MATERIALS AND METHODS

Animals

Male C57BL6/J mice (10- to 12-weeks old) (Jackson Laboratories) were used for all experiments. Mice were kept in clear plastic cages (29.2 x 19 x 12.7 cm, N10 cage, Ancare) in groups of five with *ad libitum* food (Prolab IsoPro RMH3000, PMI Nutrition International LLC.) and water (autoclaved tap water), and on a 12-hour day/night cycle. All behavioral experiments were carried out during the light phase. All animal procedures were executed in accordance with institutional guidelines. All mice were naive prior to all experimental procedures, unless otherwise stated.

Drug treatment

Cocaine hydrochloride (Sigma) (20mg for behavioral experiments and 30mg/kg for molecular and electrophysiological experiments) was dissolved in sterile saline and injected i.p. (-)-Nicotine hydrogen tartrate (Sigma) (50µg/ml for behavioral experiments and 10 µg/ml for molecular and electrophysiological experiments) was dissolved in distilled water and administered in the drinking water, which was supplied in light-protected bottles and changed every 48 hrs. SAHA 25mg/kg (kindly provided by Dr. Ronald Breslow) was dissolved in DMSO and administered i.p. Theophylline 200mg/L (Sigma) was dissolved in water and administered in the drinking water.

ChIP assay

The ChIP assay was carried out as described in (*1*) with some modifications. The striatum was dissected from mice anesthetized with 100 mg/kg ketamine 20 minutes after the last drug dose and perfused with 4% paraformaldehyde for 30 min, washed in 0.125M glycine and PBS, each for 12 min at 4°C, and homogenized. After sonication,

we centrifuged the sample and used the supernatant as the chromatin solution. The average size of DNA fragments was 300-500 bp. For the experiments described above, chromatin was used for immunoprecipitation with specific antibodies, anti-acetylated histone H4 and H3 (Upstate Biotechnology). To control for the specificity of antibody binding, we immunoprecipitated chromatin samples with nonimmune IgG, which precipitated negligible levels of the various genes studied. The presence of the FosB promoter in the precipitate was analyzed by real-time PCR with the promoter-specific primer pair GGTCCCGGAGGCATAAATTC (forward) and TCACGCCTCCAAGAAGAAGAA (reverse), using actin sequences to control for specificity of histone acetylation and transcription (TCGATATCCACGTGACATCCA (forward) TGGACACTGCCCCATTCAAT (reverse). Real-time PCR was performed with the Gene-Amp 5700 Sequence Detection System (PE Corporation). Amounts of specific histone modifications at the FosB promoter were determined by measuring the amount of acetylated histone-associated DNA using quantitative real-time PCR with actin as control. We also used the promoter of the GAPDH gene as another control with the following primers: Fwd: GTACACCTCCTGGGCTTCGA Rev: TTTCTGGAGGCCTACTTCCTTG. Input and immunoprecipitated DNA amplification reactions were run in triplicate in the presence of SYBR-Green (Applied Biosystems). Normalized reporter Rn values (fluorescence detected during PCR with GeneAmp 5700 Sequence Detection System manual, PE Corporation) from each sample were obtained by using SEQUENCE DETECTOR 1.1 software. Relative quantification of templates was carried out as described by the manufacturer (Applied Biosystems). Values (mean SEM) were analyzed by two-tailed paired t- tests (adjusted for multiple

comparisons). Statistical significance was set at ($p < 0.05$). PCR was run in triplicate for each brain sample, and at least three independent samples were used for each statistical analysis.

Measuring mRNA by Real-Time PCR

Two hours after the last drug administration, animals were guillotined. The striatum was dissected. RNA was extracted from the striata of mice that had been injected with various dosages of nicotine and cocaine with Trizol reagent (Invitrogen) and precipitated with isopropanol. mRNA was reverse transcribed by using a SuperScript III First-Strand Synthesis kit (Invitrogen). The amount of cDNA was quantified using real-time PCR. The primers that were used to amplify specific cDNA regions of the transcripts of interest were FosB 5 -ACAGATCGACTTCAGGCGGA-3 and 5 -GTTTGTGGGCCACCAGGAC-3 (these primers measure levels of the region coding for both FosB and it's splice variant • FosB); as internal control for normalization actin, 5 -ATGGTGGGAATGGGTCAGAAG-3 and 5 -TCTCCATGTCGTCCCAGTTG-3. We also used the GAPDH gene with these specific primers: 5-AACGACCCCTTCATTGAC-3 Rev: 5-TCCACGACATACTCAGCAC-3. Fold differences of mRNA over control values were calculated by using the Rn method as described by the manufacturer (Applied Biosystems).

Local infusion of SAHA and theophylline in the NAc

To test the effect of SAHA and theophylline on FosB transcription in the NAc, mice were anesthetized (Ketamine 80 mg/kg plus Xylazine 7 mg/kg i.p.) and placed into a stereotaxic apparatus (Kopf Instruments). Stereotactic procedures were used to implant stainless steel guide cannula (Alzet Brain Infusion Kit 2, Durect Corp.) into right or left

NAc. The stereotactic coordinates were +1.6mm A/P, +1.5mm Lateral to the sagittal line, -4.4mm D/V relating to dura 1.6 mm posterior to the Bregma. SAHA (100 μM) and theophylline (0.2,1 and 5μM), were delivered over 7 days by means of Alzet pumps (model #1007D), which contain 100μl. After 7 d treatment, mice were injected with cocaine or saline and NAc was dissected and RNA was extracted using Trizol.

Behavior: Open field studies

Locomotor activity was assessed using 5 Plexiglas open field boxes 43 times 43 cm2 (MED Associates). Two sets of 16 pulse-modulated infrared photobeams were placed on opposite walls 2.5-cm apart to record x–y ambulatory movements. Activity chambers were computer interfaced for data sampling at 100-ms resolution. Overall locomotor activity was quantified as the total distance traveled (cm). Mice were divided into 4 groups (n=10-12 each): drinking water + saline i.p.; oral nicotine administration + saline i.p.; drinking water + cocaine i.p.; oral nicotine administration + cocaine i.p. Locomotor activity was measured by an automated video tracking system. On days 5, 6 and 7 of nicotine administration, all mice were habituated to the Open Field test arena and the injection procedure. On day 8 cocaine (or saline as control), treatment was started and continued for four days (days 8, 9, 10 and 11). On each day, mice were injected (i.p.) with 20mg/kg of cocaine and the locomotor activity (measured as total distance traveled) was recorded by an automated tracking system. In additional behavioral experiments, mice were treated with nicotine for 1 day followed by 4 days of cocaine treatment. An additional experiment included a nicotine free hiatus: animals were treated with nicotine for 7 days, then nicotine was stopped for 14 days, and cocaine was administered for 4 days (days 15, 16, 17 and 18). Data were analyzed by two-way

ANOVA (nicotine × cocaine) for main effects and interaction effect. For multiple comparisons, one-way ANOVA with post hoc tests was performed.

CPP

C57BL6/J mice underwent conditioned place preference testing. Mice were given nicotine for 7 days and on day 8 of nicotine treatment, they were allowed 30 minutes free access to both sides of the place preference chamber (Med Associates) to determine initial preference. On days 9, 10, and 11, mice were conditioned (30 minutes) to saline on the initially preferred side, and 4 hours later, to cocaine on the least-preferred side. On day 12, mice were again given free access to both sides of chamber for 30 minutes. Data were expressed as time spent in the preferred chamber minus time spent in the less preferred chamber and analyzed by two-way ANOVA (nicotine × cocaine) for main effects and interaction effect. For multiple comparisons, one-way ANOVA with post hoc tests was performed.

Electrophysiology

Experiments were performed on coronal slices (400 µm) of brain prepared from C57BL6/J mice 6-8 weeks old. Slices were submerged and constantly perfused with ACSF at a rate of 2 ml/min and bubbled with 95% O2 and 5 % CO2. The composition of ACSF was (in mM): 124 NaCl, 1.2 MgSO4, 4 KCl, 1.0 NaH2PO4, 2 CaCl2, 26 NaHCO3, and 10 D-glucose. The GABA-A receptor antagonist picrotoxin (100 µM) was present in the perfusion solution in all experiments. The temperature of the slices was maintained at 27 ºC. Stimuli were delivered at a rate of one per minute (0.017 Hz, 0.1 ms pulse duration) through concentric bipolar stainless steel electrodes positioned in the NAc immediately adjacent to the anterior commissure (core), and the extracellular recording

electrodes were placed near the stimulation electrodes (Fig. 2) (*2*). In some experiments (Fig. 5B), parasagital slices containing NAc were prepared; the stimulation electrodes were placed at the prefrontal cortex-NAc border and the extracellular recoding electrodes were placed in the core region of NAc closest to the anterior commissure (*3*). To elicit LTP, four trains of 100 Hz tetanus (1 second, 0.2 ms pulse duration) 3 min apart were used. Any changes in synaptic strength were expressed relative to normalized baseline (mean±SEM). Statistical comparisons were performed using Student's t test.

Antibodies and immunoblotting

Mice were treated with cocaine and nicotine according to our sequential treatment paradigm. Striata were dissected in PBS on ice and homogenized in 0.25% Triton X-100, 0.5% Nonidet P-40, 10 mM EDTA, 0.5 mM EGTA, 10 mM Tris·HCl (pH 8.0), and 1 mM PMSF. Protein was measured by a Lowry-based assay (Bio-Rad); 30 µg of protein was used for detection of acetylated histones and separated by SDS/12.5% PAGE. After electrophoresis, gels were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell) and probed with acetylated Histone H3 and H4 antibodies (Millipore), overnight at 4°C. Blots were incubated with an anti-rabbit IgG-HRP as secondary antibody (1:5,000) (Sigma) for 1 h at room temperature and quantified by using ECL (Amersham Pharmacia). Some blots were later stripped with 0.2 M glycine (pH 2.8) and 0.5 M NaCl for 25 min at room temperature and reprobed as indicated. To verify the accuracy of sample loading, selected blots were reprobed with a monoclonal antibody to • -tubulin (1:20,000; Sigma). Acetylated H4 bands were measured at 12 kDa and acetylated Histone H3 at 17kDa. Relative optical density readings for the acetylated

Histone H3 and H4 and • -tubulin bands were determined by using a computer-assisted densitometry program (ImageJ) from four independent sample pairs. The optical density of components corresponding to the 12 and 17 kDa for acH4 and acH3 protein, respectively, was normalized with the optical density of • -tubulin-specific bands for each sample by dividing the optical density obtained from acH4 and acH3-specific bands by the optical density obtained from • -tubulin-specific bands for each animal.

HDAC activity in the striatum

Mice were treated with nicotine or cocaine for 7-10 days, then brains were removed and striata were dissected. The cytosolic and nuclear fractions were prepared using a nuclear extraction kit (Sigma). The two fractions from each mouse striatum were mixed and referred to as total cell lysates. HDAC activity (class I and II HDACs) was determined according to the manufacturer's instructions for the colorimetric HDAC activity assay kit (BioVision) by measuring the deacetylation of acetylated lysine side chains. The OD of the samples was measured with an ELISA plate reader at 405 nm (Spectra MR; Dynex Technologies). The results were calculated as OD per milligram of protein and then converted to percentage of control.

REFERENCES

1. A.A. Levine, Z. Guan, A. Barco, S. Xu, E.R. Kandel, J.H. Schwartz, CREB-binding protein controls response to cocaine by acetylating histones at the fosB promoter in the mouse striatum. *Proc Natl Acad Sci.* 27, 19186-19191 (2005).

2. A. F. Hoffman, M. Oz, T. C. Caulder, C. R. Lupica, Functional tolerance and blockade of long-term depression at synapses in the nucleus accumbens after chronic cannabinoid exposure. *J Neurosci.* 23, 4815-4820. (2003).

3. S. M. Nicola, S. B. Kombians, R. C. Malenka, Psychostimulants depress excitatory synaptic transmission in the nucleus accumbens via presynaptic D1-like dopamine receptors. *J. Neurosci.* 16, 1591-1604 (1996).