDNF, FosB, and so on.

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DISCLOSURE

The authors declare no conflict of interest.

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